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FULL PAPER *Immunology*

Costimulatory Effects of Complement Receptor Type 3 and Fc Receptor for IgG (FcγR) on Superoxide Production and Signal Transduction in Bovine Neutrophils

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ABSTRACT. The present study evaluated the costimulatory effects of complement receptor type 3 (CR3) and Fc receptor for IgG (FcγR) on superoxide production and intracellular signal transduction in bovine neutrophils. Stimulation with opsonized zymosan (OPZ) and heat-aggregated bovine IgG (Agg-IgG) resulted in much greater superoxide production and chemiluminescent (CL) responses in normal neutrophils compared with those stimulated with OPZ or Agg-IgG only. Superoxide production and CL response were closely associated with the stimulant-induced rise of the intracellular calcium ($[Ca^{2+}]_i$) concentration, amount of tyrosine phosphorylated 100 kDa protein, and activation of p38 mitogen-activated protein kinase (p38 MAPK). No costimulatory effect was found for these receptors on superoxide production in CR3-deficient neutrophils. Costimulation of CR3 and FcγR on bovine neutrophils leads to enhancement of superoxide production and their signaling pathways and appears to be associated with enhancement of neutrophil functions.
KEY WORDS: bovine neutrophils, costimulation, CR3, FcγR, signaling pathway.

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Neutrophils are critically important for host defence against bacterial infection. Invading bacteria are opsonized by serum proteins such as complement fragments and Ig [1]. Opsonization by complement C3 fragments or IgG recognized by the specific surface receptors of phagocytic cells augments phagocytosis of bacteria [1]. Down-modulation of complement receptor type 3 (CR3, CD11b/CD18) and Fc receptor for IgG (FcγR) is accompanied by a decrease in leukocyte functions as expressed by decreased release of oxygen metabolites, reduced FcγR-mediated phagocytosis, and impaired killing of bacteria [14]. FcγR and CR3 act cooperatively to stimulate intracellular signaling pathways to not only initiate the actin polymerization necessary for phagocytosis but also to activate NADPH oxidase [4, 19]. Cross-linking of FcγR also stimulates tyrosine phosphorylation of phospholipase Cγ, which leads to an increase in the concentration of diacylglycerol, an activator of protein kinase C (PKC) [3, 11]. Although the importance of CR3 for leukocytes has been well characterized in CR3-deficiency in Holstein cattle with leukocyte adhesion deficiency [7], the synergistic function of CR3 and FcγR in relation to intracellular signal transduction of bovine neutrophils and their interactions have not been well elucidated. Costimulation of these receptors appears to play a key role in activation of bovine neutrophils and subsequent ingestion of microbial pathogens.

The purpose of this study was to evaluate the effects of costimulation of CR3 and FcγR expression on bovine neutrophils on superoxide production, the changes in intracellular calcium $[Ca^{2+}]_i$, and phosphorylation of the signaling pathways.

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MATERIALS AND METHODS

Animals: Three clinically healthy Holstein heifers that were 11 to 15 months of age and a 19-month old heifer with leukocyte adhesion deficiency (LAD) diagnosed based on CD18 expression and polymerase chain reaction analysis were used [7]. Forty milliliters of blood was collected from the jugular vein into tubes containing heparin (20 IU/m*l*).

Isolation of neutrophils: Neutrophils were isolated from heparinized peripheral blood by Ficoll-Conray gradients, as described previously [8]. The isolated neutrophils were washed once with phosphate-buffered saline solution (PBS, pH 7.2) without Ca^{2+} and Mg^{2+}, and were resuspended in Hanks' balanced salt solution (HBSS, Nissui Pharmaceutical, Tokyo, Japan) containing 0.1% bovine serum albumin (BSA) at a concentration of 1×10^7 cells. The resulting cell populations comprised 90–95% neutrophils as determined by morphological evaluation, and >99% of the cell population was viable when assessed by trypan blue dye exclusion.

Preparation of stimulants: Opsonized zymosan (OPZ) was prepared by incubating zymosan A (Sigma Chemical, St. Louis, MO, U.S.A.) with fresh bovine serum at a concentration of 10 mg/m*l* as described previously [7]. Bovine IgG (ICN Biomedicals, Aurora, OH, U.S.A.) was dissolved in PBS at a concentration of 10 mg/m*l*, incubated at 63°C for 20 min, and used as a heat-aggregated bovine IgG (Agg-IgG) [7].

Assay for O_2^- production: Cytochrome C reduction of neutrophils was measured according to the method described previously [9]. The reaction mixture contained 150 μl of 538 μM cytochrome C (Sigma Chemical, St. Louis, MO, U.S.A.) in HBSS, 50 μl of neutrophils (2×10^7/

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m*l*), and 10 μl of each stimulant, unless otherwise stated. After incubation for 30 min at 37°C, the microplate was put into an ice-bath for 10 min. The optical density of the supernatant at 550 nm was determined with a microplate reader (MTP-32, Corona Electric Co., Ltd., Japan). The results were expressed as nmoles of O_2^- produced by 10^6 neutrophils in 30 min at 37°C using an excitation coefficient of 21.1×10^{-3} M/cm.

CL assay: The luminol-dependent CL response of neutrophils was measured according to the method described previously [7]. Five hundred μl of neutrophil suspension (2×10^6 cells/m*l*) was incubated at 37°C for 10 min in a luminometer (Aloka BLR 102, Aloka Ltd., Tokyo, Japan), and then 20 μl of luminol (final concentration 1×10^{-4} M) was added. This suspension was equilibrated for 5 min, and then 2 μl of OPZ together with 3.3 or 10 μl of Agg-IgG was added unless otherwise stated. The results were expressed as the peak CL (cpm) and peak time.

Assay of the intracellular Ca_2^+($[Ca_2^+]i$)concentration: The concentration of cytosolic free Ca^{2+} was measured after loading the cells with the Ca^{2+} indicator Fura-2, as described previously [9]. Fura-2/acetoxymethyl ester-loaded neutrophils (5×10^6 cells) in 500 μl HBSS containing 0.1% BSA were added to a cuvette. Ten μl of OPZ only or 10 μl of OPZ together with 3 or 10 μl of Agg-IgG was added 5 min after initiation of the assay. Fluorescence ratios were recorded by measuring the light emission at 510 nm that was elicited when the excitation wavelength alternated between 340 and 380 nm (CAF-110, Jasco, Tokyo, Japan). The $[Ca^{2+}]_i$ concentration was determined from the peak value, as described previously [9].

Measurement of phosphorylation: Phosphorylation of stimulated neutrophils was determined according to the procedure described previously [9]. Briefly, neutrophils (2×10^6 cells) were stimulated with 2 μl of OPZ only or 2 μl of OPZ together with 3.3 μl or 10 μl of Agg-IgG at 37°C for 3 min. The stimulated cells were then sedimented, and the pellet was immediately resuspended in 50 μl of ice-cold Tris buffer (pH 7.4) containing 50 mM Tris, 1% Nonidet P-40, 20 mM ethylenediaminetetraacetic acid, 0.2 mM sodium orthovanadate, 10 μg/m*l* aprotinin, 10 μg/m*l* leupeptin and 1 mM phenylmethylsulfonyl fluoride. The suspension was subsequently mixed with 100 μl of sample buffer containing 9% sodium lauryl sulfate, 15% glycerol, 15 mM Tris, and 0.05% bromophenol blue. The samples were then boiled at 100°C for 10 min. Insoluble debris was removed by centrifugation, and 15 μl aliquots were run on 10% SDS-polyacrylamide gels under reducing conditions, as described previously [5]. These gels were immunoblotted for phosphotyrosine using anti-phosphotyrosine antibody PY-20 (Santa Cruz Bio., La Jolla, CA, U.S.A.), anti-phosphorylated p38 MAPK, and MAPK antibodies (New England Biolabs, Beverly, MA, U.S.A.), and detection was conducted using a peroxidase substrate system.

Statistics: Statistical comparisons were conducted using the Kruskal-Wallis test. Values of P<0.05 were regarded as significant.

RESULTS

The O_2^- production of neutrophils was determined when they were stimulated with OPZ only or OPZ and Agg-IgG at varying concentrations ranging from 120 μg/m*l* to 400 μg/m*l*, respectively (Fig. 1). The peak levels of O_2^- production in neutrophils stimulated with 280 μg/m*l* to 400 μg/m*l* OPZ showed a plateau-like response. Following stimulation with Agg-IgG at 120 μg/m*l* or 400 μg/m*l* and OPZ ranging from 120 μg/m*l* to 400 μg/m*l*, the O_2^- production of neutrophils increased significantly (P<0.05) compared with OPZ.

O_2^- production of normal and CR3-deficient neutrophils stimulated with OPZ and Agg-IgG was compared (Fig. 2). The O_2^- production of CR3-deficient neutrophils stimulated with OPZ and Agg-IgG was markedly lowered than that of normal neutrophils stimulated with the same stimulants. The O_2^- production of CR3-deficient neutrophils stimulated with both OPZ and Agg-IgG was similar to that of normal neutrophils stimulated with Agg-IgG.

The luminol-dependent CL response of neutrophils stimulated with OPZ and Agg-IgG was increased compared with neutrophils stimulated with OPZ (Fig. 3). The peak CL response time was shortened in association with an increased concentration of Agg-IgG.

The changes in the $[Ca^{2+}]_i$ concentration of neutrophils were measured by stimulation with OPZ and/or Agg-IgG (Fig. 4). The sustained phase of increased $[Ca^{2+}]_i$ in neutrophils stimulated with OPZ and Agg-IgG was greater than that of neutrophils stimulated with OPZ.

Stimulant-induced phosphorylation in the bovine neutrophils was determined by western blotting. Neutrophils were stimulated with OPZ or OPZ and Agg-IgG at varying con-

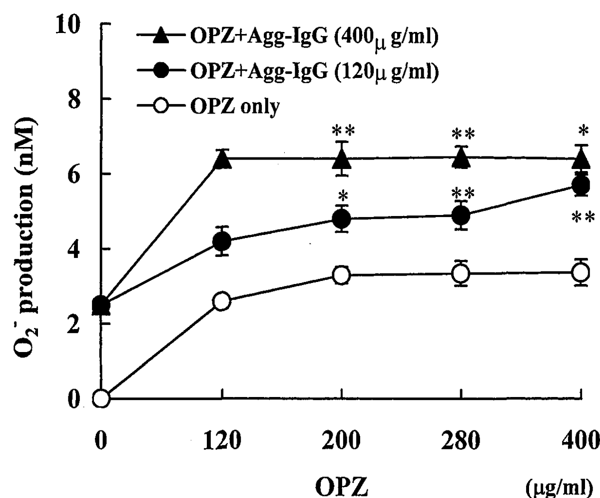

Fig. 1. Superoxide production of bovine neutrophils stimulated with OPZ or OPZ and Agg-IgG. Open circles: Neutrophils stimulated with 120 to 400 μg/m*l* OPZ. Closed circles: Neutrophils stimulated with Agg-IgG (120 μg/m*l*) and 120 to 400 μg/m*l* OPZ. Closed triangles: Neutrophils stimulated with Agg-IgG (400 μg/m*l*) and 120 to 400 μg/m*l* OPZ, respectively. Data are shown as the mean ± SE of triplicate determinations. * Significantly (P<0.05) different from the values for each concentration in the OPZ-stimulated neutrophils as shown by the open circles.

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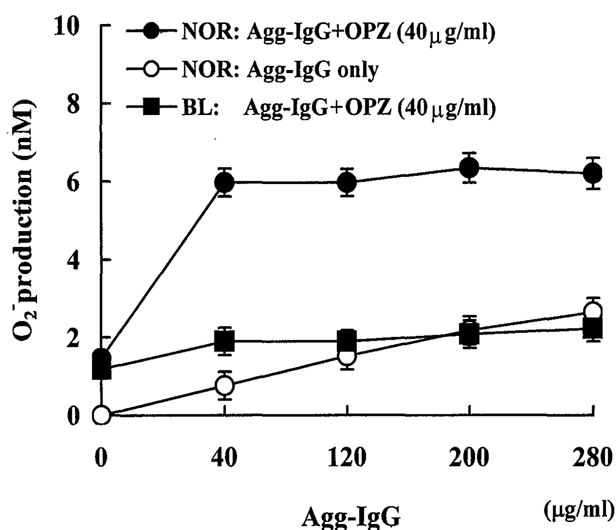

Fig. 2. Superoxide production of normal and CR3-deficient bovine neutrophils stimulated with Agg-IgG only or with Agg-IgG and OPZ. Closed circles: Normal neutrophils stimulated with 40 to 280 *μ*g/m*l* Agg-IgG and OPZ (40 *μ*g/m*l*). Open circles: Normal neutrophils stimulated with 40 to 280 *μ*g/m*l* Agg-IgG. Closed squares: CR3-deficient bovine neutrophils stimulated with 40 to 280 *μ*g/m*l* Agg-IgG and OPZ (40 *μ*g/m*l*). Data are shown as the mean ± SE of triplicate determinations.

centrations (Fig. 5). Western blotting of the stimulated neutrophils revealed a 100-kDa tyrosine-phosphorylated protein (Fig. 5). The amount of 100-kDa tyrosine-phosphorylated protein in the OPZ-stimulated neutrophils increased with the increase in concentration of Agg-IgG. Stimulant-induced activation of MAPK and p38 MAPK was detected in the bovine neutrophils, and the costimulatory effects of CR3 and FcγR were found in their phosphorylations when neutrophils were stimulated with OPZ and Agg-IgG compared with neutrophils stimulated with OPZ or Agg-IgG. (Fig. 6).

DISCUSSION

CR3 and FcγR are involved in various biological functions of phagocytic cells [2, 6, 16]. CR3 is a glycoprotein of the leukocyte integrin family that consists of a two-chain $\alpha\beta$ heterodimer expressed on polymorphonuclear neutrophils, monocytes, and natural killer cells [6, 14]. The FcγR expressed on neutrophils recognizes the Fc regions of different types of antibody molecules and may possess distinct transmembrane and cytoplasmic domains that are coupled to different signal transduction systems in order to elicit the appropriate response [1].

Superoxide production and the CL response of bovine neutrophils increased greatly when CR3 and FcγR were costimulated with OPZ and Agg-IgG compared with stimulation with OPZ. These results indicate that costimulation of CR3 and FcγR on neutrophils produces more O_2^- than stimulation with CR3 or FcγR. We also showed that costimulation with these receptors results in enhanced activation of bovine neutrophils. CR3-deficient neutrophils produced a diminished amount of O_2^-, even in neutrophils stimulated with both OPZ and Agg-IgG. A previous study showed a clear difference between the O_2^- production of neutrophils from normal heifers and a LAD heifer and determined that impairment of costimulation resulted from CR3 deficiency [8]. These findings indicate that neutrophil functions medi-

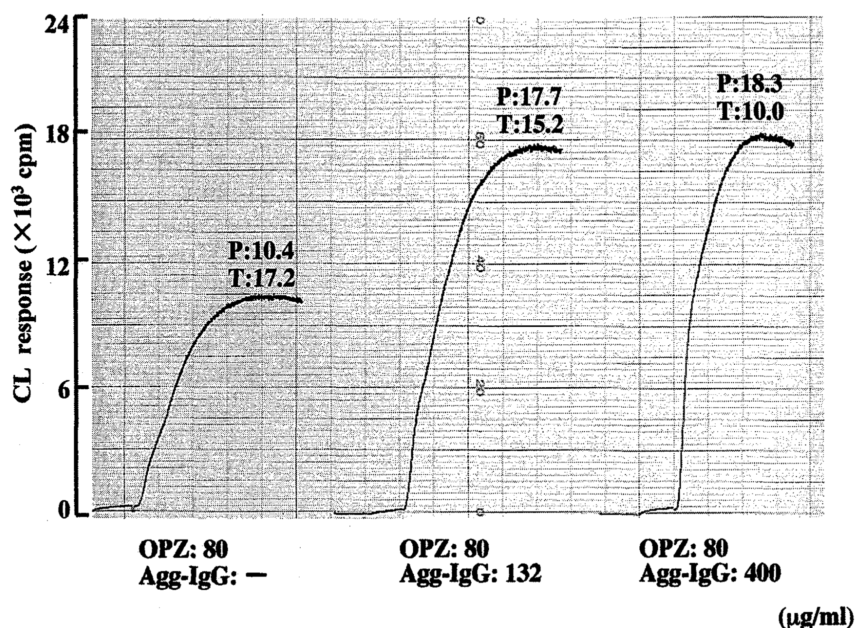

Fig. 3. Chemiluminescent (CL) response of bovine neutrophils stimulated with OPZ only or OPZ and Agg-IgG. Arrows show addition of the indicated stimulants. Data are expressed as the peak CL (P, cpm) and time (T) of the peak CL.

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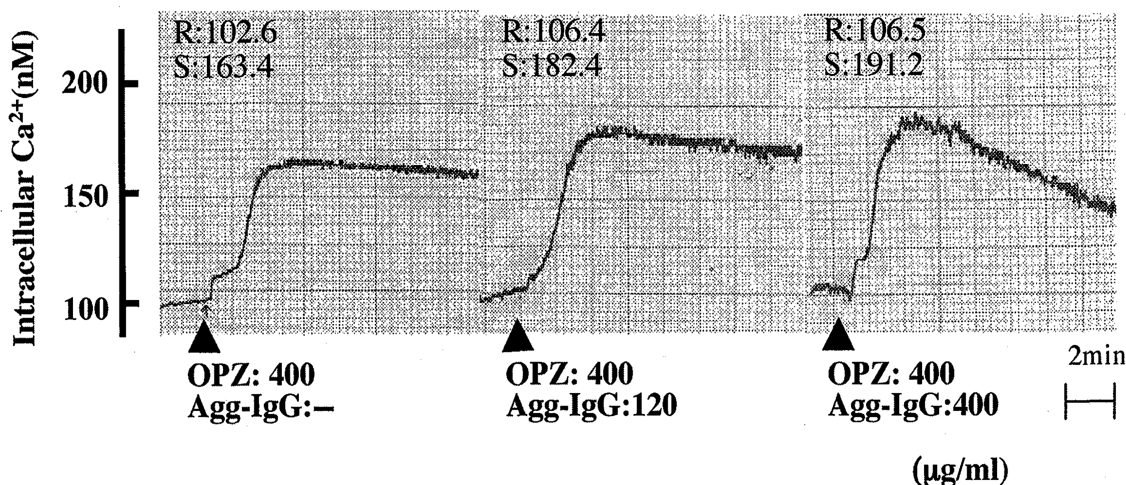

Fig. 4. Changes in the intracellular calicium ($[Ca^{2+}]_i$) concentrations of neutrophils. Neutrophils were stimulated with OPZ only or OPZ and Agg-IgG, as indicated in Fig. 4. Arrows show addition of the indicated stimulants. R: resting phase. S: sustained phase.

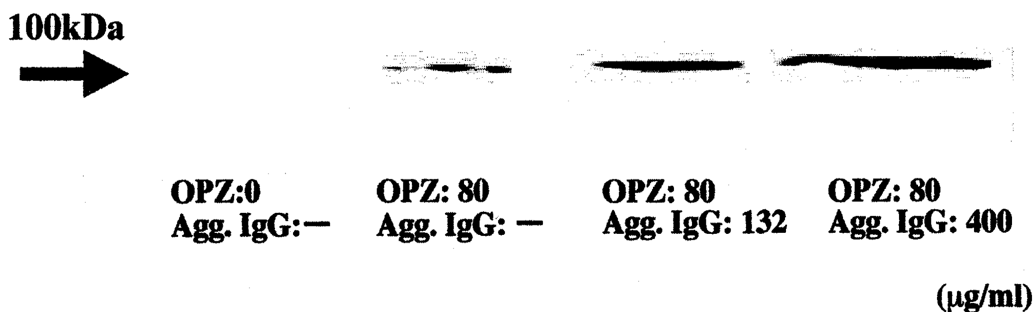

Fig. 5. Tyrosine phosphorylation in bovine neutrophils stimulated with OPZ only or with OPZ and Agg-IgG. Lane, arrow, shows the phosphorylated 100 kDa protein.

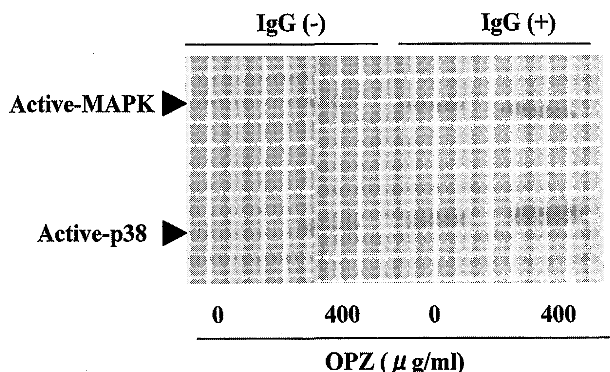

Fig. 6. Activation of mitogen-activated protein kinase (MAPK) and p38 MAPK in bovine neutrophils stimulated with OPZ or Agg-IgG only and with OPZ and Agg-IgG. Active MAPK: mitogen-activated protein kinase. p38 MAPK: p38 mitogen-activated protein kinase. OPZ: opsonized zymosan (400 μg/m*l*). IgG (+): heat-aggregated bovine IgG (400 μg/m*l*).

ated by the receptors for iC3b and FcγR act synergistically to fulfill neutrophil functions, as shown in previous studies [10, 16, 20].

The luminal-dependent CL response of neutrophils stimulated with OPZ and Agg-IgG increased compared with that of neutrophils stimulated with OPZ. The peak time of the peak CL response shortened in association with an increased concentration of Agg-IgG, indicating that costimulation of CR3 and FcγR on neutrophils accelerated the respiratory burst more than stimulation with CR3 or FcγR.

An increase in $[Ca^{2+}]_i$ in neutrophils is known to be an important second messenger in the signaling pathways leading to neutrophil activation [12]. The $[Ca^{2+}]_i$ concentration of the sustained phase in neutrophils stimulated with OPZ and Agg-IgG increased more than in those stimulated with OPZ. This result was consistent with the findings for the O_2^- production and CL response of neutrophils stimulated with CR3 and FcR in the present study.

Tyrosine kinase activation is considered to be an important step in the signaling pathway of the O_2^- generating system [15, 20]. A gradual increase of phosphorylated 100 kDa protein was detected in neutrophils when they were stimulated with increasing concentrations of OPZ and Agg-IgG, indicating that costimulatory signals led to enhanced tyrosine phosphorylation.

A previous study showed that p38 MAPK, a Ser/Thr kinase belonging to the MAPKs family, was demonstrated

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to be present in bovine neutrophils [17]. In the present study, we showed that p38 MAPK was involved in the signaling pathways of superoxide production in bovine neutrophils. In addition, we demonstrated that p38 MAPK activity in bovine neutrophils increased when the neutrophils were stimulated with FcγR and CR3.

Sehgal *et al.* [13] and Zhou *et al.* [18] suggested that there is extracellular interaction between CR3 and FγR on human neutrophils. In bovine neutrophils, Agg-IgG-induced $[Ca^{2+}]_i$ signaling of neutrophils was inhibited in a dose-dependent manner by different concentrations of D-mannose [8]. It has been suggested that there is extracellular interaction between CR3 and FcγR on bovine neutrophils; however, the detailed signaling pathways of CR3 and FcγR have yet to be elucidated.

In conclusion, enhanced superoxide production and CL response induced in neutrophils induced by costimulation of the CR3 and FcγR receptors were found to be closely associated with an increase in $[Ca^{2+}]_i$ concentration and enhanced phosphorylation in bovine neutrophils. Costimulation of CR3 and FcγR in bovine neutrophils leads to enhancement of superoxide production and their signaling pathways and appears to be associated with enhancement of neutrophil functions.

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