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Differential involvement of reactive oxygen species and myeloperoxidase in oxygen-dependent killing of urinary tract bacterial isolates by polymorphonuclear leukocytes

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Abstract We examined the involvement of reactive oxygen species (ROS) and myeloperoxidase (MPO) in bacterial killing by human polymorphonuclear leukocytes (PMN) by means of a chemiluminescence assay. Using four bacterial strains, including *Pseudomonas aeruginosa* strain ATCC 27853 and three other strains of *P. aeruginosa* that were isolated from patients with urinary tract infections, we examined whether the production of ROS and changes in MPO activity altered the numbers of bacteria in contact with PMN. For three strains (*P. aeruginosa* strain ATCC 27853, strain 40, and strain 64), a decrease in number of viable bacteria was observed with PMN addition over three 20-min intervals that differed significantly from observations in these strains without the addition of PMN, and residual MPO activity of these three strains correlated significantly with decreases in numbers of viable bacteria. We concluded that MPO-dependent processes are strongly favored by human PMN for the oxidative killing of bacteria.

Keywords Polymorphonuclear leukocytes · Bactericidal activity · Reactive oxygen species · Myeloperoxidase activity · Chemiluminescence assays

Introduction

The human organism is equipped with a variety of defense mechanisms against invasion by microbes. These defenses may be classified into two major categories: a specific system based on cellular immunity that involves complement, immunoglobulin, and lymphocytes; and a nonspecific system based on phagocytosis by polymorphonuclear leukocytes (PMN), monocytes, and macrophages. Phagocytic actions occur in three stages, including chemotaxis, phagocytosis, and killing. At the time of phagocytosis by PMN, the reactive oxygen species (ROS) are generated [1]. The cytoplasmic membrane of human PMN is known to have a simple electron delivery system producing O_2^- through the activation of NADPH oxidase [16]. When phagosomes are formed through ingestion of bacteria and foreign bodies, a large amount of O_2^- is produced within PMN and is changed to H_2O_2. Additionally, these primary phagosomes are united with cytoplasmic granules in PMN containing lysozyme and myeloperoxidase (MPO) to form secondary phagosomes. An important consequence is the formation of HOCl, which has a strong bactericidal action within PMN [3]. This sequence is considered more important than the generation of ROS in the bactericidal function of phagocytes. To our knowledge, previous authors have not investigated how concurrent production of ROS by PMN and alteration of MPO activity might act together to reduce numbers of viable bacteria by a bactericidal action. We exposed PMN to bacteria in vitro to assess production of ROS and the alteration of MPO activity at the time of bacterial phagocytosis and killing by PMN.

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Materials and methods

Isolation of PMN

Samples of peripheral venous blood were obtained from healthy volunteers following informed consent. PMN were isolated using Mono-Poly Resolving Medium (MPRM; ICN Biomedicals Tokyo, Japan). Co-existing erythrocytes were lysed with a modified ammonium chloride Tris (ACT) solution [9]. PMN were centrifuged in Hanks balanced salt solution (HBSS) without Ca_2^+ or Mg_2^+ (Sigma, St. Louis, Mo.). PMN viability, assessed by fluorescent testing of the cell membrane integrity with ethidium bromide and

acridine orange, was greater than 95% [5]. The purified PMN were resuspended in HBSS to give a cell density of approximately 5×10^6/ml. Suspensions were used immediately for measurement of bactericidal activity.

Preparation of bacteria and exposure to PMN

We used a total of four bacterial strains, consisting of *Pseudomonas aeruginosa* strain ATCC 27853 and three strains of *P. aeruginosa* isolated from patients with urinary tract infections. Bacteria were cultured overnight in a brain–heart infusion agar slant (BHIA; Oxoid, Basingstoke, UK) and then suspended in tryptic soy broth (TSB; Difco, Detroit, Mich.). The density of inoculation was determined by absorbance. After inoculation into 40 ml of TSB, bacteria were cultured at 37°C for approximately 4 h with continuous mechanical stirring. After the absorbance of the culture suspension was measured, the suspension was centrifuged at 2000 *g* for 15 min. The bacteria in the resulting pellet were suspended in sufficient HBSS to yield a bacterial density of about 6.6×10^7 CFU/ml according to absorbance. The bacterial suspension was stored at 4°C until used the same day. At the time of use, before the suspension was exposed to PMN, inactivated serum from the same PMN donor was added as an opsonin (final donor serum concentration, 10%) [7]. Then the suspension was diluted to a bacterial density of about 6.6×10^6 CFU/ml using HBSS. The suspension was then combined with PMN to result in a 50:1 ratio of PMN to bacteria.

Quantitaton of viable bacteria over time after introduction of PMN

After PMN were mixed with bacteria, the mixture was incubated at 37°C with slow mechanical shaking. In other bacterial suspensions PMN were omitted for this incubation. Numbers of viable bacteria were determined in samples taken before the incubation and at intervals of 20 min up to 1 h. To include viable intracellular bacteria within PMN in the assessment of samples containing PMN, these samples were added to 250 volumes of distilled water at pH 11 for 5 min at 37°C to osmotically lyse PMN [6]. Mueller-Hinton broth (MHB; Difco, Detroit, Mich.) was used to prepare tenfold serial dilutions of all samples. After further dilution with BHIA containing 0.2% KNO_3, each sample dilution was poured onto a plate and cultured overnight at 37°C, followed then by enumeration of colonies. The inhibitory effect of PMN on bacterial proliferation was evaluated based on the difference between the numbers of viable bacteria (i.e., CFU) during the 1-h incubation with PMN present or absent. Numbers of CFU were expressed logarithmic ally. All experiments were performed in triplicate.

Measurement of ROS and residual MPO activity

Production of OCl^- was determined in luminol-dependent chemiluminescence (LDCL) using luminol (Sigma) as an indicator [8]. O_2^- production was determined using a *Cypridina* luciferin analog, 2-methyl-6-[p-methoxyphenyl] -3,7-dihydroimidazo [1,2-a] pyrazin-3-one (MCLA; TCI, Tokyo), as an indicator (MCLA-dependent chemiluminescence, or MDCL) [14]. Luminol concentrations were adjusted to 17.7 mg/ml using dimethyl sulfoxide (Wako Chemical, Osaka) while the MCLA concentration was adjusted to 400 μM using distilled water. Aliquots of indicator preparations were prepared and stored frozen at –80°C until needed [14]. PMN–bacterial suspensions with the ratio of PMN to bacteria adjusted to 50:1 were combined with 10% donor serum as an opsonin plus either 5 μl of luminol or 25 μl of MCLA; the maximum intensity of luminescence was determined over 60 min with a luminometer (model 1251; BioOrbit, Turku, Finland).

Residual MPO activity was extracted and measured by chemiluminescence using the following protocol [12]. After the mixture of opsonized PMN and bacteria was allowed to react for 60 min, PMN were isolated, thoroughly washed with HBSS, and combined with 2 ml of 0.02% cethyltrimethyl ammonium bromide (CTAB; Sigma). The suspension was sonicated with a Branson Sonifier (model 250; Branson Ultrasonics, Danbury, Conn.). Sonification was performed at 30 W in 0.5-s bursts alternating with 0.5-s intervals of stillness for 30 s. The sonicated preparation was centrifuged at 40,000 *g* for 20 min at 4°C, and the supernate was used for measurement [13]. After preparation in advance, the following materials were placed in a cuvette in the following order: 1,000 μl of buffered 0.2 M acetic acid solution (pH 4.5; CH_3COOH-CH_3COONa), 765 μl of distilled water, 20 μl of 2 mM desferoxamine mesylate (Sigma), 20 μl of 50 mM KBr (Sigma), 20 μl of 50 μM superoxide dismutase (SOD; Sigma), 100 μl of MCLA, and 25 μl of 40 mM H_2O_2 (Wako Chemical). Finally, the sample for measurement was added, and MDCL was measured over 60 min to obtain residual MPO activity based on a linear calibration plot constructed using known MPO concentrations [12]. Residual MPO activity were measured five times per bacterial strain.

Statistical analysis

Statistical assessment of OCl^- production, O_2^- production, and MPO activity was performed using analyses of variance and Fisher's PLSD method as a post hoc test. In addition, the change in viable bacterial numbers over time was evaluated using repeated measurement analysis of variance, while single regression analysis was employed to examine the relationships between the change in number of viable bacteria over time and OCl^- production, O_2^- production, or residual MPO activity among the different bacterial strains. A probability of $P<0.05$ was considered statistically significant.

Results

Direct observations: changes in number of viable bacteria

Prior to incubation, numbers of viable bacteria in the prepared suspensions ranged from 5.44 to 6.00 (log CFU/ml). The three strains of *P. aeruginosa* (ATCC 27853, 40, and 64) showed increasing numbers of viable bacteria with time in samples without added PMN (Fig. 1). In samples of these strains with added PMN, a time-dependent decrease in the number of viable bacteria was observed. The differences in numbers between PMN and non-PMN preparations at each 20-min interval were statistically significant ($P<0.05$). For the one other strain of *P. aeruginosa*, 179, no decrease was apparent in the number of viable bacteria over time even with PMN addition and the number of viable bacteria during 60 min of exposure to PMN actually increased from the baseline. With this latter strain, no significant inhibition of bacterial growth resulted from the addition of PMN.

OCl^- production

Chemiluminescence resulting from OCl^- production by PMN after 60 min in the presence of the various bacterial strains ranged from 28.3 to 38.4 mV, with no significant differences observed among the strains tested. Two strains of *P. aeruginosa,* strain 40 (38.386 ± 47.411 mV; mean ± SD) and strain 64 (35.596 ± 32.231 mV), were associated with somewhat more OCl^- production by

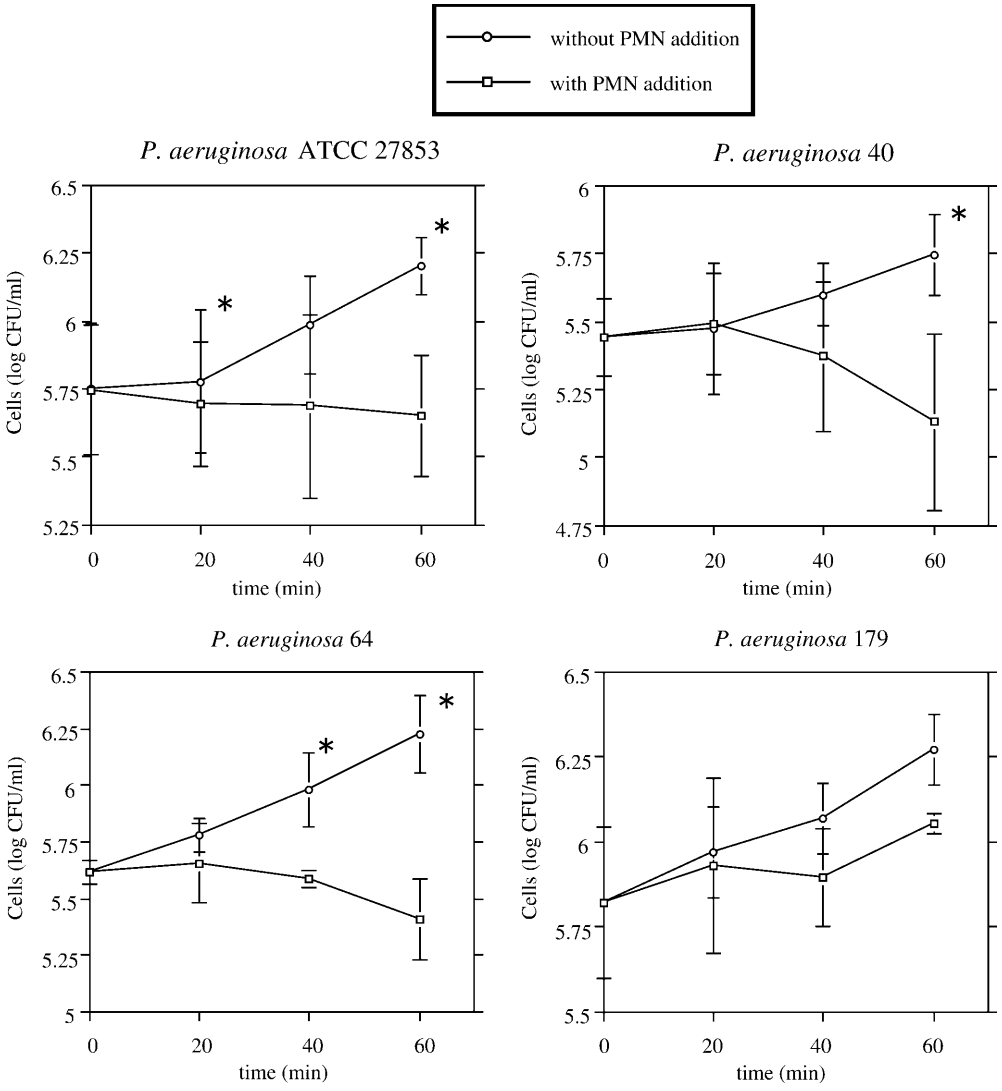

Fig. 1 Changes in the numbers of viable bacteria with and without polymorphonuclear leukocytes (PMN) addition. These differences were compared between bacterial strains. For three strains (*Pseudomonas aeruginosa* strain ATCC 27853, *P. aeruginosa* strain 40, and *P. aeruginosa* strain 64), a decrease in the number of viable bacteria was observed with PMN addition over three 20-min intervals that differed significantly from observations in these strains without PMN addition (*, $P < 0.05$)

PMN than the amount seen with the remaining two strains, although the differences were not statistically significant.

O_2^- production

Chemiluminescence resulting from O_2^- production by PMN after 60 min in the presence of the various bacterial strains ranged from 491.7 to 603.7 mV, with no significant differences among the strains. Three strains of *P. aeruginosa*, strain ATCC 27853 (598.220 ± 297.596 mV), strain 40 (601.840 ± 307.009 mV), and strain 64 (603.660 ± 319.853 mV) were associated with somewhat more O_2^- production by PMN than was seen with the remaining strain. However, the differences were not statistically significant.

Residual MPO activity

MPO activity in PMN in the absence of bacteria was approximately 200,000 pM (199,381.2 ± 19,080.6). MPO activity following exposure to individual strains (residual MPO activity) had decreased to 7.3%–13.9% of pre-exposure MPO activity. Furthermore, residual MPO activity varied by strain (Fig. 2). The residual MPO activity with three *P. aeruginosa* strains, strain ATCC 27853 (26,611.5 ± 8,976.8 pM), strain 40 (24,993.7 ± 5,677.8 pM), and strain 64 (27,693.1 ± 5,985.4 pM) was greater than with the other one strain, *P. aeruginosa* strain 179 (14,638.9 ± 4,815.2 pM). Statistically significant differences in residual MPO activity were observed between *P. aeruginosa* ATCC 27853 and strain 179 ($P = 0.0107$), strain 40 and strain 179 ($P = 0.0238$), and also strain 64 and strain 179 ($P = 0.0062$).

Analysis of factors contributing to the changes in number of viable bacteria and relationship between change in number of viable bacteria and ROS/MPO activity at 60 min

Simple regression analysis was performed to determine whether the difference in number of viable bacteria

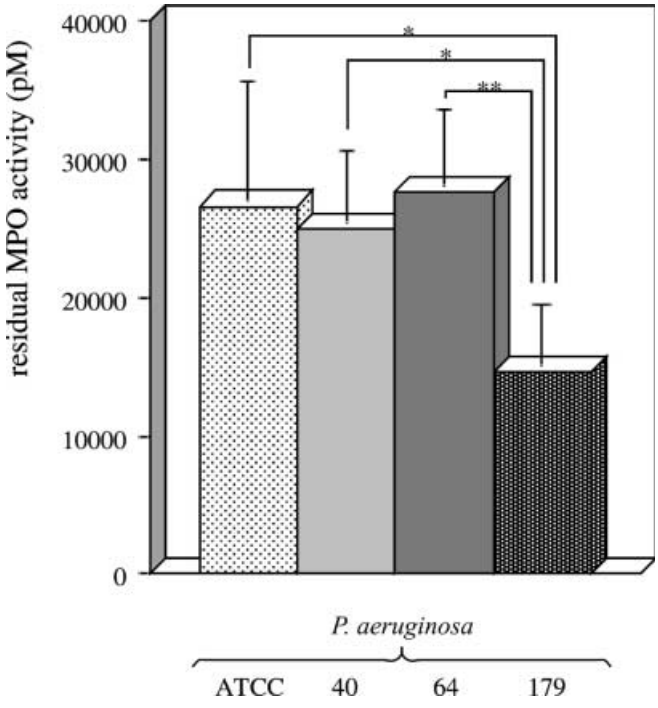

Fig. 2 Residual myeloperoxidase (MPO) activity in polymorphonuclear leukocytes (PMN) in the absence of bacteria was approximately 200,000 pM (199,381.2 ± 19,080.6). Residual MPO activity in PMN following bacterial exposure had decreased to 7.3%–13.9% of pre-exposure MPO activity. Residual MPO activity varied between strains. Statistically significant differences in residual MPO activity were observed between *P. aeruginosa* ATCC 27853 and strain 179 ($P = 0.0107$), strain 40 and strain 179 ($P = 0.0238$), and also strain 64 and strain 179 ($P = 0.0062$). (*, $P < 0.05$; **, $P < 0.01$)

between strains with and without PMN addition was related to any of the three biochemical variables measured (OCl^- production, O_2^- production, and residual MPO activity). Statistically significant correlations were not evident between the difference in number of viable bacteria and OCl^- production ($P = 0.9862$, $r = 0.006$, $n = 12$), O_2^- production ($P = 0.2039$, $r = 0.395$, $n = 12$), and residual MPO activity ($P = 0.3275$, $r = 0.310$, $n = 12$).

Change in number of viable bacteria and residual MPO activity for individual strains

Simple regression analysis was performed to examine possible associations between changes in numbers of viable bacteria and residual MPO activity at 60 min for individual strains. A statistically significant association was noted between residual MPO activity and bacterial numbers for three *P. aeruginosa* strains: strain ATCC 27853 ($P = 0.0473$), strain 40 ($P = 0.0347$), and strain 64 ($P = 0.0084$). No significant association was observed for the remaining one isolate: *P. aeruginosa* strain 179 ($P = 0.3304$). Residual MPO activity with the former three strains was somewhat greater than with the latter one strain.

Correlation between residual MPO activity, OCl^- and O_2^- production

We found no correlation between residual MPO activity and OCl^- production by PMN for any strain assessed in this study ($P = 0.4774$, $r = 0.169$, $n = 20$). However, correlations were observed when we took account of differences in residual MPO activity and OCl^- production between strains ($P = 0.0324$, $r = 0.696$, $n = 20$). When the relationship between residual MPO activity and O_2^- production was similarly investigated, although no significant correlation was observed for any strain ($P = 0.4575$, $r = 0.176$, $n = 20$), statistically significant correlations were noted when differences in residual MPO activity between strains were taken into account ($P = 0.0179$, $r = 0.726$, $n = 20$). A tendency toward a definite correlation with residual MPO activity was not clear for either OCl^- production or O_2^- production. After all, OCl^- production and O_2^- production also showed strong specificity among strains with various residual MPO activity.

Discussion

Oxygen-dependent and oxygen-independent systems are involved in the bactericidal mechanisms of PMN [4]. Particularly important mechanisms are those involving MPO-catalyzed halogenation, beginning with the formation of large amounts of O_2^- in the oxygen-dependent MPO-H_2O_2-Cl system following phagocytosis of bacteria or foreign bodies. Formation of OCl^- also occurs, as a result of the oxidation of halogen ions [11]. In the present study we investigated factors contributing to the phagocytotic and bactericidal actions of PMN against viable bacteria.

We found that O_2^- production by stimulated PMN, which showed no clear differences between various bacterial strains, correlated with residual MPO activity between strains. These results suggest that a bactericidal mechanism involving O_2^- participated in MPO-dependent bactericidal processes.

Residual MPO activity varied between strains and was closely related to the change in number of viable bacteria with strain specificity. In three strains of *P. aeruginosa* (ATCC 27853, 40, and 64), which showed differences in numbers between PMN and non-PMN preparations at each 20-min interval, were statistically significant, with differences in the number of viable bacteria correlating negatively with residual MPO activity. On the other hand, in one strain of *P. aeruginosa* 179, no decrease was apparent in the number of viable bacteria over time even with PMN addition and differences in number of viable bacteria did not correlate with residual MPO activity. Furthermore, the former three strains showed high residual MPO activity compared with the latter strain. These results suggest that bacterial killing may reflect differences in the intensity of the MPO-catalyzed response provoked by individual strains

in PMN, at least among *P. aeruginosa* strains. The finding that residual MPO activity was the most useful index of the phagocytic and bactericidal effect of PMN is consistent with the hypothesis that MPO-catalyzed halogenation is an important bactericidal mechanism. Hampton et al. [10] investigated oxygen-dependent killing of bacteria using *Staphylococcus aureus*, finding the MPO-dependent system to be the most important bactericidal mechanism. These authors also concluded that direct killing of bacteria by O_2^- occurred in relation to the MPO-dependent system. The results of the present study support their findings.

We found no significant differences between bactericidal mechanisms mediated by OCl^-, but correlated with residual MPO activity between strains. OCl^- would be expected to differ between strains because residual MPO activity varied. Chloramine, which is produced by the reaction of a nitrogen compound in PMN with OCl^-, also has bactericidal effects [8,15]. OCl^- is a strong oxidant and destroys bacteria by oxidation. However, OCl^- is merely one of a number of bactericidal substances produced by MPO-catalyzed reactions, and a complex relationship seems likely between residual MPO activity and OCl^- production. In our study, OCl^- production tended to increase as more MPO activity was consumed, but only among samples with higher residual MPO activity. These results suggest that reserve OCl^- production was greater when residual MPO activity was greater.

In the present study involving only four strains of *P. aeruginosa*, we could not detect an overall difference in PMN bactericidal function among *Pseudomonas*. However, the individual *P. aeruginosa* strains showed differences in relation to residual MPO activity and changes in the number of viable bacteria. Investigation of the differences in PMN bactericidal function between larger numbers of strains of bacteria is needed.

In addition, the examination of bactericidal properties of PMNs, such as carried out in our research, require dividing bacteria which are usually grown to logarithmic phase, and then refrigerated. It might have been better to have used the bacteria immediately. This might have influenced the response of a particular bacterium.

Various studies have evaluated the bactericidal function of PMN by chemiluminescence using luminol or MCLA, or to assess MPO activity with respect to the bactericidal function of PMN. Our results indicate that chemiluminescence is a useful way to measure O_2^- and OCl^- production as well as MPO activity. Molecular biological studies of mechanisms involved in the activation of NADPH related O_2^- producing enzymes as well as in the killing of bacteria by PMN-produced oxygen radicals will result in more detailed understanding.

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