Bb Fragment of Bovine Complement Factor B: Stimulation of the Oxidative Burst in Bovine Monocytes

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ABSTRACT

In a previous study, we reported that fragment Bb of bovine complement factor B activated bovine monocytes, as demonstrated by the uptake of ^{3}H-deoxyglucose. In the present study, the effects of Bb on the production of superoxide anion and hydrogen peroxide by bovine monocytes was investigated. The production of superoxide was measured by the superoxide dismutase inhibitable reduction of cytochrome c. The change in absorbancy was determined by a spectrophotometer at a wavelength of 550 nm. Hydrogen peroxide production was measured by the horse-radish peroxidase-dependent oxidation of phenol red. The resulting color change was measured by a spectrophotometer at a wavelength of 620 nm. Fragment Bb (20 μg/mL) induced the generation of 0.96 $\pm$ 0.2 (mean $\pm$ SEM) nanomoles of superoxide/2.5 x 10^5 monocytes at 5 min. The production of superoxide peaked at 15 min (1.24 $\pm$ 0.3 nanomoles). The production of hydrogen peroxide was also rapid: 0.195 $\pm$ 0.05 nanomoles/ 2.5 x 10^5 monocytes at 5 min with a peak at 15 min (0.250 $\pm$ 0.04 nanomoles). These observations show that fragment Bb, which has serine protease activity, induces bovine monocytes to generate reactive oxygen intermediates such as superoxide and hydrogen peroxide.

RÉSUMÉ

Une étude antérieure a rapporté que le fragment Bb du facteur B du complément active les monocytes bovins tel que démontré par l'augmentation de la captation du 3H-désoxyglucose. Le but de cette étude était d'évaluer la production d'anions superoxydes et de peroxyde d'hydrogène par les monocytes en présence du fragment Bb. La production de superoxyde se fait lorsqu'il y a réduction du cytochrome c par la superoxyde dismutase mesurée par spectrophotométrie (550 nm). La production du peroxyde d'hydrogène a été mesurée par oxydation du phénol rouge relié à l'activité peroxidase du raifort mesurée par spectrophotométrie (620 nm). Le fragment Bb (20 μg/ml) a induit la production de 0.96 $\pm$ 0.20 nanomole de superoxyde à l'aide de 2.5 $\times$ 10^5 monocytes après cinq minutes atteignant sa production maximal après 15 minutes (1.24 $\pm$ 0.3 nanomole). Après cinq minutes, la production de peroxyde d'hydrogène était de 0.195 $\pm$ 0.050 nanomole atteignant un pic de production après 15 minutes à 0.250 $\pm$ 0.040 nanomole à l'aide de 2.5 $\times$ 10^5 monocytes. Ces résultats montrent que le fragment Bb, lequel possède une activité sérine protéase, induit les monocytes à produire du superoxyde et du peroxyde d'hydrogène. ***(Traduit par Dr Pascal Dubreuil).***

INTRODUCTION

The role of the mononuclear phagocyte in host defence is unparalleled in versatility. It includes phagocytic, microbicidal, tumoricidal, secretory and antigen presenting activity (1, 2). The products known to be secreted include the reactive metabolites of oxygen (3). This unique oxidative metabolic pathway, called the respiratory burst, consists of a rapid increase in the consumption of oxygen, triggered when cells are phagocytosing or activated by soluble agents. In the process, molecular oxygen is converted to superoxide anion. As the superoxide is shortlived, it undergoes a rapid spontaneous dismutation to yield hydrogen peroxide and hydroxyl radicals. The enzyme and the electron system responsible for this have been identified and have been implicated in microbicidal activity (4, 5). The enhanced oxidative metabolism has been shown to be an expression of macrophage activation (6).

Organisms of great phylogenetic diversity including marine algae (7), bombardier beetle (8), and the vertebrate phagocyte use the oxidative burst as a defence mechanism when triggered by microorganisms, immune complexes or other soluble stimuli (9, 10).

The generation of superoxide and hydrogen peroxide by bovine poly-

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morphonuclear leukocytes, monocytes and alveolar macrophages has been studied *in vitro* (11, 12).

In a recent report it was shown that fragment Bb of bovine complement factor B could activate bovine monocytes as demonstrated by the enhanced uptake of ^{3}H-deoxyglucose (13). This report is a continuation of the studies being undertaken to investigate the functional role of bovine factor B. Factor B is a protein of the alternative complement pathway (ACP) (14). Fragment Bb is a cleavage product of factor B and, in association with complement component C3b, forms the C3 convertase ($\overline{C3b.Bb}$) (14). It retains its serine protease activity after decay dissociation from C3b (15).

The present study reports the production of superoxide and hydrogen peroxide by bovine monocytes following stimulation by the Bb fragment. The assays for the detection of superoxide and hydrogen peroxide were semi-automated microassays. The measurement of superoxide was based on the superoxide dismutase-inhibitable ferricytochrome c reduction measured at 550 nm. The quantitation of hydrogen peroxide was based on the horse-radish peroxide (HRPO)-mediated oxidation of phenol red by hydrogen peroxide and assayed at 620 nm.

MATERIALS AND METHODS

ANIMALS

A group of ten Hereford-Angus heifers were maintained at the University of Saskatchewan Goodale farm. These animals were used as a source of monocytes. The guidelines of the "Guide to the Care and Use of Experimental Animals" of the Canadian Council on Animal Care were followed.

FRAGMENT Bb OF COMPLEMENT FACTOR B

Fragment Bb of bovine complement factor B was affinity-purified and tested for endotoxin as described previously (13). Endotoxin (0.25 endotoxin units (EU/mL) was detected to be present in the purified preparation of Bb, as determined by the limulus amebocyte lysate assay (Whittikar M.A. Bioproducts, Walkersville, Maryland). This concentration of endotoxin was used in all assays as a control.

ENDOTOXIN

Escherichia coli O111.B4 endotoxin was purchased from Whittikar M.A. Bioproducts, Walkersville, Maryland. It contained 24 EU/mL.

MONOCYTES

Monocytes were obtained by the centrifugation of bovine blood on Ficoll-hypaque (Pharmacia (Canada), Quebec) as described previously (13). The purified monocytes were suspended in Hanks' balanced salt solution (HBSS) without phenol red (Gibco, Burlington, Ontario). Monocytes of $> 90\%$ purity and $> 95\%$ viability were used for the experiments.

CHEMICALS AND MEDIA

Superoxide dismutase (SOD) (3000 units/mg, from bovine erythrocytes), cytochrome c (Cyto C) type VI, from horse heart, phenol red (PR), peroxidase from horse-radish (HPRO) type II, 210 purpurogallin units/mg, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co. (St. Louis, Missouri). Hydrogen peroxide (50%) was obtained from Fisher Scientific, Winnipeg, Manitoba, and Linbro 96-well flat-bottom microtiter plates with lids from Flow Laboratories, Mississauga, Ontario. Phenol red solution (PRS) contained 140 mM NaCl, 10 mM potassium phosphate buffer, pH 7.0, 5.5 mM dextrose, 0.2 g/L PR, and 19 U/mL of HRPO as described (16). Phenol red and HRPO were added just prior to commencing the experiments.

APPARATUS

An automated spectrophotometer (Multiskan Type 310 C, Flow Laboratories, Mississauga, Ontario), was used to record the absorbance values. It is an eight channel photometer which reads absorbance vertically through individual wells of a 96-well microtiter plate. The interference filters used were 550 nm for the superoxide assay and 620 nm for the hydrogen peroxide assay.

SUPEROXIDE ASSAY

The method of Pick and Mizel (16) was followed after slight modification. Briefly, bovine monocytes (2.5×10^5/well) were plated in 50 μL volumes in HBSS. After 30 min incubation at 38°C, 50 μL/well of a 100 μM solution of Cyto C in HBSS without phenol red were added to the adherent monocytes and further incubated at 38°C in a humidified chamber. The total volume/well was 100 μL. For reproducibility, the stimulants were dissolved in the Cyto C solution prior to addition to the monocytes. At various time intervals, the microtiter plate was read at 550 nm and the amount of superoxide generated per well was calculated using a correction factor, as described (16), expressed by the formula:

Nanomoles of superoxide/well =
absorbance at 550 nm x 100/6.3

HYDROGEN PEROXIDE ASSAY

For the measurement of hydrogen peroxide production the method of Pick and Mizel (16), after a slight modification, was used. It is based on the HRPO-dependent conversion of PR by hydrogen peroxide into a compound with increased absorbancy at 620 nm. Monocytes (2.5×10^5/well) in 50 μL volumes of PRS without PR and HRPO, pH 7.0 were added to each well of a microtiter plate. After a 30 min incubation at 38°C, PRS (50 μL) containing the stimulants was added to the wells according to the design of the experiment. At the required time, 10 μL of 1N NaOH were added to bring the pH to high alkalinity and induce cell death. The plates were then read at 620 nm. For the expression of the results in nanomoles of hydrogen peroxide produced/well, a standard curve for hydrogen peroxide was established. The hydrogen peroxide generated by 2.5×10^5 monocytes was then extrapolated from the standard curve.

HYDROGEN PEROXIDE STANDARD

Hydrogen peroxide (50%) was diluted in distilled water to give a stock concentration of 10 mM. For the determination of the standard curve, appropriate dilutions of the stock were made to obtain a final concentration of hydrogen peroxide ranging from 0.5 μM to 10 μM corresponding to 0.5 to 10 nanomoles/mL.

BLANKING PROCEDURE

The instrument is designed such that sample blanks can be positioned in the first vertical row of the microtiter plate. The instrument then reads the absorbance of each horizontal row against the selected blank (well 1) in the same row.

For the superoxide assay, blanking was performed by placing in the first vertical row the following: monocytes (2.5×10^5/well), Cyto C and SOD, with no stimulant.

For the hydrogen peroxide assay, blanking was performed by placing in the first vertical row: monocytes (2.5×10^5/well) and PRS, made alkaline by addition of 10 μL in 1N NaOH.

For the standard curve for hydrogen peroxide, the first vertical row contained PRS, no exogenous hydrogen peroxide and was made alkaline by adding 10 μL of 1N NaOH.

STATISTICS

Data were analyzed using one-way analysis of variance. Each experiment was done using eight replicate wells. Values represent the mean $\pm$ SEM of four experiments.

RESULTS

SUPEROXIDE ASSAY

It was found that bovine monocytes alone (control) did not produce detectable superoxide. Superoxide dismutase, when added with PMA, totally inhibited the generation of superoxide by monocytes (data not shown). The PMA (0.15 μg/mL) was able to generate 1.8 ± 0.3 nanomoles superoxide/2.5×10^5 monocytes at 60 min. The PMA was used as a positive control as it is a potent stimulator of the oxidative burst (2) (Fig. 1). Comparatively, fragment Bb (20 μg/mL) at 60 min could stimulate the generation of 0.64 nanomoles of superoxide. In contrast, the generation of superoxide elicited by fragment Bb was very rapid at 5 min (0.96 ± 0.2 nanomoles) and increased at 15 min (1.24 ± 0.3 nanomoles). At 30 min, Bb-stimulated superoxide production decreased and further dropped at 60 min (Fig. 1). Endotoxin (0.25 EU/mL) did not cause any production of superoxide.

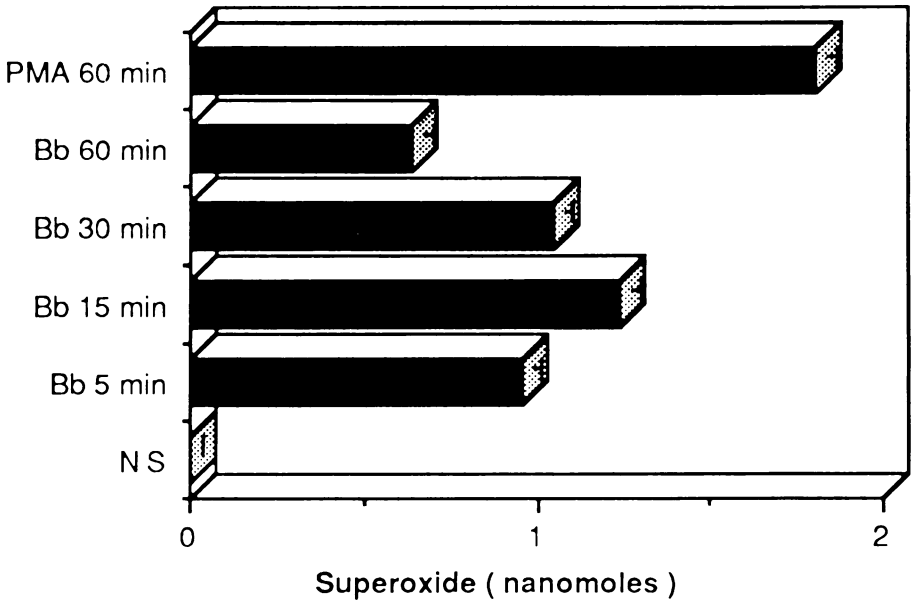

Fig. 1. Production of superoxide by bovine monocytes incubated with bovine complement fragment Bb. Monocytes (2.5×10^5/well) were plated in 96-well tissue culture plates. Bb (20 μg/mL) and phorbol 12-myristate 13 acetate (PMA 0.15 μg/mL) were tested in eight wells/experiment. Results represent the mean $\pm$ SEM of four experiments. Blank wells contained monocytes, cytochrome c solution without stimulants, but in the presence of superoxide dismutase (SOD). Values are significantly different from nonstimulated cells (NS).

STANDARD CURVE FOR HYDROGEN PEROXIDE

A standard curve for hydrogen peroxide was established using concentrations of known molarity of hydrogen peroxide. There was a positive correlation between the concentration of hydrogen peroxide and the absorbance ($r = 0.99$) (Fig.2).

PRODUCTION OF HYDROGEN PEROXIDE

Bovine monocytes alone in PRS did not produce detectable hydrogen peroxide. Endotoxin (0.25 EU/mL) also did not stimulate production of hydrogen peroxide (data not shown). The PMA (0.15 μg/mL) at 60 min generated 0.415 ± 0.04 nanomoles of hydrogen peroxide/2.5×10^5 monocytes (Fig. 3). Comparatively Bb (20 μg/mL) did not stimulate a detectable level at 60 min (data not shown). In contrast, there was rapid production of hydrogen peroxide by bovine monocytes at 5 min (0.195 ± 0.05 nanomoles) and this was greatest at 15 min (0.250 ± 0.4 nanomoles) on stimulation by Bb. At 30 min there was no detectable production of hydrogen peroxide on stimulation by Bb.

DISCUSSION

Superoxide dismutase-inhibitable reduction of cytochrome c is widely used as a reliable quantitative measure of superoxide anion synthesis by phagocytes triggered into oxidative metabolism. The production of toxic oxygen metabolites is one of the principal functional responses by which phagocytes kill invading organisms and tumor cells. The interaction of a number of soluble factors such as complement component C5a, lectins, chemotactic peptides, phorbol esters, calcium ionophores, leukotrienes and platelet activating factors result in the oxidative burst (2).

Subsequent to our finding that bovine fragment Bb had the ability to activate bovine monocytes (13), we expanded our studies on the functional role of fragment Bb. One of our studies has shown that bovine fragment Bb of complement factor B had the ability to stimulate bovine monocytes in intracellular killing of *Staphylococcus aureus* (17). Then the question arose: what biochemical events might be triggered by fragment Bb to enhance the microbicidal activity of bovine monocytes? As one of the possible answers, it could be

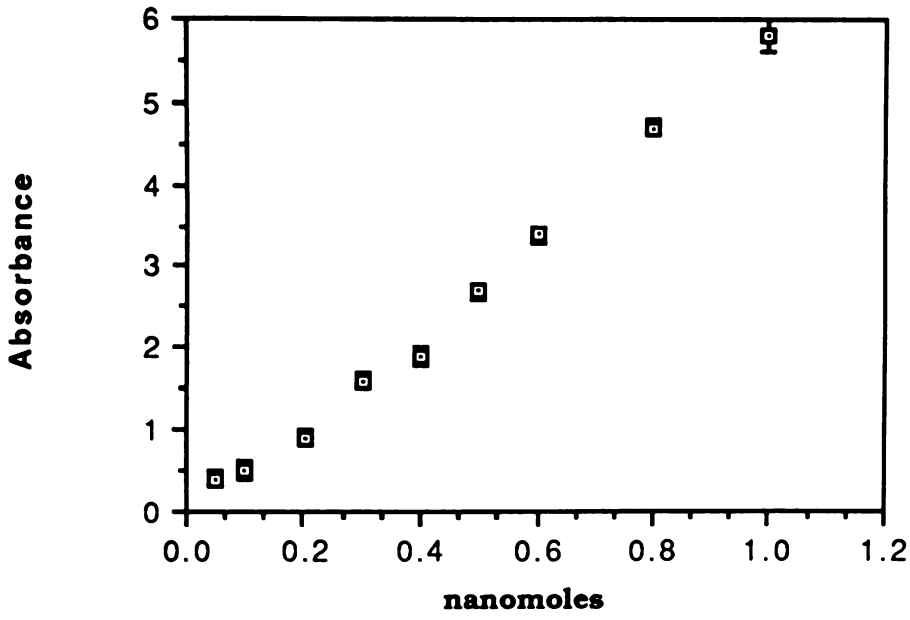

Fig. 2. Standard curve for hydrogen peroxide (r = 0.99). To 1 mL aliquots of phenol red solution (PRS), 10 μL of hydrogen peroxide were added so as to obtain a final concentration of hydrogen peroxide ranging from 0.5 to 10 nanomoles/mL. To plot the standard curve, 100 μL of the PRS-hydrogen peroxide mixture were dispensed into one vertical row (eight replicate wells) of a 96-well microtiter plate for each concentration. After 5 min, 10 μL of 1 N NaOH/well were dispensed to make the mixtures alkaline and the absorbance was read at 620 nm in the automated spectrophotometer.

postulated that monocytes generated reactive oxygen intermediates such as superoxide and hydrogen peroxide. A major antimicrobial mechanism of macrophages is the production and release of intracellular reactive oxygen intermediates. Destruction of various pathogens, including *Toxoplasma, Trypanosoma cruzi, Mycobacterium, Leishmania* and *Candida*, have been correlated with the ability of stimulated macrophages to secrete hydrogen peroxide, albeit the exact mechanism of microbial killing is not clear (10).

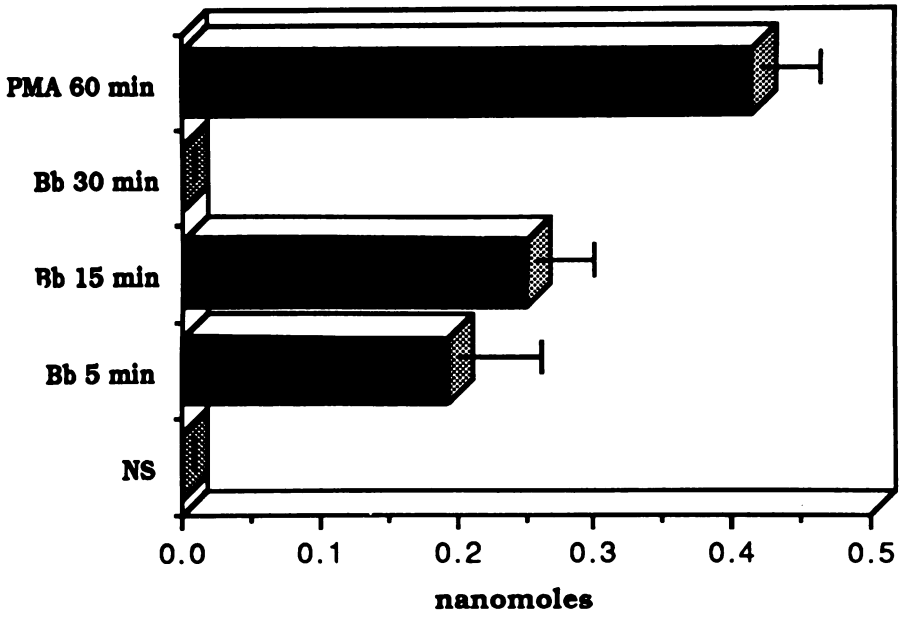

Fig. 3. Production of hydrogen peroxide by bovine monocytes incubated with bovine complement fragment Bb. Monocytes (2.5 x 10^5/well) were plated in 96-well tissue culture plate. Bb (20 μg/mL) and phorbol 12-myristate 13-acetate (PMA, 0.15 μg/mL) were tested in eight wells/experiment. Results represent the mean ± SEM of four experiments and were extrapolated from the hydrogen peroxide standard curve (Fig. 2.) Blank wells contained monocytes, phenol red solution without stimulants plus NaOH at time 0 min. Values are significantly different from nonstimulated cells (NS).

Early studies on human monocytes have shown that complement and immunoglobulins stimulated the production of superoxide independent of phagocytosis (18). There is a report that human complement protease Bb induced an increased release of glucosaminidase, hydrogen peroxide and superoxide from human monocytes (19). Recently it has been shown that there is a complement mediated enhancement of IgA-induced hydrogen peroxide release by human polymorphonuclear leukocytes, and the activation of the ACP was essential for this cooperative effect of complement and IgA (20). Fragment Bb has serine protease activity (15), and serine proteases have been implicated in superoxide production by human neutrophils, monocytes and basophils (21, 22).

The present study has established that fragment Bb (20 μg/mL) stimulated bovine monocytes to generate superoxide and hydrogen peroxide. The generation of superoxide was quite rapid and short. It was detected at 5 min and was greatest at 15 min and then decreased to low levels at 30 and 60 min. Nonstimulated monocytes did not produce detectable superoxide or hydrogen peroxide. Similarly, bovine monocytes generated hydrogen peroxide on stimulation by Bb (20 μg/mL). Here too, the production was rapid, being detected at 5 min and was greatest at 15 min, after which it was undetectable at 30 and 60 min.

The rapid generation of both superoxide and hydrogen peroxide by bovine monocytes, on stimulation by fragment Bb during an infection, appears physiologically plausible. The production of these reactive oxygen species would suggest that they could be directly microbicidal to invading organisms. The indirect detrimental effect of the respiratory burst has been associated with a rapid rise in vacuolar pH, which is postulated to facilitate

killing and bacteriolysis by granule proteins (23).

In the same context, acute phase proteins like C-reactive protein have been shown to activate peritoneal macrophages of guinea pigs and cause the production of superoxide (24). The C-reactive protein has also been shown to inhibit migration of guinea pig macrophages (25). Human factor B has also been classified as an acute phase protein (26, 31) and has been shown to cause spread of macrophages and to inhibit their migration as determined by the capillary tube assay for macrophage migration inhibition (27-29). The interaction of factor B/Bb with monocytes/macrophages could involve a receptor for factor B/Bb on monocytes. A recent study has shown that human monocytes do express binding molecules for factor B/Bb which are pronase sensitive (30).

In summary, affinity-purified fragment Bb of bovine factor B, a protein of the ACP, has been shown to stimulate the production of superoxide and hydrogen peroxide from bovine monocytes. It could be postulated then, that factor B/Bb along with its critical role in the formation of the C3 convertase of the alternative complement pathway, may play a functional role at the site of tissue damage and inflammation by activating monocytes/macrophages, and participating in the microbicidal activity of monocytes.

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