Kinetics of peroxidases in guinea pig bone marrow under immunostimulation

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Abstract Eosinophil peroxidase and myeloperoxidase play an important role in the host defense. Both enzymes are present in bone marrow, synthesized by blood progenitor cells. This research investigated the kinetic properties of peroxidases under immunostimulation in guinea pig bone marrow. Results suggest that there are at least two myeloperoxidase isozymes and at least three eosinophil peroxidase isozymes in guinea pig bone marrow and that some of these isozymes are expressed upon immunostimulation.

Key words: Peroxidase; Kinetics; Isozyme; Immunostimulation; Bone marrow; Guinea pig

1. Introduction

Two peroxidases (donor: hydrogen peroxide oxidoreductase, EC 1.2.1.7), eosinophil peroxidase (EPO) and neutrophil peroxidase (myeloperoxidase: MPO) are present in bone marrow [1], synthesized by eosinophil and neutrophil cells. Eosinophils, like neutrophils, are cytotoxic to mammalian tumor cells and this cytotoxicity is mediated by a peroxidase (EPO or MPO)-H_2O_2-halide system [2]. In contrast, human basophils do not contain EPO [3]. Both MPO and EPO play an important role as anti-infection agents. However, both enzymes may also be detrimental and cause damage to host tissues like EPO does in inflammatory disease states [4,5] and MPO in some leukemia [6]. EPO can also injure cells and tissues directly in addition to its capacity to produce free radical species [7]. Because of the importance of EPO and MPO in the human health and disease, both enzymes are now under intense investigation [6,8–11]. Both MPO and EPO have been purified by various authors and their properties have been investigated [12,13]. Furthermore, isozymes of peroxidase have been found in plant [14,15] and MPO isozymes have been detected in neutrophils [16]. Inactivation of peroxidases in bone marrow by repeated administration of propylthiouracil has been reported [17]. Moreover, quantitative assessments of hematopoiesis in experimental animals has shown both a decrease in the cellularity of bone marrow after irradiation [18] or an increase in that cellularity upon subcutaneous injection of granulocyte colony stimulating factor [19,20]. Various methods for the mobilization of peripheral blood progenitor cells into the circulation have also been described [20,21]. But, to our knowledge, stimulation of the peroxidases biosynthesis in bone marrow by an immunostimulant and their kinetic properties have not yet been reported. Thus, the purpose of this research was to investigate the kinetic properties of peroxidases in guinea pig bone marrow under immunostimulation. Our results showed that an increase in the granulocyte population was accompanied by an increase in the activity of peroxidases and the existence of multiple kinetically distinct MPO and EPO in guinea pig bone marrow under immunostimulation. A preliminary result of this research was published in [22].

2. Materials and methods

2.1. Chemicals

KCN, sodium azide, KI, cetyltrimethylammonium bromide (CTAB) and Triton X-100 were from Sigma (St. Louis), all other chemicals, including *o*-dianisidine, guaiacol and 3-amino-1H-1,2,4-triazole (aminotriazole) were from Merck (Darmstadt, Germany) and all were of the best grade available.

2.2. Immunostimulation

Immunostimulation was performed as follows: 1 ml sheep blood was injected subcutaneously to 3–4 months old guinea pigs, daily, for 12 days. Injection of sheep blood was originally used by Winquist [23] to induce basophilia in guinea pigs. However, we found that such an injection produced also an increase in the number of eosinophils and neutrophils.

2.3. Sample preparation

At various times ranging from 4 to 30 days after the first injection of sheep blood, animals were killed by cervical dislocation after general anesthesia. Front and hind legs were removed and bone marrow smears were prepared and stained essentially according to [24]. Four unstimulated animals were used as control. To remove and lyse the red cells, the bone marrow was suspended in 150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA and centrifuged at $800\times g$. The pellet was suspended in 9‰ NaCl and pelleted at 3000 rpm ($1000\times g$). The pellet was frozen at −20°C for up to 48 h. After two cycles of freeze-thawing, cells were suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 200 mg/l phenylmethylsulfonyl fluoride and homogenized with 20 passes in a Potter homogenizer (with tight pestle). The homogenate was centrifuged at $20\,000\times g$ for 15 min, the pellet was discarded and the supernatant was used for the determination of peroxidase activity. The protein concentration was determined by the Biuret method.

2.4. Peroxidase activity

EPO activity was measured using two different substrates. (1) Guaiacol oxidation was measured at 470 nm, using an extinction coefficient of 26.6 mM^{-1}/cm [25]. (2) KI oxidation was measured at 350 nm, using an extinction coefficient of 26 mM^{-1}/cm [26]. MPO activity was measured by following the *o*-dianisidine oxidation at 450 nm [27], using an extinction coefficient of 11.3 mM^{-1}/cm [28]. In all cases, the reaction mixture consisted of 3 ml 0.1 M citrate buffer, pH 5.0, 10 μl sample, the appropriate substrate and H_2O_2 to a final concentration of 0.33 mM. The final concentrations of substrates were as follows: 33 mM for guaiacol, 5 mM for KI and 10 μM for *o*-dianisidine. In some experiments, CTAB or Triton X-100 were present at a final concentration of 0.05%.

All assays were carried out at room temperature (∼22–25°C) using Aminco-DW2 and Milton-Roy spectrophotometers. Results were

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averages of two experiments (four controls and 2×6 animals). All measurements were done in triplicate.

The velocity constant for the reaction between the enzyme-substrate complex and the electron donor molecule, k_4, was calculated according to [25], as follows. Under our experimental conditions, given the respective final concentrations of H_2O_2 and of substrate, k_4 could be expressed as follows:

$$k_4 = 1/a_0 e \times \Delta x/\Delta t$$

where $\Delta x/\Delta t$ is the rate of substrate disappearance over the measured time interval, a_0 is the initial concentration of donor and e is the concentration of enzyme (which was expressed as mg protein in the sample).

3. Results and discussion

Fig. 1 shows variations in the absolute number of various granulocytes in guinea pig bone marrow, at different times after the onset of immunostimulation. The number of basophil cells rose progressively with a 20-fold increase at day 12 after the first injection of sheep blood (from 0.5% at day 0 to 10.5% at day 12). Then, the basophils number dropped to roughly the initial control value at day 16. The number of eosinophil cells decreased up to day 4, then, increased progressively up to day 20. After that, the number of eosinophils dropped again to reach the initial value at day 30. Thus, a 3-fold increase in the number of eosinophil cells was seen at the peak on day 20 (from 0.6×10^6 at day 0 to 1.6×10^6 at day 20). For neutrophil cells, after an initial decrease until day 4, the number of cells rose progressively until day 12 and remained at a plateau until day 20. It then decreased progressively to reach approximately the initial value at day 30. Thus, the results showed an increase in the total number of bone marrow granulocytes under our stimulation conditions (Fig. 1, total granulocytes). This was similar to results obtained upon subcutaneous injection of granulocyte colony stimulating factor [9,20]. The decrease seen on day 4 in the population of eosinophils (from 0.6×10^6 at day 0 to 0.2×10^6 at day 4) and neutrophils (from 2.1×10^6 at day 0 to 1.7×10^6 at day 4) was due to their discharge in the blood circulation upon immunostimulation.

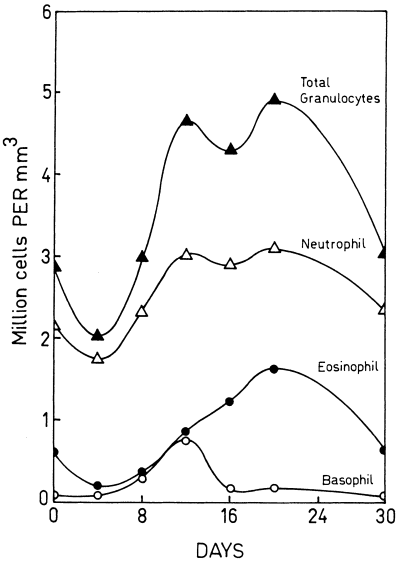

Fig. 1. Changes in granulocyte populations in bone marrow during and after immunostimulation.

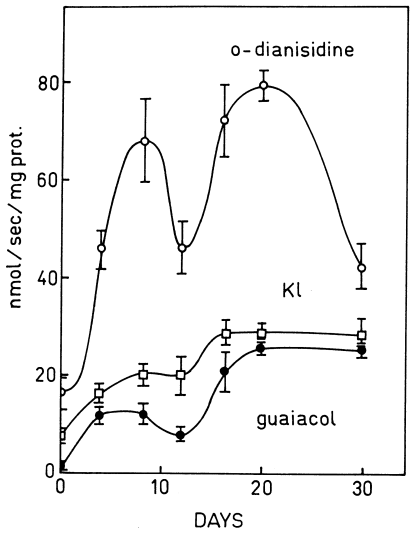

Fig. 2. Determination of peroxidase activity in bone marrow at various times during and after immunostimulation. Activity was expressed as nmol substrate (*o*-dianisidine, guaiacol or KI) peroxidized per s. Values were averages of two experiments (four controls and 2×6 animals). Measurements were done in triplicate.

Fig. 2 shows the activity of bone marrow peroxidases at different times after immunostimulation as measured by the oxidation of *o*-dianisidine, guaiacol and KI. All substrates showed roughly the same pattern of enzyme activity. With *o*-dianisidine which is more sensitive to oxidation by MPO [27], the peroxidase activity increased up to day 8, decreased slightly at day 12, increased again at days 16 and 20 and decreased at day 30. Thus, two peaks of peroxidase activity at days 8 and 20 were seen and the maximum activity was nearly 7-fold that of the control at day 0 (~80 nmol/s/mg proteins for day 20 versus 12 nmol/s/mg proteins for day 0). With guaiacol as substrate, however, the maximum increase in activity at day 20 was over 30-fold the value of the control at day 0 (~27 nmol/s/ mg proteins for day 20 versus 0.8 nmol/s/ mg proteins for day 0) while with KI as substrate, the maximum increase in activity at day 20 was about 4.5-fold the control value (~29 nmol/s/mg proteins for day 20 versus 6.5 nmol/s/mg proteins for day 0). Since KI is a specific substrate for EPO [26], the increase in the rate of its oxidation represents a true increase in the level of EPO by immunostimulation. On the other hand, since guaiacol was reported to be more sensitive than *o*-dianisidine to oxidation by EPO [27],

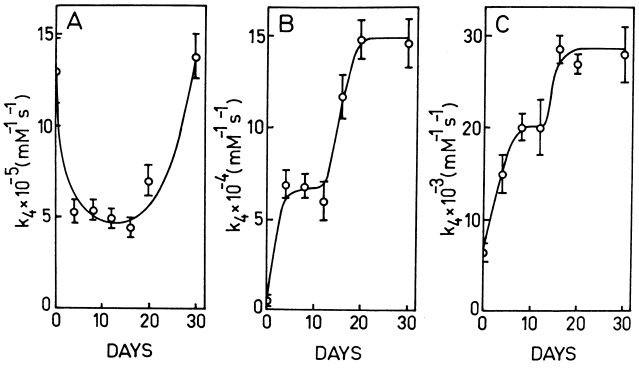

Fig. 3. Variation of k_4 values during and after immunostimulation using *o*-dianisidine (A), guaiacol (B) or KI (C) as substrate.

Table 1
Values of V_{MAX} and K_M obtained with *o*-dianisidine at various times after the onset of immunostimulation

	Control	Day 4	Day 8	Day 12	Day 16	Day 20	Day 30
V_{MAX}	27.0 ± 1.0	36.0 ± 1.5	28.5 ± 1.0	21.0 ± 2.4	24.6 ± 0.5	48.0 ± 1.5	45.0 ± 3.0
K_M	4.0 ± 0.5	19.0 ± 3.0	10.5 ± 1.5	8.5 ± 1.0	16.0 ± 2.0	8.5 ± 1.0	9.0 ± 1.0

V_{MAX} is expressed in nmol/s/mg protein, K_M is expressed in mM.

the differential increase in the oxidation rate of guaiacol indicated a preferential increase of EPO rather than MPO. In the presence of 0.05% CTAB or 0.05% Triton X-100, the activity of MPO and EPO was 30% higher than in the absence of detergent. However, in order to remain as close as possible to the in vivo situation, we did not base our kinetics studies on detergent-stimulated peroxidases.

Table 1 shows the values of V_{MAX} and K_M obtained with *o*-dianisidine. V_{MAX} was lowest at day 12 (21 nmol/s/mg protein) and highest at day 20 (48 nmol/s/mg protein). K_M was lowest for the control at day 0 (4 μM) and highest at day 4 (19 μM). But a consistent pattern was not found, neither for V_{MAX} nor for K_M. Similar results were found with KI and guaiacol. As it was pointed out [29], there is no true V_{MAX} for peroxidase systems because the value depends upon the choice of H_2O_2 concentration [29]. Thus, the values of V_{MAX} and K_M obtained were not interpretable in terms of changes in the amount or kinetic properties of peroxidases. In contrast, determination of k_4, as suggested in [29], might show a more accurate picture of the changes in peroxidases under immunostimulation. The reaction scheme was as follows:

$$E + H_2O_2 \xrightarrow{k_1} EO + H_2O$$

$$EO + \text{electron donor} \xrightarrow{k_4} E + O - \text{electron donor}$$

Fig. 3 shows changes in the value of k_4 under our experimental conditions when *o*-dianisidine, guaiacol and KI were respectively used as substrates. With *o*-dianisidine, k_4 was similar at days 0 (control) and 30, suggesting that the oxidation of *o*-dianisidine was by the same kind of isozyme. In contrast, under immunostimulation, another value of k_4 was observed for days 4–20, suggesting that another isozyme was synthesized during that period. With guaiacol, k_4 exhibited roughly similar values for days 4, 8 and 12 and higher but similar values for days 16, 20 and 30. With KI, the results obtained paralleled those obtained with guaiacol, k_4 exhibited roughly similar values for days 4, 8 and 12 and higher but similar values for days 16, 20 and 30. Since guaiacol and KI are more sensitive than *o*-dianisidine to oxidation by EPO [27], the k_4 under our experimental conditions suggested the existence of at least three EPO isozymes, one characterized by the k_4 value found for the control and the other two characterized by the k_4 values found for stimulated eosinophils. On the other hand, *o*-dianisidine is more sensitive for the determination of MPO and showed possibly two isozymes with two different k_4s, one for control and for day 30, and one for all others. The increases in peroxidase activity (Fig. 2) paralleled the increase in the populations of both eosinophils and neutrophils (Fig. 1). On the fourth day after the onset of stimulation, even though the populations of both types of granulocytes decreased, peroxidase activities increased. This might be due to the appearance of young granulocytes exhibiting a burst of peroxidase synthesis. Furthermore, it appeared that the newly synthesized peroxidases were isozymes compared with the unstimulated cells. From day 4 to 12, the isozymes continued to be synthesized. Interestingly, after the last subcutaneous injection of whole sheep blood cells on day 12, a new isozyme was synthesized with an increased activity (Fig. 2).

The sensitivities to either MPO or EPO of the substrates used are differential rather than exclusive, although KI is almost exclusively oxidized by EPO [26]. Thus, results reflected oxidation by a mixture of enzymes with differential activities. However, results obtained in the presence of inhibitors helped in the differentiation between MPO and EPO. Substrate oxidation was completely inhibited by 5 mM KCN or 100 μM azide under all experimental conditions. 200 μM aminotriazole inhibited KI oxidation by 90% while it inhibited guaiacol and *o*-dianisidine oxidation by 60 and 10%, respectively. It has been well-established that EPO is highly sensitive to aminotriazole while MPO is not [30]. Thus, oxidation of KI represented the true value of EPO activity while guaiacol was preferentially oxidized by EPO, assuming that the MPO and EPO substrate specificities were conserved in the isozymes. Characterization of isozymes of EPO and MPO from bone marrow necessitates large amounts of bone marrow cells and tedious procedures of purification, electrophoresis and ancillary methods. Thus, a prohibitive amount of time would be required to purify and study MPO and EPO at various times during immunostimulation (in this study, samples were studied at seven different times during and after immunostimulation). Furthermore, purification might also alter, e.g. degrade, the protein and produce inaccurate kinetic properties. However, a kinetic approach like that described in this work is a relatively simple procedure, reproducible, requiring small amounts of material and it gave results consistent with the presence of isozymes of peroxidase in plant [14,15] and isozymes of myeloperoxidase in human neutrophils [16].

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