Kinetics and Characteristics of Bovine Neutrophil Alkaline Phosphatase During Acute *Escherichia coli* Mastitis

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ABSTRACT

Alkaline phosphatase activity of isolated bovine blood neutrophils was investigated before and during experimentally induced *Escherichia coli* mastitis. Activities markedly increased 1 wk after infection in neutrophils of cows suffering from moderate or from severe disease. Elevated neutrophil alkaline phosphatase activity did not correlate with a specific stage of maturing postmitotic neutrophils appearing in circulation during mastitis. Neutrophil alkaline phosphatase from healthy cows before infection and from mastitic cows was characterized by means of thermostability, specific inhibitor patterns, slab gel electrophoresis, and kinetic parameter analysis. The leukocyte enzyme from healthy and mastitic cows displayed very similar characteristics, suggesting that the increase in activity during mastitis is most probably related to the enhanced expression of the normal alkaline phosphatase enzyme.

(Key words: neutrophil leukocytes, alkaline phosphatase, *Escherichia coli* mastitis)

Abbreviation key: G-CSF = granulocyte colony-stimulating factor, NAP = neutrophil alkaline phosphatase.

INTRODUCTION

Large variations in alkaline phosphatase activities have been demonstrated in leukocytes from man and several mammalia. For example, mature neutrophils of the dog, cat, and mouse are inherently deficient in alkaline phosphatase (13, 23), but considerable neutrophil alkaline phosphatase (NAP) activities have been shown in porcine, equine, and bovine circulating cells (27).

Although no physiological function of NAP is obvious, NAP activity fluctuates significantly in a variety of physiological and pathological conditions (11, 17, 32) and is not related to variations in serum alkaline phosphatase (28). Williams (35) suggested that fluctuations of NAP reflect a function of cell age. However, a relationship between NAP activity and neutrophil maturity is still unclear and controversial.

During the course of acute *Escherichia coli* mastitis in cattle, a massive migration of neutrophils from the blood toward the inflamed udder is regularly observed. At the same time, several types of neutrophil precursor cells frequently appear in the circulation in great numbers. Under controlled conditions, the outcome, course, and severity of an experimentally induced inflammation of the udder can be followed and related to the appearance and kinetics of immature neutrophils in the blood (22).

This study was undertaken to evaluate the relationship between alterations in NAP activity and maturation stage and time of emergence of neutrophils from bone marrow. In addition, several characteristics of NAP activity from neutrophils before infection and from cells with elevated NAP during mastitis were compared to determine whether the enzyme from mastitic cows represents a qualitative change in activity or a different form of NAP.

MATERIALS AND METHODS

Animals and Induction of *Escherichia coli* Mastitis

Eight healthy cows of the East Flemish Red Pied breed, in their second lactation and yield-
After centrifugation at 1000 g for 20 min at room temperature, the plasma layer anduffy coats were removed. The packed erythrocytes containing most of the neutrophils were lysed in deionized water, and isotonicity was restored after 1 min. After centrifugation, the remaining cell suspension contained mostly neutrophils, nonlysed erythrocytes, and variable numbers of eosinophils. The cell suspension was kept on ice, and all further steps were performed at 0 to 4°C. The cell preparation was then carefully layered on top of a preformed continuous Percoll® (Pharmacia Biosystems, Uppsala, Sweden) gradient (average density 1.103) and centrifuged at 800 × g for 20 min. This procedure separated the neutrophils from the other cells. Only samples containing more than 95% neutrophils were used for experiments. The viability of the purified neutrophils exceeded 98% measured by exclusion of 0.2% trypan blue in phosphate-buffered saline. Neutrophil differential counts were obtained by means of May-Grünwald-Giemsa-stained (Merck, Darmstadt, Germany) duplicate smears on glass slides. On each slide, 200 cells were differentiated. Freshly purified neutrophils were immediately assayed for NAP activity or stored in liquid nitrogen for maximally 2 wk. The latter procedure did not influence either NAP activity or electrophoretic pattern of the enzyme. Before analysis, fresh or thawed cells were sonicated for 1 min at 100 W with an ultrasonic cell disruptor (Measuring & Scientific Equipment Ltd., London, England). Neutrophils were kept on ice during sonication.

Assay for Alkaline Phosphatase

Assays for NAP were performed in duplicate at 37°C on sonicates of purified neutrophils by the method of DeChatelet and Cooper (7), measuring the conversion of p-nitrophenylphosphate to p-nitrophenol. Five millimoles per liter of p-nitrophenylphosphate in 100 mmol/L of 2-amino-2-methyl-1-propanolhydrochloride buffer, pH 10.0, containing 1 mmol/L of Mg²⁺ and .1% Triton X-100 (Sigma Chemical Co., St. Louis, MO), were the standard reaction mixture in all cases. The reaction was initiated by the addition of 10⁵ sonicated cells. After 10 min of incubation at 37°C, the reaction was stopped by the addition of .1 M NaOH. The p-nitrophenol released during the course of the incubation was measured from its absorbance at 410 nm in a Gilford 240 spectrophotometer (Gilford Instrumentation, Cleveland, OH).
Alkaline phosphatase activity was expressed in milliunits: 1 mU represents the amount of enzyme in total sonicates necessary to split 1 nmol of substrate in 1 min.

**Enzyme Solubilization and Electrophoresis**

Extraction of NAP from a neutrophil suspension in phosphate-buffered saline was carried out in the presence of 1% (vol/vol) Triton X-100. The cells were sonicated (three exposures of 30 s and 100 W) and kept on ice during sonication. The homogenate was then spun at 25,000 x g at 2°C for 1 h. The clear supernatant was collected; the ghost pellet was resuspended in 1% Triton X-100 and subjected to a new cycle of sonication. Extraction yield of NAP activity was calculated; it ranged from 80 to 90%. Cell extracts were kept frozen at -20°C before biochemical determination.

Slab gel electrophoresis was performed in 7.5% acrylamide containing .05% Triton X-100 essentially as described by Wray and Harris (36). Electrophoresis was run at a constant current of 40 mA at 10°C for 4 h. Activity of NAP was located after incubation and staining with .1% α-naphthylphosphate as substrate and .1% fast blue salt as coupling dye in 2-amino-2-methyl propanol buffer pH 10.0 containing 1 mM Mg²⁺.

**Inhibition and Heat Inactivation Studies**

Inhibition studies of NAP were performed on cellular sonicates and neutrophil extracts with inhibitor concentrations between 0 and 20 mM for L-phenylalanine, L-histidine, and imidazol and ranging from 0 to .1 mM for levamisol. A mixture of each inhibitor and enzyme was incubated for 15 min at 37°C prior to assay. Triplicate inhibition curves were determined for each enzyme sample.

Thermolability of NAP was calculated from the decrease in activity after incubating neutrophil sonicates at 45 and 56°C for 5, 10, and 20 min using unheated samples as reference controls.

**Chemicals**

Chemicals of the highest purity available were purchased from Merck; biochemicals were from Sigma Chemical Co. Levamisol was a gift from Janssen Pharmaceutica (Beerse, Belgium).

**Statistical Analysis**

Means, standard deviations, standard errors, correlations, and Student's t test were computed according to Snedecor and Cochran (25). Statistical significance was determined at P < .05.

**RESULTS**

Cows were classified in two groups according to the severity of the disease developed after intramammary inoculation of E. coli (12). Cows demonstrating a decrease in milk and α-lactalbumin production of less than 45% of prechallenge levels during 2 d following infection were called moderate responders; cows showing a higher milk loss were classified as severe responders. Severe responders showed the clinical symptoms of severe mastitis: a swollen and painful udder, a strongly diminished or lost secretory function of the infected quarter, discolored milk containing fibrin clots, fever, rumen stasis, and diarrhea.

At the onset of mastitis, a reduction in blood neutrophil numbers and a concomitant shift to the left occurred in both groups 1 d after inoculation. In moderate responders, this initial neutropenia was followed by a return to basal levels after 2 to 3 d. However, 1 wk after infection severe responders developed a neutrophilia during which metamyelocytes and myelocytes appeared frequently in the blood and even became predominant cell types (Figure 1).

The NAP activity of isolated blood neutrophils was followed during the development of both types of mastitis. No major differences in pattern were observed between NAP activity of cows suffering from moderate or severe mastitis (Figure 2). One or 2 d after intramammary inoculation of E. coli, NAP activity increased in all cases. The NAP reached maximal activity after 5 to 6 d. Maximal activity exceeded prechallenge activity by about three times in moderate responders (P < .05) and five times in severe responders (P < .01). Total NAP activity was significantly different between moderate and severe responders during

**Figure 1.** Blood neutrophil number (10³/mm³) and differential count during an experimentally induced *Escherichia coli* mastitis. Infection started at d 0 as indicated by arrow. Cows were classified in moderate (n = 3) and severe (n = 3) mastitis groups. Counts of segmented and band cells differed from metamyelocytes and from myelocytes (P < .05).
Figure 2. Alkaline phosphatase activity of blood neutrophils isolated from four cows with moderate and four cows with severe *Escherichia coli* mastitis. Infection started at d 0 as indicated by arrow. Assays on each neutrophil sample were done in triplicate. Values are means with standard deviations. Neutrophil alkaline phosphatase activity is expressed as mU/1 x 10⁶ neutrophils.
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the 2nd wk after inoculation ($P < .05$). Basal activity was regained after about 2 wk in moderate responders but lasted for several weeks in severely affected cows. Expression of NAP activity was maximal in neutrophil populations composed of both mature and immature cell types (Figures 1 and 2). Maximal NAP during mastitis did not correlate either with the total neutrophil number or with a specific type of immature cells ($r = .18$, $n = 30$).

To detect functional differences between NAP activity of circulating neutrophils before (basal NAP activity) and after (enhanced NAP activity) the induction of mastitis, the relative inhibition of both enzymatic reactions by a series of known inhibitors was compared. As shown in Figure 3, no significant differences ($P < .01$) could be detected in the inhibiting activity of different amounts of levamisole, imidazol, L-phenylalanine, or L-histidine. Furthermore, thermal inactivation studies revealed that, in both cases, the enzymes were stable at $45^\circ C$ for 20 min and lost 50% of their initial activity after incubation at $56^\circ C$ for 10 min (Figure 4).

Next, effects of pH and kinetic parameters of NAP from healthy and mastitic cows were compared. For both enzymes, activity was maximal at pH 10.0 with either $p$-nitrophenylphosphate or phenylphosphate as a substrate. The Michaelis constant and maximal velocity were estimated at pH 10.0 by Lineweaver-Burk plots. Although values of the apparent Michaelis constant of both NAP activities for $p$-nitrophenylphosphate were not significantly different ($P > .1$), the maximal velocity of the enzyme from mastitic cows was approximately three times higher than preinfection value (Table 1). Electrophoresis on polyacrylamide gels revealed only one similar isoenzyme for alkaline phosphatase from neutrophils isolated before and after induction of...
mastitis. The electrophoretic mobility of NAP differed from that of the bovine intestinal and liver isoenzymes.

DISCUSSION

Little is known about the variation of NAP activity in animals. However, alterations of NAP activity have been shown in human patients suffering from a variety of disorders. Increased NAP activity has been described in polycythemia vera, leukocytosis, gout, leukemoid reactions, trauma (14, 17), Down's syndrome (2), and acquired immune deficiency syndrome (11). Concentrations of NAP are also elevated during pregnancy (9), various stress conditions, and during some infections (33, 34). No relationship of NAP activity to serum alkaline phosphatase has been demonstrated, indicating that the two are completely independent parameters (28). The results of the present study demonstrate that an acute experimentally induced E. coli mastitis in cows evokes a significant increase in NAP activity. Increased NAP activity in individual bovine neutrophils during the disease might explain the earlier noticed enhancement of alkaline phosphatase activity in milk with elevated cell counts from mastitic cows (3).

Acute inflammation of the udder results in a mobilization of tremendous numbers of mature neutrophils from the circulating and marginal pools and often causes release of immature polymorphonuclear leukocytes from bone marrow into the blood. Because cows demonstrate great variability in their response pattern during experimentally induced mastitis, a broad variety of different maturing stages of neutrophils could be recovered from the blood during the course of the disease. Two days after inoculation of bacteria into the mammary gland, NAP activity was not considerably altered. During those days, young immature neutrophils were most prominent in the blood. Between 5 and 7 d after infection, NAP activity increased markedly and reached maximal activity. Although the magnitude of this increase correlates slightly with the severity of the disease, no relationship between NAP activity and kinetics or composition of the neutrophil population could be demonstrated. Also, the observed increase of NAP did not correlate with the formerly noticed enhanced stimulation of the respiratory burst during recovery from mastitis (12). This supports the idea that NAP is not required for the normal bactericidal activity of neutrophils (6), a mechanism at least partially dependent on generation of reactive oxygen species.

Conflicting data on cell maturity and NAP activity have been published. Some authors (4, 8, 30) observed that NAP activity is highest in functionally and chronologically older neutrophils. Others (26, 35) stated that NAP is highest in the youngest cells, so elevation of

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$^1$ The Michaelis constant ($K_{\text{m}}$) is expressed in millimolar $p$-nitrophenol and maximum velocity ($V_{\text{max}}$) as nanomoles of $p$-nitrophenol per min $\times 1.10^6$ neutrophils. Preinfection values are from neutrophils isolated from cows developing a severe mastitis 5 d after inoculation of Escherichia coli into the udder. Data are from triplicate experiments on four cows.
NAP in some diseases might be the result of a hastened entry of neutrophils into circulation. The present results indicate that NAP activity is not correlated with a specific stage of the maturating postmitotic neutrophil, and, thus, it is not a real measure for maturity. Data suggest that the enhanced NAP activity noticed 1 wk after onset of mastitis was the result of a stimulating effect of inflammatory mediators on the expression of enzyme activity during cellular maturation in bone marrow. Sato et al. (21) have shown that a factor present in cystic fluid of human carcinoma cells has the capacity to induce neutrophil alkaline phosphatase in postmitotic granulocytes. Recently, this factor, the NAP-inducing factor, was shown to be identical to granulocyte colony-stimulating factor (G-CSF) (20). A specific receptor for human G-CSF on human neutrophils has been demonstrated (31). The G-CSF gene can be turned on in a number of cell types in the presence of activating signals. The gene product is made by cells of the monocyte-macrophage lineage exposed to endotoxins or by T lymphocytes in the presence of immune stimuli. Neutrophils respond in vitro to lipopolysaccharides by up-regulation of alkaline phosphatase activity on the cell surface (1). Interestingly, NAP-inducing ability of G-CSF was suppressed by granulocyte-macrophage colony-stimulating factor in vitro (29). We propose that increased NAP activity following inflammation of the udder may reflect an enhanced proliferation and differentiation of bone marrow granulocytes mediated by G-CSF. In 1973, Keleman (15) claimed that a high turnover rate of the marrow store furnished more alkaline phosphatase positive neutrophils, whereas release of already stored cells delivered more negative cells.

The nature of NAP is still a matter of discussion. In one study (16), human NAP has been suggested to be distinct from other organ-specific alkaline phosphatases; others (10, 24) have supplied evidence that NAP is the product of the same structural gene that codes for the liver, bone, and kidney group of alkaline phosphatases. It has also been unclear whether the enzyme in disease states with increased NAP activity represents the alkaline phosphatase present in normal neutrophils or whether it might represent activation of another alkaline phosphatase (19). In accordance with the results of Miller et al. (16), the data obtained in this study by use of a series of inhibitors, by heat inactivation, by kinetic parameter analysis, and by electrophoresis provide evidence that elevated NAP activity during inflammation of the mammary gland is not related to expression of a different isoenzyme or to qualitative changes in the enzyme molecule. Therefore, enhanced activity is most probably due to increased expression of the normal NAP enzyme under direct or indirect influence of inflammatory mediators.

ACKNOWLEDGMENTS

This study was supported by a grant from the Belgian Nationaal Fonds voor Wetenschappelijk Onderzoek (Brussels, Belgium, Grant Number 3.0091.89). We thank Daniele Monbaliu for expert technical assistance.

REFERENCES

12 Heyneman, R., C. Burvenich, and R. Vercauteren. 1990. Interaction between the respiratory burst activ-
ity of neutrophil leukocytes and experimentally induced *Escherichia coli* mastitis in cows. J. Dairy Sci. 73:985.