0014-4754/96/020167-08$1.50 + 0.20

Bactericidal activity against *Pseudomonas aeruginosa* is acquired by cultured human monocyte-derived macrophages after uptake of myeloperoxidase

M. Mathy-Hartert*, G. Deby-Dupont[a, b], P. Melin[c], M. Lamy[a, b] and C. Deby[a]

[a]*Centre for the Biochemistry of Oxygen, Institut de Chimie B6, Domaine universitaire du Sart Tilman, B-4000 Liège (Belgium), Fax + 32 41 662866, and* [b]*Department of Anesthesiology and Intensive Care, CHU B35, Domaine universitaire du Sart Tilman, B-4000 Liège (Belgium) and* [c]*Medical Microbiology CHU B23, Domaine universitaire du Sart Tilman, B-4000 Liège (Belgium)*

Received 5 April 1994; received after revision 26 June 1995; accepted 3 July 1995

Abstract. Myeloperoxidase (MPO) is an enzyme located within polymorphonuclear neutrophils capable of producing cytotoxic oxidant species that are particularly active against bacteria with polysaccharide capsules. *Pseudomonas aeruginosa* (10^6 bacteria per 1 ml) are killed within 1 h in vitro by a MPO/H_2O_2/Cl^- system (48 mU = 132 ng of MPO). The question arose as to whether human macrophages would acquire cytotoxic activity when loaded with this enzyme. Monocytes were therefore isolated from human blood and cultured for up to ten days to induce maturation to macrophages. These cells lost endogenous MPO within five days while H_2O_2 production in response to stimulation by phorbol myristate acetate (10^{-6} M) decreased to 23% within ten days. On the other hand, their capacity to take up exogenous MPO increased fourfold from day three to day ten. Human macrophages cultured from eight days (when both H_2O_2 production and MPO uptake were sufficient) were therefore used to study the effects of MPO uptake on cytocidal activity against *Pseudomonas aeruginosa*. After a 1 h MPO loading period, macrophages (5×10^5 cells per ml) were incubated in the presence of bacteria (0.5 to 2×10^6 bacteria per ml) for 2 h at 37 °C. At a bacteria/macrophage ratio of 1, only 34.8 ± 7.0% of bacteria survived (compared to killing by non-loaded macrophages), while 74.4 ± 9.3% survived at a ratio of 4. From these results, we conclude that loading macrophages with exogenous MPO could enhance their microbicidal activity, suggesting a potentially useful therapeutic application.

Key words. Myeloperoxidase; hydrogen peroxide; monocytes-macrophages; bactericidal activity; *Pseudomonas aeruginosa*.

When phagocytes are activated by appropriate stimuli, their membrane-bound NADPH oxidase complex catalyzes the one-electon reduction of oxygen to superoxide anion ($O_2^{\cdot-}$). Superoxide then dismutates to hydrogen peroxide (H_2O_2). H_2O_2 is a substrate for myeloperoxidase (MPO), a haem protein contained in the azurophil granules of polymorphonuclear neutrophils (PMN). MPO catalyzes the production of hypochlorous acid, a strong oxidant compound from which other toxic molecules (e.g. aldehydes and chloramines) are derived[1–3]. The components of the MPO system are thus the enzyme itself, H_2O_2, and an oxidizable species which is a halide anion (Cl^-, I^-). This system possesses potent antimicrobial, antiparasitic, and cytotoxic properties, and plays a key role in host defenses against microorganisms[2–5]. It is particularly active against polysaccharide bacterial capsules, which are resistant to other leucocyte-derived proteases and hydrolases.

In vitro studies with purified MPO have demonstrated a cytotoxic effect on *Candida albicans* and *tropicalis*[6,7]; the binding of MPO to the surface of *Candida albicans* via a mannose receptor is necessary to achieve cytotoxicity[6]. The candidacidal activity of MPO and the role of cell wall mannan were demonstrated in a murine model of candidiasis[8].

MPO is present in monocytes, but at considerably lower concentrations than in PMN. Macrophages are devoid of MPO and are no longer able to destroy intracellular protozoa[9]. Locksley et al.[9] and Nogueira et al.[10] reported that MPO-depleted macrophages almost entirely recovered their antiprotozoal activity when coated with eosinophil peroxidase prior to phagocytosis.

When pulmonary inflammation was induced by Calmette-Guérin bacillus in rats, alveolar macrophages acquired peroxidase activity within cytoplasmic inclusions[11]. Similarly, isolated alveolar macrophages took up purified MPO but an increase in bactericidal activity was not demonstrated. MPO is apparently taken up by macrophages after binding to a mannose receptor[12] and, after internalization, is delivered to the lysosomal compartment where it remains enzymatically active. Endocytosis of PMN granules containing MPO by macrophages has been demonstrated by electron microscopy[13]. After

* Corresponding author.

yeast infection, MPO was detected in the newly formed phagosomes containing the infecting organisms[13].

Because their polysaccharide capsules are resistant to both protease and hydrolase digestion, *Pseudomonas aeruginosa* are only killed with difficulty by macrophages. We therefore investigate a possible enhancement of pseudomonicidal activity by MPO-loaded macrophages.

Material and methods

Purification of human myeloperoxidase. MPO was purified from human polymorphonuclear neutrophils isolated as follows from buffy coats (blood collected from 500 healthy doners – Blood Transfusion Centre, University Hospital of Liège)[14]. To buffy coats were added 0.5 volume of Ficoll-Paque (Pharmacia), which were then centrifuged at room temperature (20 min at 300 g). The supernatant was diluted with 0.5 volume of 0.9% NaCl and centrifuged (1000 g, 10 min). The collected cells were washed with a hypotonic solution (155 mM NH_4Cl, 170 mM Tris-HCl, pH 7.4) to lyse any remaining erythrocytes. After centrifugation (800 g, 15 min), the collected cells were stored at −20 °C until MPO purification. Frozen cells were suspended in ice-cold acetate buffer (1 M NaCl, 0.2 M sodium acetate, pH 4.7) and disrupted with a teflon-glass homogenizer. After centrifugation (5000 g, 10 min, +4 °C), MPO activity was found in the supernatant. This extraction procedure was repeated twice. The combined supernatants were dialysed at +4 °C against a Tris-HCl buffer (0.05 M Tris-HCl, pH 8:0, 0.1 M NaCl). The precipitate, containing cathepsin G and elastase, was removed by centrifugation at 2000 g for 10 min. The supernatant was adjusted to 55% saturation with solid $(NH_4)_2SO_4$ and centrifuged (1000 g, 15 min); the precipitate was discarded and the supernatant was dialysed at +4 °C against buffer A (0.2 M NaCl, 0.02 M sodium acetate, pH 4.7). MPO was further purified by two chromatography steps (Fast Protein Liquid Chromatography-FPLC Pharmacia). The first step was an ion exchange chromatography on a High Performance Hiload S Sepharose (Pharmacia) column (1.6 × 10 cm) equilibrated with buffer A. After loading, the column was washed with buffer A and MPO was then eluted with a NaCl gradient (0.2 M to 1 M, 160 ml) at a flow rate of 2 ml/min. The peaks with the highest MPO activities were loaded on a gel filtration chromatography Sephacryl S-200 (Pharmacia) column (2.6 × 60 cm) and eluted with buffer A at a flow rate of 3 ml/min.

Purity of MPO was assayed by measuring the Reinheitzahl ratio (Rz: absorbance ratio A_{430}/A_{280}) and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie Blue. Protein concentration was determined by the Folin-Ciocalteu procedure and enzymatic activity by the ortho-dianisidine test (see below).

Assays for myeloperoxidase. MPO was assayed by two different techniques, an assay using enzymatic activity and an immunological assay.

Enzymatic assay. MPO activity was assayed at 25 °C by measuring the absorbance increase at 460 nm due to the oxidation of ortho-dianisidine[15]. The reaction buffer consisted of pH 5.5 Sörensen buffer, with 50 mM ortho-dianisidine. MPO (50 µl) was added to 3 ml of the reaction buffer and the reaction was started by addition of H_2O_2 at a final concentration of 15 mM. One unit of activity was defined as the amount of MPO increasing the absorbance by one optical density unit per min at 460 nm.

Immunological assay. MPO concentration was measured using a double antibody radioimmunoassay described previously[16,17], using human ^{125}I labelled MPO as tracer, human MPO as an unlabelled standard molecule and a polyclonal antiserum obtained from rabbit. The sensitivity limit of the method was 2 ng/ml.

***Pseudomonas aeruginosa* cultures.** The strain was provided by the Medical Microbiology Department of the University Hospital of Liège. Stock cultures of *Pseudomonas aeruginosa* were grown at 37 °C on L Broth agar plates (L Broth consisted of 10 g/l NaCl, 10 g/l Bacto Peptone Difco, 5 g/l yeast extract Difco, 1 g/l glucose, pH 7.4, added to 15 g/l agar) and stored at +4 °C until use. Before each experiment, a single colony was inoculated into 10 ml of L Broth and incubated at 37 °C with agitation. Bacterial growth was followed by the measurement of optical density at 590 nm; the culture was used when an optical density of 0.2 was reached (approximately 10^8 CFU/ml). The bacteria were then washed twice with Dulbecco's Phosphate-Buffer Saline (PBS: KH_2PO_4 0.2 g/L, Na_2HPO_4 1.15 g/L, KCl 0.2 g/L, NaCl 8 g/L), and resuspended in PBS.

Bactericidal activity of the $MPO/H_2O_2/Cl^-$ system. *Pseudomonas aeruginosa* (1×10^6) in 1 ml of PBS were incubated in the presence of varying amounts of MPO (4 mU = 11 ng to 48 mU = 132 ng). H_2O_2 (5×10^{-5} M) was added and the mixture was incubated for 1 h at 37 °C. Serial dilutions were prepared in PBS and 100 µl were spread onto L Broth agar plates (pour-plate assay). After an overnight incubation at 37 °C, colonies were counted. Each assay was performed in triplicate and the entire experiment was repeated three times.

Isolation of monocytes and culture of monocyte-derived macrophages. Monocytes were isolated from buffy coats obtained from the blood of ten healthy donors (Blood Transfusion Centre – University Hospital of Liège). The monocytes were collected after standard Ficoll-Paque (Pharmacia) density gradient centrifugation[18]. The cells were washed twice with PBS and then resuspended in RPMI 1640 medium (Gibco Laboratories) containing 20% heat-inactivated foetal calf serum (Gibco Laboratories). After two h incubation at 37 °C

in 5% CO_2-humidified air, nonadherent cells were removed by washing with warm (37 °C) PBS. The monolayering cells were covered with fresh media and returned to the incubator. The media were renewed every 48 h until the cells were used for experimental assays. Transformation of monocytes into macrophages during culture was confirmed by characteristic morphology[19,20].

H_2O_2 production by macrophages: ferrithiocyanate method. Adherent macrophages were washed three time with Hanks' balanced salt solution (HBSS: KH_2PO_4 0.06 g/L, Na_2HPO_4 0.048 g/L, $NaHCO_3$ 0.35 g/L, KCl 0.4 g/L, NaCl g/L, $MgSO_4 \cdot 7H_2O$ 0.1 g/L, $MgCl_2 \cdot 6H_2O$ 0.1 g/L, $CaCl_2 \cdot 2H_2O$ 0.185 g/L, glucose 1 g/L, pH 7.4). They were scraped gently with a rubber policeman into HBSS. Viable cells, assayed by trypan blue exclusion, were counted. The amount of H_2O_2 produced by 2×10^6 phorbol myristate acetate-stimulated cells (PMA, at a final concentration of 10^{-6} M) was measured in the presence of 1 mM azide according to the ferrithiocyanate method[21]. After 1 h at 37 °C, the reaction was stopped by addition of trichloroacetic acid (10%). After centrifugation (500 g, 10 min) ferrous ammonium sulfate (2 mM) and potassium thiocyanate (0.25 M) were added to the supernatant. The absorption of the ferrithiocyanate complex formed in the presence of hydrogen peroxide was measured at 480 nm. The amount of H_2O_2 produced by the cells was calculated from a standard curve and expressed as nmoles of H_2O_2 generated per hour and per 10^6 cells (nmoles/h 10^6 cells). Each assay was done in triplicate and the entire experiment was repeated three times.

MPO content and MPO uptake by cultured monocyte-macrophages. Monocyte-macrophages (5×10^6 cells), obtained as described above, were suspended in 1 ml of water. A 200 µl aliquot of this suspension was used for MPO measurement by radioimmunoassay after ultrasonication of the suspension. To the remaining cell suspension, cetylethylammonium-bromide (Sigma) (0.02%) was added. After a 20 min incubation at room temperature, the cell suspension samples were freeze-thawed twice and assayed for MPO enzymatic activity by the ortho-dianisidine oxidation assay as previously described. For the determination of MPO uptake, monocyte-macrophages (5×10^6 cells) were suspended in 5 ml of HBSS containing 1% bovine serum albumin (BSA) and were incubated with MPO (5.6 U, 15 µg) for 1 h at 37 °C. After washing three times, the cells were suspended in 1 ml of water and were treated as described above. Each assay was done in triplicate and the experiment was repeated three times.

Histochemical peroxidase assay. MPO uptake by macrophages was demonstrated by peroxidase activity using a modification of Graham and Karnovsky technique[22]. Adherent macrophages cultured for eight days in six well multidishes were incubated with MPO (1 µg) in 1 ml of HBSS containing 1% BSA for 1 h at 37 °C. Cells were washed three times in Tris-HCl buffer (100 mM, pH 7.6) and were incubated for 30 min at room temperature in the same buffer containing 3,3′-diaminobenzidine tetrahydrochloride (Sigma DAB, 0.05%) and with H_2O_2 (0.01%). Cells were fixed for 15 min at 4 °C with 2.5% glutaraldehyde in 100 mM cacodylate buffer, pH 7.2, containing 0.1% $CaCl_2$. Cells were then stained with toluidine blue (1%) and examined by light microscopy. Controls for the specificity of the peroxidase reaction were obtained by the omission

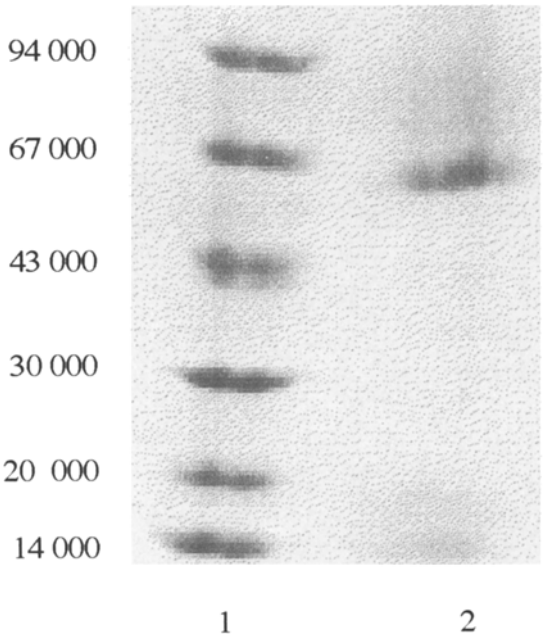

Figure 1. SDS-polyacrylamide gel electrophoresis of purified MPO. Lane 1: calibrating proteins; Lane 2: human MPO reduced by dithiothreitol (5 mM) prior to electrophoresis in order to demonstrate the two MPO subunits.

Table 1. Bactericidal activity of the MPO/H_2O_2/NaCl system against *Pseudomonas aeruginosa*.

Added compound H_2O_2 (M)	MPO (mU)	Viable bacteria (% of control)
0	0	100 (control value)
0	48	95 ± 7*
5.10^{-5}	0	91 ± 18*
5.10^{-5}	4	93 ± 7*
5.10^{-5}	12	32 ± 8**
5.10^{-5}	48	0 ± 0**

Pseudomonas aeruginosa (10^6) were incubated for 1 h at 37 °C in the presence of different compounds. The data are presented as percent of viable bacteria relative to control incubation (without H_2O_2 and without added MPO) and are the arithmetic means ±S.D of three different experiments each done in triplicate (n = 9).

* no statistical difference ($p > 0.05$) and ** statistical difference ($p < 0.05$) versus control when analysed with two-tailed Student's t-test.

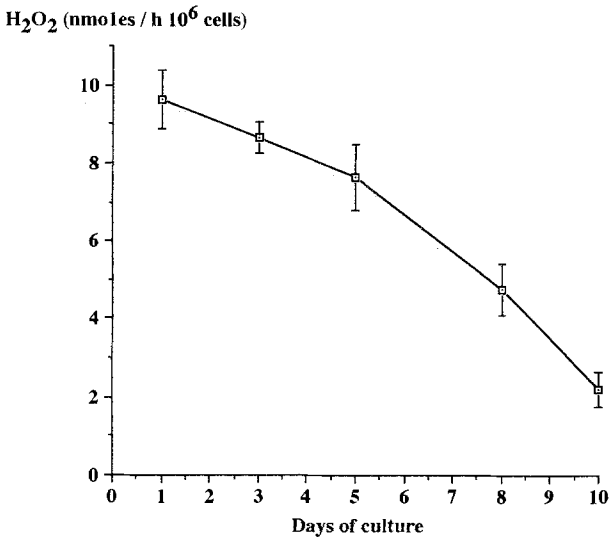

Figure 2. H_2O_2 production of stimulated monocytes-macrophages cultured for various times. On different days of culture, the cells (2×10^6) were stimulated by PMA (10^{-6} M) and H_2O_2 production (nmoles H_2O_2 per h and per 10^6 cells) was estimated after 1 h incubation at 37 °C. Data are the arithemic means ± SD of three independent experiments realized with three cell batches, and in each experiment assays were conducted in triplicate (n = 9).

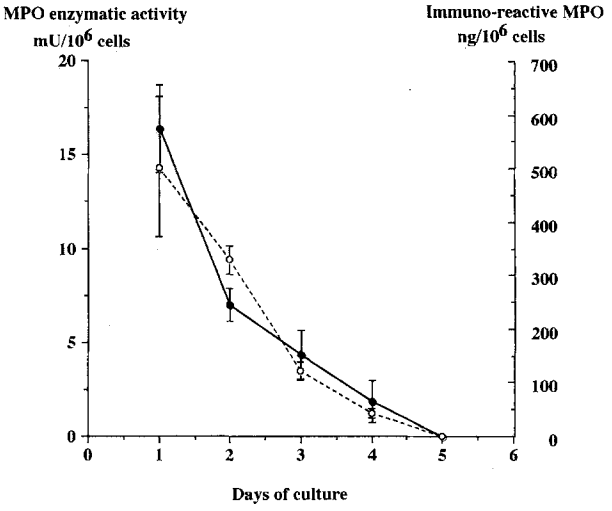

Figure 3. Content of endogenous MPO in cultured monocytes-macrophages. The endogenous MPO content of the cells was determined after different days of culture:
● = MPO content measured by enzymatic activity (mU/10^6 cells)
○ = MPO content measured by immunoreaction (ng/10^6 cells).
Data are the arithmetic means ± SD of three independent experiments realized with three cell batches, and in each experiment assays were conducted in triplicate (n = 9)

of H_2O_2 or DAB from the histochemical incubation medium; control macrophages, with no MPO added, were treated similarly.

Bactericidal activity of MPO loaded macrophages. Macrophages cultured for eight days were washed and gently scraped with a rubber policeman into HBSS containing 1% BSA. The cells were counted and their concentration adjusted to 1×10^6 cells/ml. MPO (1 µg = 0.37 U/10^6 cells) was added to the suspended macrophages. After 1 h incubation at 37 °C, the cells were centrifuged and washed three times to remove unincorporated MPO. Control macrophages were treated in the same manner, but without added MPO. Macrophages (5×10^5 cells) in 1 ml of culture medium were infected with *Pseudomonas aeruginosa* (5×10^5 to 2×10^6 bacteria). After 2 h incubation at 37 °C, aliquots were removed and serial dilutions were carried out with 0.1% Triton. A 100 µl aliquot was spread onto L Broth agar plates and bacterial colonies were counted after an overnight incubation at 37 °C. Each assay was done in triplicate and the entire experiment was repeated three times.

Results

Human MPO purification. Pure MPO from human polymorphonuclear leucocytes was obtained after the second chromatographic step (gel filtration). The electrophoretic analysis showed two bands of MW 65,000 and approximately 15,000, corresponding respectively to the heavy and light subunits of MPO (fig. 1). Specific activity was 370 U/mg protein and Rz was 0.8.

Bactericidal activity of the MPO/H_2O_2/Cl^- system on *Pseudomonas aeruginosa*. The results of the bactericidal assay, estimated by the pour-plate method, are shown in

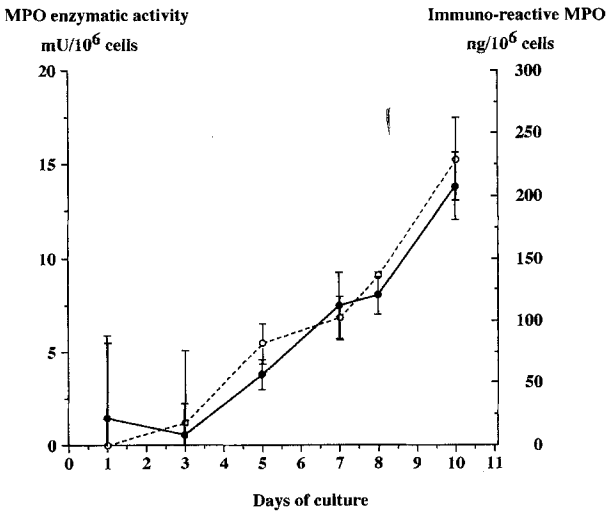

Figure 4. Uptake of exogenous MPO by cultured monocytes-macrophages. The cells (5×10^6), after culture for different periods, were incubated for 1 h with exogenous MPO (15 µg, 5.6 U) and MPO uptake was measured after washing.
● = MPO content measured by enzymatic activity (mU/10^6 cells)
○ = MPO content measured by immunoreaction (ng/10^6 cells).
Data are the arithmetic means ± SD of three independent experiments realized with three cell batches, and in each experiment assays were conducted in triplicate (n = 9)

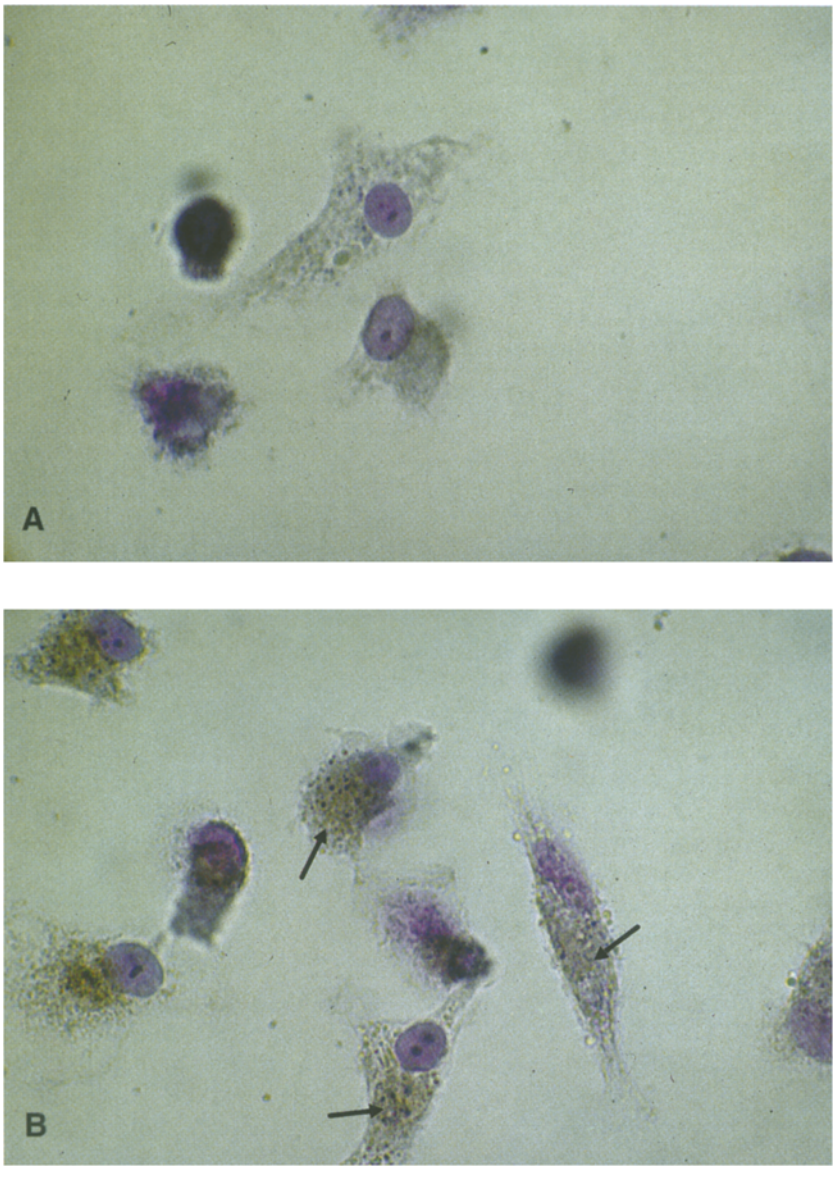

Figure 5. Detection of MPO uptake in monocytes-macrophages by peroxidase histochemistry. After eight days in culture, MPO was detected by incubating macrophages with DAB and H_2O_2 for 30 min. The monolayers were fixed in glutaraldehyde and stained with toluidine blue.
A) Control macrophages, *B*) MPO-loaded macrophages; arrows: arrows indicate accumulation of greenish inclusion of MPO.

table 1. The number of bacteria incubated in PBS containing neither MPO nor H_2O_2 was taken as reference (100%). Supplementation with either H_2O_2 (5×10^{-5} M) or with MPO (48 mU = 132 ng) alone had no significant bactericidal activity ($p > 0.05$). In the presence of H_2O_2 (5×10^{-5} M), MPO at 4 mU (11 ng) had no bactericidal activity ($p > 0.05$); with 12 mU (32.5 ng) the percentage of viable organisms fell to 32% and a complete cytotoxic effect was obtained with 48 mU (132 ng).

H_2O_2 production and MPO content of cultured macrophages. The cells could be maintained in culture for more than 14 days after their isolation from blood. After eight days in culture, the maturation of monocytes into macrophages was complete. Cellular capacity to produce H_2O_2 in response to stimulation by PMA (10^{-6} M) was tested until day ten of culture (fig. 2). During culture, the cells progressively lost their ability to produce H_2O_2. On the tenth day of culture, the production of H_2O_2 (2.20 ± 0.44 nmoles/h 10^6 cells) was reduced to 23% of the initial value (9.62 ± 0.76 nmoles/h 10^6 cells).

We also determined the time course of the MPO content of cultured monocytes-macrophages (fig. 3) by

Table 2. Bactericidal activity of MPO-loaded macrophages against *Pseudomonas aeruginosa*.

Bacteria/macrophage ratio	Viable bacteria in % (bacteria number $\times 10^6$)	
	control	MPO-loaded macrophages
1/1	100 ± 14 (5.14 ± 0.73)	34.8 ± 13.6 (1.79 ± 0.7)
2/1	100 ± 12 (7.53 ± 0.92)	50.5 ± 6.3 (3.80 ± 0.47)
4/1	100 ± 12 (11.49 ± 1.43)	74.4 ± 9.3 (8.54 ± 1.06)

Monocyte-derived macrophages cultured for eight days were incubated for 1 h at 37 °C with MPO (1 µg/10^6 cells) and after washing, were tested for their bactericidal activity. Cells (5×10^5) were incubated with different numbers of *Pseudomonas aeruginosa*. After an incubation of 2 h at 37 °C, viable bacteria numbers were estimated by pour-plate assay. Control value of viability was obtained with macrophages not submitted to MPO loading (100% viable bacteria).
Data are the arithmetic means ± S.D. of three independent experiments realized with three macrophage batches, and in each experiment, assays were conducted in triplicate (n = 9).

enzymatic and immunological assay. After five days in culture, the cells were totally devoid of endogenous MPO.

Exogenous MPO uptake by cultured macrophages. After a 1 h incubation at 37 °C in the presence of MPO (15 µg, 5.6 U), the cells (5×10^6) were washed and MPO content was estimated by immunological and enzymatic assay. The values presented in figure 4 were obtained after substraction of endogenous MPO content (see fig. 3). On day one, the cells did not take up MPO, but over the following days uptake progressively increased until day ten. The MPO thus taken up maintained its enzymatic activity; the specific activity was 0.053 ± 0.016 mU/mg, close to that of endogenous MPO (0.0340 ± 0.0096 mU/mg). Only traces of MPO activity were found in the supernatant of the first washing, and no MPO activity was found in the fluid used for the second washing. The enzymatic activity of exogenous MPO in the test medium was also measured and remained greater than 83% after two hours of incubation at 37 °C.

Because macrophages were still capable of producing H_2O_2 after PMA stimulation on day eight (see fig. 2), had lost their endogenous MPO activity at that time, and were able to incorporate exogenous MPO (see fig. 4), we chose day eight as a reliable time point to test the effects of uptake of MPO on macrophage killing activity against *Pseudomonas aeruginosa*.

Peroxidase histochemistry of MPO-loaded macrophages. To confirm that exogenous MPO was indeed taken up by macrophages, we used classical DAB histochemical staining for peroxidase[22]. After eight days in culture, macrophages were negative for peroxidase activity (fig. 5A), whereas following MPO uptake, the macrophages became strongly peroxidase-positive (fig. 5B). Peroxidase activity was visible as greenish inclusions into the cytoplasm. In control preparations, where we omitted the peroxidase substrate (DAB) or H_2O_2, no peroxidase activity was detected (data not shown).

Bactericidal activity of MPO loaded macrophages against *Pseudomonas aeruginosa*. Macrophages cultured for eight days were divided into two groups: controls (no exogenous MPO added) and MPO-loaded macrophages (uptake of exogenous MPO performed as previously described). The two groups were infected with *Pseudomonas aeruginosa*. Several ratios of bacteria to macrophages were used: 1/1, 2/1, and 4/1 (table 2). After infection of control macrophages, the growth of bacteria was not impaired; this number of viable bacteria was taken as reference (100%). For each ratio of bacteria to macrophages, the MPO-loaded macrophages showed an acquired killing activity relative to control macrophages (table 2). The highest cytocidal activity was obtained when the bacteria/macrophage ratio was equal to 1. Under these conditions, the number of viable bacteria was reduced to 34.8 ± 7.0% of control.

Discussion

As suggested by the results of SDS-PAGE analysis, it appeared that MPO isolated from human PMN was pure, with the expected molecular weights of 65,000 and 15,000 for the heavy and light subunits respectively, and with a Rz ratio comparable to data previously published[14].

MPO has been demonstrated to be particularly active against microorganisms with polysaccharide envelopes. These are resistant to proteases and hydrolases, but not to the MPO/H_2O_2/Cl^- system[2–4]. The cytotoxic activity of MPO against *Candida* is well documented[6–8]. Wright et al.[6] demonstrated that preincubation of *Candida* with MPO is required for in vitro cytotoxicity. In vivo, intraperitoneal administration of MPO increased survival of mice with chronic renal candidiasis[8]. Casentini-Borocz and Bringman[7] used MPO and glucose oxidase-conjugated antibodies together against *Candida tropicalis*, and demonstrated significant killing with binding of both conjugates to the yeast.

Pseudomonas aeruginosa is another microorganism with a polysaccharide capsule frequently found in immunodepressed patients[23]. *Pseudomonas aeruginosa* is frequently responsible for severe pulmonary infections in patients with cystic fibrosis[24]. The MPO/H_2O_2/Cl^- system was bactericidal against these bacteria: 68 ± 8% and 100% bacterial killing were obtained with low levels of MPO, respectively 12 mU/ml (32.5 ng/ml) and 48 mU/ml (132 ng/ml). Our results are in agreement with those obtained by Rosen and Klebanoff[25] who tested the direct cytotoxic activity of canine MPO on *Escherichia coli* under similar experimental conditions.

When monocyte-derived macrophages are cultured in sterile conditions, they quickly lose their capacity to produce active oxygen species after stimulation[26]. We observed a progressive loss of their capacity to produce H_2O_2 from day three to day ten of culture. On day eight, when complete maturation to macrophages was achieved, H_2O_2 production was still 50% of the value measured on day one.

Locksley et al.[9] demonstrated that cultured macrophages lose their endogenous MPO as a function of time. We found that endogenous MPO progressively decreased from day one to day five of culture; on day five, macrophages were devoid of MPO.

Shellito et al.[11] reported that tissue macrophages were able to trap MPO at sites of inflammation. Leung and Goren[13] showed that MPO in neutrophil granules was endocytosed by mouse peritoneal macrophages. In the presence of live opsonized yeast, MPO was transported to phagosomes with a half life of six to nine h. Shepherd and Hoidal[12] have shown that exogenous MPO was taken up by rat macrophages and delivered to lysosomes. They demonstrated that this uptake was dependent on a mannose receptor-specific process. Endothelial cells and fibroblasts, but not monocytes, also bind and internalize MPO[27].

We demonstrate here that cultured monocyte-macrophages began to take up exogenous MPO on day three; this uptake progressively increased with the maturation of monocytes into macrophages. By day ten, both the amount of exogenous MPO taken up by the cells and its specific activity were similar to initial endogenous levels and specific activities measured in freshly isolated monocytes.

On day eight of culture (when macrophages were still capable of both exogenous MPO uptake and production of H_2O_2) we compared the pseudomonacidal activity of MPO-depleted cells (controls) to that of MPO-loaded cells. In the presence of control macrophages, the growth of bacteria was not stopped. Loading of macrophages with MPO, on the other hand, resulted in bactericidal activity. This killing activity was maximal at a ratio of bacteria/macrophages of 1 (34.8% of viable bacteria compared to control) and was still observed at a bacteria/macrophages ratio of 4 (74.4% of viable bacteria compared to control). Because of the short incubation (two hours), bacterial phagocytosis may have been incomplete; our measurement of the ability of MPO-loaded macrophages to kill *Pseudomonas aeruginosa* may underestimate the true capacity. Newman et al.[28] showed that cultured macrophages only have a bacteriostatic action against *Escherichia coli* and *Staphylococcus aureus*, while monocytes which still have endogenous MPO are able to kill these bacteria. Locksley et al.[9] demonstrated that the decay in monocyte antiprotozoal activity was correlated with the decrease in their MPO content, and that coating the microorganisms with eosinophil peroxidase prior to phagocytosis restored antiprotozoal activity. Nogueira et al.[10] observed that killing of *Trypanosomas cruzi* by macrophages was rendered possible by pre-coating the microorganism with eosinophil peroxidase. To our knowledge, killing of *Pseudomonas aeruginosa* by exogenous MPO-loaded macrophages has not yet been demonstrated.

The uptake of exogenous MPO by macrophages could be of interest in certain pathological situations. In cystic fibrosis patients, alveolar macrophages loaded with MPO could become capable of killing *Pseudomonas aeruginosa*, the predominant respiratory pathogen infecting these patients[24]. In immunodepressed patients, who are particulary sensitive to polysaccharide-coated microorganisms[29], loading of macrophages with MPO could also be helpful. From this perspective, a therapeutic role from MPO loading would be possible; the production of recombinant human MPO (identical to the native enzyme extracted from human neutrophils)[30] would allow synthesis of clinically useful quantities, and could create interesting therapeutic possibilites.

Acknowledgements. This work was supported by FRSM grant 3.4554.93. The authors are indebted to Mrs Monique Dister, Josiane Bourdon and Anne Dethier for their skilled technical assistance, and to the Blood Transfusion Centre (University Hospital of Liège) for providing human blood. We gratefully thank Dr G. Hartstein for his assistance with the English language.

1 Harrison, J. E., and Schultz, J., J. biol. Chem., *251* (1976) 1371.
2 Klebanoff, S. J., J. Bacteriol *95* (1968) 2131.
3 Klebanoff, S. J., in: Phagocytic Cells: Products of Oxygen Metabolism. Inflammation: Basic Principles and Clinical Correlates, pp. 391–444 Eds J. I. Gallin, I. M. Goldstein and M. D. Snyderman Raven Press, Ltd., New York 1988.
4 Klebanoff, S. J., and Hamon, C. B., J. reticuloendothel. Soc. *12* (1972) 170.
5 Odell, E. W., and Segal, A. W., Biochim. biophys. Acta *971* (1988) 266.
6 Wright, C. D., Bowie, J. U., Gray, G. R., and Nelson, R. D., Infection and Immunity *42* (1983) 76.
7 Casentini-Borocz, D., and Bringman, T., Antimicrob. Agents Chemother *34* (1990) 875.
8 Wright, C. D., and Nelson, R. D., Infection and Immunity *47* (1985) 363.
9 Locksley, R. M., Nelson, C. S., Fankhauser, J. E., and Klebanoff, S. J., Am. J. trop. Med. Hyg., *36* (1987) 541.
10 Nogueira, N. M., Klebanoff, S. J., and Cohn, Z. A., J. Immunol. *128* (1982) 1705.
11 Shellito, J., Sniezek, M., and Warnock, M., Am. J. Pathol *129* (1987) 567.
12 Sheperd, V. L., and Hoidal, J. R., Am. J. Respir. Cell. Molec. Biol. *2* (1990) 335.
13 Leung, K. P., and Goren, M. B., Cell Tissue Res *257* (1989) 653.
14 Bakkenist, A. R. J., Wever, R., Vulsma, T., Plat, H., and Van Gelder, B. F., Biochim. biophys. Acta *524* (1978) 45.
15 Worthington Enzyme Manual, pp 43–45. Worthing Biochemical Corp. Freehold, NJ, 1972.
16 Deby-Dupont, G., Pincemail, J., Thirion, A., Deby, C., Lamy, M., and Franchimont, P., Experientia *47* (1991) 952.
17 Pincemail, J., Deby-Dupont, G., Deby, C., Thirion, A., Torpier, G., Faymonville, M. E., Damas, P., Tomassini, M., Lamy, M. and Franchimont, P., J. immunol. Methods *137* (1991) 181.

18 Boyum, A., Scand. J. Immun. *5* (1976) 9.
19 Hanifin, J. M., and Cline, M. J., J. Cell Biol. *46* (1970) 97.
20 Wilson, C. B., Tsai, V., and Remington, J. S., J. expl Med. *151* (1980) 328.
21 Thurman, R. G., Ley, H. G., and Scholz, R., Eur. J. Biochem *25* (1972) 420.
22 Graham, R. C., and Karnovsky, M. J., J. Histochem. Cytochem. *14* (1966) 291.
23 Abraham, E., and Stevens, P., Crit. Care Med. *20* (1992) 1127.
24 Speert, D. P., Eftekhar, F., and Puterman, M. L., Infection and Immunity *43* (1984) 1006.
25 Rosen, H., and Klebanoff, S. J., J. biol. Chem., *257* (1982) 13731.
26 Pabst, M. J., Hedegaard, H. B., and Johnston, R. B., J. Immunol., *128* (1982) 123.
27 Zabucchi, G., Soranzo, M. R., Menegazzi, R., Bertoncin, P., Nardon, E., and Patriarca, P., J. Histochem. Cytochem., *37* (1989) 499.
28 Newman, S. L., and Tucci, M. A., J. clin. Invest., *86* (1990) 703.
29 Malech, H. L., and Gallin, J. I., N. Engl. J. Med., *317* (1987) 687.
30 Jacquet, A., Deby, C., Mathy, M., Moguilewsky, N., Deby-Dupont, G., Thirion, A., Goormaghtigh, E., Garcia-Quintana, L., Bollen, A., and Pincemail, J., Archs Biochem. Biophys. *291* (1991) 132.