

## Original Article

# The effect of conglutinin on production of reactive oxygen species in bovine granulocytes

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**Conglutinin is a high molecular-weight lectin originally detected in bovine serum. It belongs to the family of collectins that bind sugar residues in a Ca<sup>2+</sup>-dependent manner and are effector molecules in innate immunity. Conglutinin appears to play an important role in immune defense mechanisms, showing antiviral and antibacterial activities when tested *in vivo* and *in vitro*. The present study evaluated the effect of conglutinin on the respiratory bursts in bovine peripheral phagocytes. Using nitroblue tetrazolium and hydrogen peroxide assays, we showed that sugar ligand-bound conglutinin stimulated the production of superoxide and H<sub>2</sub>O<sub>2</sub> in granulocytes whereas the non-sugar-bound form of conglutinin inhibited these processes. These results indicate that both forms of conglutinin are able to interact with surface leukocyte receptors but have opposite effects on phagocytic activity. Our findings suggest that conglutinin bound to sugar residues on microbial surfaces can induce oxygen burst in phagocytes, and thereby mediates the elimination of pathogens and prevents the spread of infection.**

**Keywords:** conglutinin, reactive oxygen species

## Introduction

Conglutinin is an oligomeric lectin present in bovine serum, colostrum, and milk. Conglutinin belongs to the collectin family, a small group of collagen-like, C-type proteins produced in vertebrates that bind sugar residues in a Ca<sup>2+</sup>-dependent manner [20]. Conglutinin is capable of binding the non-reducing terminal *N*-acetylglucosamine, mannose, and fucose residues [12], and its ability to bind zymosan, a yeast cell wall extract rich in mannan, has made

this factor suitable for isolating lectin from bovine serum [1]. Besides conglutinin, collectins also include mannan binding protein (MBP), surfactant proteins A (SP-A) and D (SP-D), bovine collectin 43 (CL-43), and several lesser known proteins. Collectins are involved in innate immune mechanisms. They bind to microbial surface carbohydrates, thereby inducing aggregation and preventing the spread of pathogens. Moreover, collectins are secretory pattern recognition receptors that can act as opsonins and cause the destruction of microorganisms by stimulating phagocytic cells [20].

The biological role of conglutinin is still not fully understood, but several reports have indicated that this protein functions in the immune system [3,6]. The antimicrobial activity of conglutinin is based on its binding to viral or bacterial surface sugars, and to complement the degradation product iC3b deposited on microbes [3]. The unique ability of conglutinin to bind iC3b is due to the selective affinity of this lectin to high mannose oligosaccharides in the  $\alpha$ -chain of iC3b [11]. It has been suggested that absorption of conglutinin by the activated complement component iC3b or directly by microorganisms is the cause of low conglutinin serum levels during acute infections like pneumonia or metritis [8]. Other study showed that conglutinin has antibacterial properties that are dependent on the presence of both intact complement and macrophages [6]. This indicates that iC3b-bound conglutinin mediates interactions between opsonized bacteria and phagocytic cells. Furthermore, the generation of oxygen metabolites by effector cells is probably one of the mechanisms underlying the antibacterial activity of conglutinin [5].

The purpose of this study was to evaluate the influence of conglutinin on the production of reactive oxygen

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intermediates (ROIs) by bovine peripheral blood polymorphonuclear leukocytes (PMNs). Generation of microbicidal ROIs is an important oxygen-dependent mechanism for cell death induced by phagocytic cells [2]. ROI production can be elicited in the presence of lipopolysaccharides, zymosan, lectins, phorbol myristate acetate (PMA), or latex beads [14]. This stimulation leads to increased consumption of oxygen whose reduction, catalyzed by membrane-bound enzyme NAD(P)H-oxidase, gives rise to superoxide ( $O_2^-$ ). Beginning with superoxide, a series of reactions leads to the production of hydrogen peroxide ( $H_2O_2$ ), singlet oxygen ( $^1O_2$ ), hydroxyl radicals (OH), and numerous other reactive products toxic to pathogens [15]. Previous study indicated that bovine conglutinin has a stimulating effect on respiratory bursts in mice spleen cells. The selection of bovine peripheral granulocytes for our experiment was dictated by the lack of information regarding the effect of conglutinin on the phagocytic activity of these cells.

## Materials and Methods

### Purification of conglutinin

Conglutinin was purified according to the method developed by Krogh-Meibom *et al.* [9] with some modifications. Bovine serum derived from Holstein cattle was batch-incubated with TSK-gel beads (Toyopearl HW-75F; Tosoh Bioscience, Japan) for 2.5 h at 37°C in a glass flask. This enabled deposition of iC3b on the beads and subsequent binding of conglutinin to iC3b. The serum-treated TSK-gel beads were packed under flow in a fast protein liquid chromatography (FPLC) system; (BioRad, USA), washed with Tris base saline [(TBS)-Ca-1M NaCl; (10 mM Tris, 140 mM NaCl, pH 7.4 containing 5 mM  $CaCl_2$  and a total of 1 M NaCl)] and eluted with TBS-EDTA (TBS with 5 mM EDTA). In the second step of purification, the EDTA eluate was applied to an ion exchange column (Macro-Prep High-Q Support; Bio-Rad, USA) and the proteins retained were separated by running a linear gradient of NaCl. Chromatographically purified conglutinin was analysed by SDS-PAGE [10] and Western blotting [17] using a monoclonal antibody against bovine conglutinin (mAb 263-01; Abcam, UK), diluted 1 : 1,000 and secondary polyclonal peroxidase-labelled rabbit anti-mouse IgG or goat anti-rabbit IgG diluted 1 : 5,000. To induce a colour reaction, 1.4 chloronaphthol was used as a substrate.

### Coating of microplates with conglutinin

The binding of conglutinin to mannan-coated wells was measured by an ELISA using anti-bovine-conglutinin monoclonal antibodies. Microtiter plates (MaxiSorp; Nunc, USA) were coated with different concentrations (0.37~100  $\mu\text{g/mL}$ , 50 mL/well) of mannan (Sigma-Aldrich, USA) or

N-acetyl-D-glucosamine (Sigma-Aldrich, USA) in coating buffer (100 mM  $Na_2CO_3/NaHCO_3$ , pH 9.6) overnight at 4°C. The plates were then blocked with TBS (10 mM Tris, 140 mM NaCl, pH 7.4) containing 3% bovine serum albumin (Sigma-Aldrich, USA) for 2 h at room temperature. Next, the plates were washed with TBS-Ca (TBS with 5 mM  $CaCl_2$ ) containing 0.05% Tween-20 (TBS-Ca-Tween) and incubated for 2 h at room temperature with conglutinin (50 mL/well) diluted (final concentration 50  $\mu\text{g/mL}$ ) with TBS-Ca containing 1% BSA (TBS-Ca-1% BSA). After washing with TBS-Ca-Tween, monoclonal anti-conglutinin antibodies (Abcam, UK) diluted in TBS-Ca-1% BSA (1 : 1,000) were added to the wells (50  $\mu\text{L/well}$ ). The plates were incubated overnight at 4°C, washed with TBS-Ca-Tween, and then incubated with rabbit anti-mouse Ig conjugated with horseradish peroxidase (HRP, 50  $\mu\text{L/well}$ ; Jackson Immuno Research, UK) diluted in TBS-Ca-1% BSA (1 : 13,000). Following 2 h of incubation, the plates were washed with TBS-Ca. Enzyme activity of HRP was detected using a 3,3', 5,5'-tetramethylbenzidine peroxidase ELISA substrate kit (Bio-Rad, USA) according to the producer's recommendations. The reaction was read on a microplate reader (Bio-Rad, USA) at 655 nm.

### Isolation of granulocytes

Blood was collected via jugular venipuncture from four clinically healthy Holstein cows aged 2~3 years and in late lactation. PMNs were isolated from heparin-treated blood by hypotonic lysis as previously described [19]. Cells were centrifuged at 400  $\times$  g for 10 min at 4°C, washed, and resuspended in Hanks balanced salt solution (HBSS) without phenol red (Sigma-Aldrich, Poland). The leukocyte preparation consisted of 80~90% granulocytes as determined by hematological staining using staining kit (Hemacolor; Merck Chemicals, Germany) according to the procedure recommended by the producer. Cell viability exceeded 95% based on trypan blue staining.

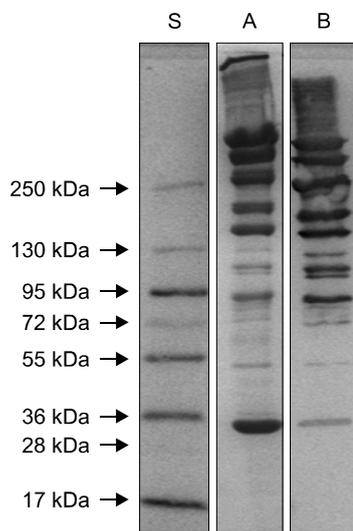
### Superoxide anion assay

The assay was performed according to the method described by Pick [16]. PMNs ( $8 \times 10^6$  cells/mL) transferred to conglutinin-coated or non-coated wells (50  $\mu\text{L/well}$ ) of 96-well microplates were covered with 50  $\mu\text{L/well}$  of nitroblue tetrazolium (NBT; Sigma-Aldrich, USA) solution (2 mg/mL) in phenol red-free HBSS. The wells received 5  $\mu\text{L}$  of conglutinin (non-coated plate, final concentrations ranged from 0.125 to 5  $\mu\text{g/mL}$ ) or 5  $\mu\text{L}$  of PMA (final concentration 200 ng/mL, positive control; Sigma-Aldrich, USA), or 5  $\mu\text{L}$  of HBSS. Wells without conglutinin served as the negative control. Reference wells (blank) contained cells suspended in NBT (1 mg/mL) with iodoacetamide (10 mM). The plates were incubated at 37°C in 5%  $CO_2$  for 60 min. The amount of formazan was measured with a microplate reader

**Table 1.** Selection of the type and concentration of sugar ligand for coating the microplate with conglutinin

Concentration (µg/mL)	100	25	6.2	1.5	0.37
Mannan*	1.012 ± 0.077	1.331 ± 0.043	0.987 ± 0.044	0.486 ± 0.004	0.133 ± 0.026
N-acetyl-D-glucosamine*	0.035 ± 0.012	0.015 ± 0.003	0.015 ± 0.022	0.007 ± 0.022	0.001 ± 0.016

The results are presented as the mean ± SD for one experiment performed in triplicate. \*Absorbance at 655 nm.



**Fig. 1.** Conglutinin purified from bovine serum using a two-step chromatographic method; (A) SDS-PAGE analysis of non-reduced conglutinin in 3 ~ 20% polyacrylamide gel stained with Coomassie brilliant blue R-250, (B) Western blotting and (S) molecular weight standard.

(Bio-Rad, USA) at 550 nm. The results are expressed as the optical density per well after subtracting the values for the blank.

### H<sub>2</sub>O<sub>2</sub> assay

The rate of H<sub>2</sub>O<sub>2</sub> production was determined by peroxidase-dependent oxidation of phenol red as described by Pick [16]. A PMN suspension ( $8 \times 10^6$  cells/mL) was added to conglutinin-coated or non-coated wells (50 µL/well) of a microplate. The cells were covered with 50 µL/well of assay solution, which was prepared on the day of the experiment and consisted of HBSS, phenol red (final concentration 0.2 g/L; Sigma-Aldrich, USA), and HRP (final concentration 20 U/mL; Sigma-Aldrich, USA). The

wells received 5 µL of conglutinin (non-coated plate, final concentrations ranged from 0.125 to 5 µg/mL), 5 µL of PMA (final concentration 200 ng/mL, positive control), or 5 µL of HBSS. The plate was incubated at 37°C in 5% CO<sub>2</sub> for 90 min. The reaction was then stopped by adding 10 µL/well of 1 N NaOH. After a 3-min equilibration interval, the plate was read at 620 nm by a microplate reader (Bio-Rad, USA). The amount of H<sub>2</sub>O<sub>2</sub> in each well was calculated according to a standard curve and expressed as µM H<sub>2</sub>O<sub>2</sub>/90 min/ $4 \times 10^5$  cells.

### Statistical analysis

Data obtained from the NBT and H<sub>2</sub>O<sub>2</sub> assays were analysed with Statistica (ver. 9.0; StatSoft, USA). The aim of the statistical analysis was to ascertain the effects of conglutinin (mannan-bound and unbound) on the generation of superoxide radicals and H<sub>2</sub>O<sub>2</sub> by bovine granulocytes. Significance of differences between the control and conglutinin-treated cells were evaluated by a one-way ANOVA using the Dunnett test for post-hoc comparisons. Prior to the analysis, data not falling within a normal distribution (Shapiro-Wilk test at the 95% confidence interval) were transformed using the Box-Cox method. Homogeneity of variance was verified by the Brown-Forsythe test. As Box-Cox transformed data were not normally distributed or if data failed Brown-Forsythe test, we applied the Kruskal-Wallis test to perform a nonparametric analysis of variance followed by rank-based multiple comparisons. The results are presented as the mean ± SD of four independent experiments. *p*-values below 0.05 were considered significant.

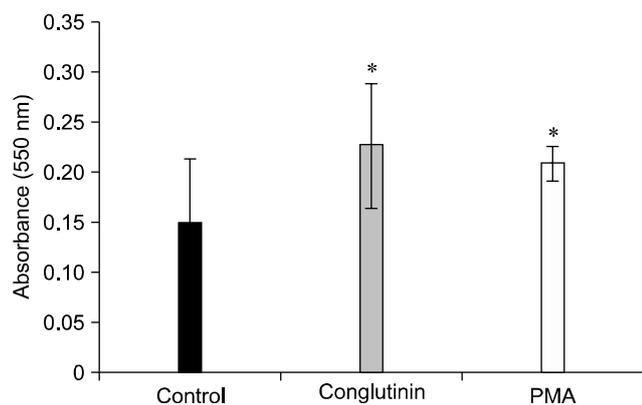
## Results

### Conglutinin purification

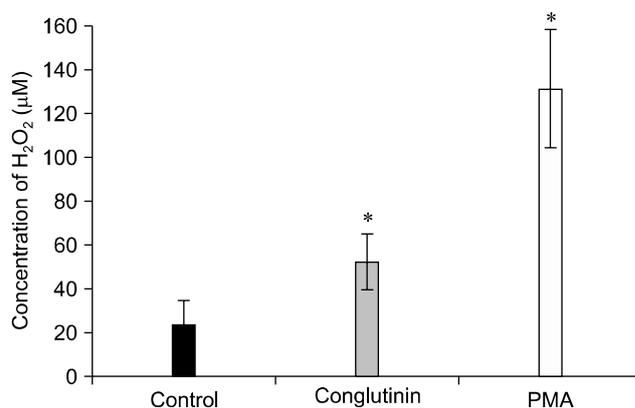
Conglutinin purified using a two-step chromatographic method was analysed by SDS-PAGE under non-reducing conditions and Western blotting using monoclonal antibodies. Comparative characterization of the conglutinin protein profiles in the gel and on PVDV membranes indicated a high level of purity for the protein obtained. In the Western blot, this appeared as a ladder composed of many bands with molecular weights ranging from 34 to 630 kDa (Fig. 1).

### Selection of the type and concentration of ligand for coating the microplate with conglutinin

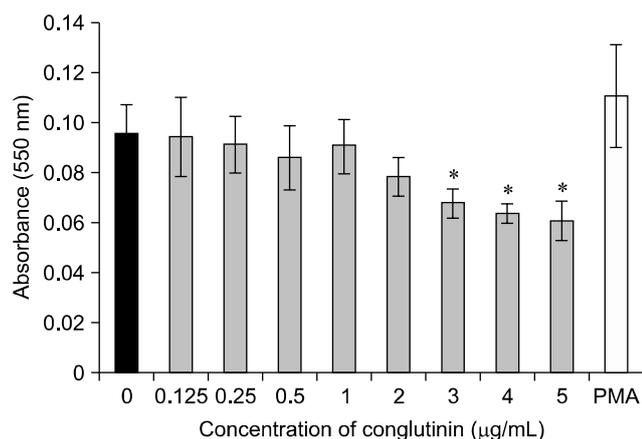
Conglutinin was observed to bind more strongly to mannan than N-acetyl-D-glucosamine (Table 1). The highest value was observed for 25 µg/mL of mannan, so this concentration of mannan was selected for further experiments using microplates coated with conglutinin.



**Fig. 2.** The effect of mannan-bound conglutinin (2.5  $\mu\text{g}/\text{well}$ ) coated onto microplate on superoxide production by bovine leukocytes as measured by a nitroblue tetrazdium (NBT) assay. Control wells contained neither mannan nor conglutinin, and positive control wells contained PMA (200  $\text{ng}/\text{mL}$ ). The results are presented as mean  $\pm$  SD for four independent experiments, each done in sextuplicate. PMA: phorbol myristate acetate. \* $p < 0.05$  vs. the control.



**Fig. 4.** The effect of mannan-bound conglutinin (2.5  $\mu\text{g}/\text{well}$ ) coated onto microplate wells on  $\text{H}_2\text{O}_2$  production by bovine leukocytes determined by the peroxidase-dependent oxidation of phenol red. Control wells contained neither mannan nor conglutinin, and positive control wells contained PMA (200  $\text{ng}/\text{mL}$ ). The results are presented as mean  $\pm$  SD for four independent experiments, each done in quintuplicate. \* $p < 0.05$  vs. the control.



**Fig. 3.** The effect of conglutinin on superoxide production by bovine leukocytes as measured by an NBT assay. Different concentrations of bovine conglutinin were added to a cell suspension in plate wells. Control wells received HBSS instead of conglutinin, and positive control wells contained PMA (200  $\text{ng}/\text{mL}$ ). The results are presented as mean  $\pm$  SD for four independent experiments, each done in sextuplicate. \* $p < 0.05$  vs. the control.

### Effect of conglutinin on the generation of superoxide anions by PMNs

To determine whether conglutinin affects superoxide anion generation in bovine PMNs, we performed an NBT microplate assay. Inside the cells water-soluble, yellow NBT dye is rapidly converted into insoluble dark blue monoformazan by two  $\text{O}_2^-$  molecules. Monoformazan concentration (and therefore the concentration of  $\text{O}_2^-$ ) is calculated based on spectrophotometric measurements at 550 nm [21].

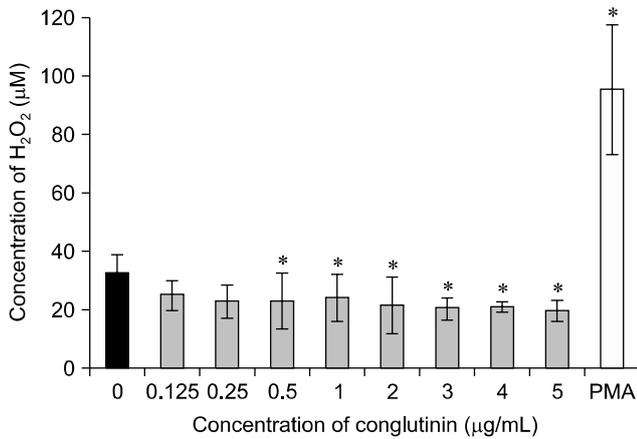
Mannan-bound conglutinin was shown to stimulate oxidation processes in the PMNs. The average absorbance of the sample with conglutinin was  $0.227 \pm 0.062$  and the difference in absorbance compared to the control (wells without mannan and conglutinin) was statistically significant. The mean absorbance of the samples with PMA (standard receptor-independent stimulus of ROIs) was  $0.208 \pm 0.017$ , similar to that of the sample with conglutinin (Fig. 2).

In the other variant of the experiment, conglutinin at concentrations ranging from 0.125 to 5  $\mu\text{g}/\text{mL}$  was added directly to a suspension of PMNs. The average absorbance of the samples containing different concentrations of conglutinin was lower than the absorbance of the control samples. The inhibitory effect of conglutinin on PMNs metabolism depended on the concentration of the protein; the higher the concentration of protein, the stronger the inhibitory effect. Statistically significant differences were observed for all PMN samples treated with conglutinin at concentrations greater than 2  $\mu\text{g}/\text{mL}$  (Fig. 3).

### Effect of conglutinin on $\text{H}_2\text{O}_2$ generation by PMNs

The phenol red colorimetric method was used to assess the effect of conglutinin on  $\text{H}_2\text{O}_2$  production by bovine PMNs. The dye undergoes oxidation in the presence of  $\text{H}_2\text{O}_2$  released from the cells. The concentration of  $\text{H}_2\text{O}_2$  is directly proportional to the absorbance of wells.

The results showed that mannan-bound conglutinin in the microplate wells stimulated the production of  $\text{H}_2\text{O}_2$  by the PMNs. The amount of  $\text{H}_2\text{O}_2$  in the sample treated with conglutinin was more than twice as high ( $51.78 \mu\text{M} \pm 12.8$ ) as in the control ( $23.3 \mu\text{M} \pm 11.0$ ). PMA proved to be a very



**Fig. 5.** The effect of conglutinin on H<sub>2</sub>O<sub>2</sub> production by bovine peripheral polymorphonuclear leukocytes measured by the peroxidase-dependent oxidation of phenol red. Different concentrations of bovine conglutinin were added to a cell suspension in plate wells. Control wells received HBSS instead of conglutinin, and positive control wells contained PMA (200 ng/mL). The results are presented as mean  $\pm$  SD for four independent experiments, each done in quintuplicate. \* $p < 0.05$  vs. the control.

strong stimulator of H<sub>2</sub>O<sub>2</sub> production; the average concentration of H<sub>2</sub>O<sub>2</sub> produced by cells treated with this reagent was  $130.9 \mu\text{M} \pm 27.04$  (Fig. 4).

In the other variant of the experiment, different concentrations of conglutinin (0.125–5  $\mu\text{g/mL}$ ) were added to cells suspended in HBSS with phenol red. An inhibitory effect on the production of H<sub>2</sub>O<sub>2</sub> by PMNs was observed in the presence of all concentrations of conglutinin. However, statistically significant differences were observed at concentrations between 0.5 to 5  $\mu\text{g/mL}$  (Fig. 5).

## Discussion

This study assessed the effect of conglutinin on the production of reactive oxygen species in bovine PMNs using NBT and H<sub>2</sub>O<sub>2</sub> assays. Results of the tests showed that the effects of conglutinin on generation of superoxide radicals and H<sub>2</sub>O<sub>2</sub> can vary (stimulatory or inhibitory) depending on whether the protein is bound to sugar residues or remains unbound. Mannan-bound conglutinin stimulated the production of superoxide radical and H<sub>2</sub>O<sub>2</sub> by PMNs. Our results suggest that after conglutinin binds to sugar residues on the surface of microorganisms, it stimulates the cidal activity of phagocytes. Hence it is likely that the binding of sugars activates conglutinin, allowing the collagen fragments to interact with receptors on phagocytic cells and inducing the synthesis of microbicidal ROIs. In contrast, conglutinin which is not bound to a sugar ligand has inhibitory effects on the production of O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> by PMNs.

Friis *et al.* [5] demonstrated that the production of oxygen metabolites by effector cells is one of the possible mechanisms underlying the antibacterial activity of conglutinin. Lectin increases the production of ROIs in mouse spleen macrophages stimulated by *E. coli*, but only in the presence of complement. In this system, a decisive role is probably played by iC3b complement fragments for which conglutinin has a specific affinity since conglutinin binds to the sugar residues of iC3b in the presence of Ca<sup>2+</sup> ions [3]. The decisive role of iC3b in ROI production by conglutinin is indicated by the fact that conglutinin-mediated enhancement of the respiratory burst is inhibited in the presence of N-acetyl-D-glucosamine, D-mannose, and N-acetyl-D-mannosamine; these are monosaccharides reported [5] to inhibit conglutinin-binding to the complement factor iC3b. Uemura *et al.* [18] showed that when human MBP, which is structurally similar to conglutinin, is bound to mannan coating a microplate, it not only stimulates the production of superoxide radicals in neutrophils but also induces cell aggregation. The biological activity of MBP correlated positively both with its concentration and the time the cells were exposed to MBP. Madan *et al.* [13] observed that conidia of *Aspergillus fumigatus* opsonized with SP-A and SP-D (1  $\mu\text{g/mL}$ ) stimulates respiratory bursts in human leukocytes.

Collectins act on target cells via C1qR, originally identified as a receptor for the complement component C1q. Collectins and C1q both have a collagen fragment and globular domains which may be responsible for transmitting various types of cellular signals. Hence it seems appropriate to relate our results to studies by other authors that examined the effects not only of collectins but also of C1q on ROI production. Eggleton *et al.* [4] showed that C1q, both purified (1–100  $\mu\text{g/mL}$ ) and opsonized on *Staphylococcus aureus* (0.1  $\mu\text{g/mL}$ ), stimulates the production of superoxide radicals in human neutrophils. Antibodies specific for C1qR substantially inhibit this process. Similarly, Goodman and Tenner [7] observed that C1q complement components coating a microplate stimulate the production of superoxide radicals in PMNs, and demonstrated that the collagen fragment of C1q is responsible for this reaction. However, the MBL and SP-A proteins used in the study, which also have collagen fragments, did not exhibit such properties [7].

The results of this study provide additional information on the effect and possible biological role of bovine conglutinin. The effects of this protein on oxygen-dependent intracellular mechanisms of cell death and the binding of conglutinin to sugar residues appear to be necessary for this protein to induce the generation of ROIs. Our results suggest that conglutinin opsonized on microbes stimulates oxidative burst in phagocytes and helps to eliminate infection with pathogens.

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