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Original article

Effect of conglutinin on phagocytic activity of bovine granulocytes

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Abstract

In the present study we investigated the effect of bovine conglutinin on the phagocytic activity of leukocytes. We measured both the chemotactic activity of conglutinin and its effect on the internalization of zymosan particles and *E. coli* by granulocytes. We also assessed the binding of conglutinin to various microorganisms isolated from clinical cases in cattle.

We showed that conglutinin binds strongly to the surface of yeast cells and to mannan-rich zymosan particles, while weak binding was observed in the case of the bacterial strains tested, including those whose O antigen is composed of mannan.

Conglutinin (1-10 μg/ml) neither acts as a chemotactic factor for peripheral blood leukocytes nor affects ingestion of *E. coli* by granulocytes. However, as flow cytometry based assay showed, conglutinin (0.1-1 μg/ml) increased ingestion of zymosan expressed as mean fluorescence intensity (MFI) of positive cells.

Key words: chemotaxis, conglutinin, microorganism binding, phagocytosis

Introduction

Conglutinin is a high molecular C-type lectin, which is present in bovine serum, fetal calf serum and in the colostrum and milk of cows (Dec et al. 2011a). It is a lectin that binds in a calcium-dependent manner to zymosan and to the complement fragment iC3b, and has exhibited antimicrobial activity when tested *in vivo* and *in vitro* (Dec and Wernicki 2006). Conglutinin belongs to the small collectin group of C-type (Ca^{2+}-dependent) lectins that occur in mammals and chickens (Hogenkamp et al. 2006) but they have also been found in fish (Nakao et al. 2006) and in invertebrates (Nair et al. 2000). Besides conglutinin, the mammal collectin family includes mannan binding protein (MBP), surfactant proteins A (SP-A) and D (SP-D) bovine collectin 43 (CL-43) and several lesser known proteins. All collectins have similar structures characterized by the presence of collagen-like sequences and globular carbohydrate recognition domains (CRDs) and they are involved in innate defence mechanisms. Collectins bind to microbial surface carbohydrates in a calcium-ion-dependent manner and hence induce aggregation and prevent the pathogens from spreading. Aggregated microbial cells are more easily eliminated, e.g. from mucous membrane of the respiratory tract. Moreover, collectins stimulate the processes of destruction of microorganisms by facilitating phagocytosis (MBP, SP-A, SP-D) and activating complement (MBP) (van de Wetering et al. 2004).

The biological role of conglutinin is still not fully understood, but several reports indicate a function in the immune system (Śliwa-Dominiak et al. 2010).

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It has been suggested that the low serum conglutinin level during acute infections such as pneumonia or metritis is caused by its absorption by activated complement component iC3b, to which conglutinin shows an affinity, or directly by microorganisms (Ingram and Mitchell 1971).

In 1959 Ingram demonstrated that the conglutinin-containing euglobulin fraction of ox serum could protect mice against pathogenic bacteria. This evidence was later confirmed by Friis-Christiansen et al. (1990), who observed that mice subcutaneously injected with conglutinin manifested extended survival following intraperitoneal injection of a highly virulent strain of Salmonella Typhimurium. *In vitro* studies have indicated that antibacterial activity of conglutinin requires the presence of both an intact complement system and mouse spleen cells (Friis-Christiansen et al. 1990), and other studies (Friis et al. 1991, Dec at al. 2012a) demonstrated that the generation of oxygen metabolites by granulocytes is one of the mechanisms of the antibacterial activity of conglutinin.

Conglutinin also displays antiviral activity, whose effect is dependent on the binding of conglutinin to surface viral glycoproteins. It displays both haemagglutination inhibition and neutralizing activity towards influenza A virus (Hartshorn et al. 1993) and rotaviruses (Reading et al. 1998). Moreover, viral aggregations formed by conglutinin appear to increase H_2O_2 production by provoking more extensive cross-linking of sialylated neutrophil receptors (Hartshorn et al. 2002).

The purpose of the present study was to investigate the effect of conglutinin on phagocytic activity of bovine granulocytes. We focused on two stages of phagocytosis – chemotaxy and ingestion.

Materials and Methods

Purification of conglutinin

Conglutinin was purified as described by Krogh-Meibom et al. (2010), with some modifications (Dec et al. 2012b). Bovine serum was batch-incubated with TSK beads (Toyopearl HW-75F, Tosh Bioscience) for 2.5 h at 37°C in a glass flask. The serum-treated TSK beads were packed under flow on a FPLC system (BioRad), washed with TBS-Ca-1M NaCl and eluted with TBS-EDTA. EDTA eluate was applied to an ion-exchange column (Macro-Prep High-Q Support, Bio-Rad) and the proteins retained were separated by running a linear gradient of NaCl.

Isolation of bovine leukocytes

Blood samples were collected via jugular venipuncture from 3 healthy Holstein cattle (Bos taurus). Leukocytes were isolated from heparin-anticoagulated blood by hypotonic lysis with 2x volume of ice-cold distilled water as previously described (Urban-Chmiel et al. 2009a). Isotonicity was restored by adding 2x volume of hypertonic (2x concentrated) Hanks' balanced salt solution (HBSS). Cells were centrifuged at 400 x g for 10 min at 4°C, washed and resuspended in HBSS without phenol red with 0.5% lactoalbumine hydrolisate (Biomed, Poland). This procedure usually produces a cell fraction containing 77% granulocytes, 19% lymphocytes and 4% monocytes, determined using the May-Grunwald/Giemsa stain. The viability of cells was >95% as determined by trypan blue exclusion.

Chemotaxis assay

Chemotaxis assays were performed using a Neuroprobe 48-well chemotaxis chamber and cellulose filters with a pore size of 5 μm (Millipore) as previously described (Urban-Chmiel et al. 2009b). The lower wells were filled with 25 μl of HBSS (containing 0.5% lactoalbumine hydrolisate), alone (spontaneous migration) or containing conglutinin (1, 5 or 10 μg/ml) or 10% zymosan activated bovine serum (ZAS) as a positive control. The upper wells were filled with 50 μl of leukocyte suspension diluted to 5 x 10^6 cells/ml. After 1.5 h incubation at 37°C, 5% CO_2, the filter was removed, fixed in absolute ethanol and stained with Harris haematoxylin. The parts of the membrane corresponding to different wells were then analysed by light microscopy to determine the number of cells that had migrated through the filter. The results were expressed as the mean value of 10 high-power fields counted at x40 magnification for each group. Samples were analysed in triplicate and averaged for each experiment.

Preparation of zymosan-activated serum

In the chemotaxis assay we used bovine serum from which the conglutinin had been removed. To remove the lectins fresh bovine serum (15 ml) was incubated with mannan-agarose chromatographic medium (5 ml) for 3 h at 4°C. The conglutinin-free serum was then aspirated and filtrated. Zymosan A from *Saccharomyces cerevisiae* (Sigma) was washed with PBS and heated at 99°C for 1h prior to use. The conglutinin-free bovine serum was incubated with zymosan (5 mg/ml) at 37°C in a shaking bath for 60 min. The zymosan particles were then removed by centrifugation (2000 x g, 10

min), and the C5a-rich serum was collected and stored at -20°C until use.

Evaluation of binding of conglutinin to bacteria, yeasts and zymosan

We selected strains of *Streptococcus sp.*, *S. aureus* and *C. albicans* from clinical cases of mastitis in cows, as well as strains of *Mannheimia haemolytica* and *Pasteurella multocida* isolated from individuals with upper respiratory infections. In the case of *M. haemolytica* serotypes 1 and 2, distinguished based on differences in their capsular antigens, were used (Katsuda et al. 2008), and in the case of *P. multocida* we used two isolates (P236 and P13) of the A3 serotype. In addition, standard strains were tested – *E. coli* O8 (PCM 409) and *K. pneumoniae* O5 (PCM 61) from the Polish Collection of Microorganisms in Wrocław and *S. cerevisiae* from our own collection.

The bacteria and yeasts were cultured overnight in trypticase soy medium (BTL, Poland) and Sabouraud medium (BTL, Poland), respectively. Microorganisms were then heat-killed for 1 h at 70°C, washed and suspended in TBS-Ca. Zymosan A (Sigma) was washed twice in PBS and then suspended in TBS-Ca. The final optical density of all suspensions was 1.0 when measured at 600 nm. The microorganisms and zymosan were incubated with conglutinin (5 µg/ml) for 1 h at 37°C, then washed and centrifuged (10,000 x g, 15 min) three times with TBS-Ca and finally suspended in 300 µl of TBS-Ca.

For Western blotting, samples were boiled at 95°C for 5 min in sample buffer (0.5 M Tris, 2% SDS (w/v), 10% glycerol (v/v), 0.1% bromophenol blue (w/v), pH 6.8), and then centrifuged at 10,000 x g, 10 min. SDS-PAGE was performed on 3-20% acrylamide gels with pre-stained PageRuler standard (Fermentas) as a molecular marker. Proteins from SDS-PAGE gel were transferred to a PVDF membrane (Bio-Rad) as described by Towbin et al. (1979). Blocked membranes were incubated overnight with monoclonal anti-conglutinin antibodies (1:1000, mab 263-01, Abcam). Polyclonal peroxidase-labeled rabbit anti-mouse IgG (1:5000, Jackson ImmunoResearch) was used as a secondary antibody. To induce a colour reaction, 1.4 chloronaphthol was employed as a substrate.

FITC labelling of zymosan particles and E. coli

Zymosan A from *Sacharomycves cerevisiae* and heat-killed *Escherichia coli* O8 were suspended in carbonate buffer, pH 9.6. Fluorescein (FITC, isomer I, Sigma) was added to the zymosan and bacteria suspensions to final concentrations of 10 mg/ml and 3 µg/ml, respectively. The suspensions were labelled in the dark at room temperature for 1 h on a rotary mixer and then washed 4 times with PBS. The zymosan and bacteria were finally resuspended in HBSS to concentrations of 2 mg/ml and 2 x 10^9 CFU/ml, respectively, and stored at -20°C until use.

Selecting the optimal concentration of conglutinin for the phagocytosis assay

FITC-labelled *E. coli* and zymosan were diluted in HBSS to a final concentration of 2 x 10^8 CFU/ml and 0.1 mg/ml, respectively, and incubated with a range of conglutinin concentrations at 37°C for 1 h. Following incubation, optical density was measured spectrophotometrically at 600 nm.

Phagocytosis assay

FITC-labelled zymosan and *E. coli* suspended in HBSS at concentrations of 0.1 mg/ml and 2 x 10^8 CFU/ml, respectively, were incubated with conglutinin at 37°C for 1 h, and then cooled to 4°C. Leukocytes (4 x 10^6 cells/ml) were mixed with an equal volume of previously prepared zymosan or bacteria suspension and incubated at 37°C in a shaking bath for 25 min. The ratio of leukocytes to bacteria was 1 : 50. For each experimental sample a control tube was prepared which was incubated at 4°C. The phagocytosis assay was stopped by adding ice-cold PBS containing 1.5 mM EDTA, and the cells were centrifuged at 400 x g for 8 min. The pellet cells were resuspended in a trypan blue solution (200 mg/ml) to quench external fluorescence. The cells were then washed with PBS and analysed in a flow cytometer (FASCalibur, Becton Dickinson, USA) using CellQuest software (Becton, Dickinson and Company, USA). Phagocytosis was measured as the percentage of granulocytes containing FITC-labelled particles or bacteria and as MFI of positive cells. Data were obtained from 4 independent experiments with duplicates for each sample.

Statistical analysis

Data obtained in chemotaxis and internalization assays were analysed in Statistica 9.0. The aim of the statistical analysis was to ascertain the effects of conglutinin on peripheral blood leukocyte migration and ingestion of zymosan particles or *E. coli* by granulocytes. Significance of differences between

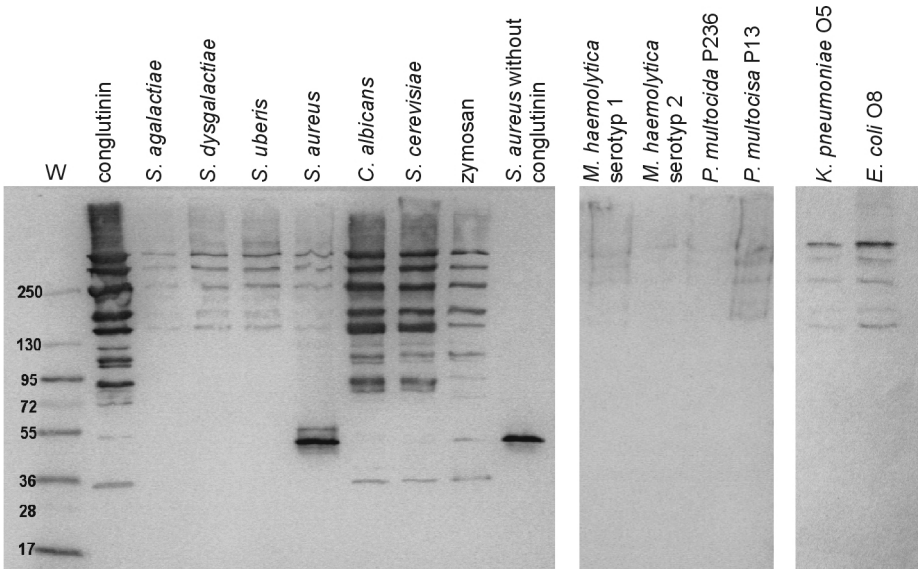

Fig. 1. Binding of conglutinin to different microorganisms and to zymosan, analysed by Western blotting. Heat-killed microorganisms and zymosan suspended in TBS-Ca were incubated with conglutinin (5 μg/ml) for 1h at 37°C, washed and placed on 3-20% acrylamide gels. Following electrophoretic separation the proteins were transferred onto a PVDF membrane. The presence of bound conglutinin was shown using monoclonal antibodies specific for bovine conglutinin.

control and conglutinin treated cells was checked by one-way ANOVA, using the Dunnett test for post-hoc comparisons. Prior to the analysis, data distributions were tested for normality with Shapiro-Wilk W test and for equality of variances with Brown-Forsythe tests. The results are presented as mean ± SD for three independent experiments. P value below 0.05 was considered significant.

Results

The effect of conglutinin on leukocyte migration

The results of the experiment showed that conglutinin at concentrations of 1, 5 and 10 μg/ml does not have chemotactic properties for bovine peripheral blood leukocytes (Table 1).

Table 1. Effect of conglutinin on migration of bovine peripheral leukocytes. Cells were allowed to migrate through a membrane with 5-μm pores toward conglutinin (1, 5 or 10 μg/ml), zymosan-activated serum (10%) or buffer control (HBSS) for 90 min. Membranes were stained and scored for the average number of cells that had migrated through the membrane per high-power field.

	% of cell migration
HBSS (control buffer)	100
Conglutinin 1 μg/ml	99.03 ± 33.94
Conglutinin 5 μg/ml	105.35 ± 35.98
Conglutinin 10 μg/ml	92.5 ± 10.61
Zymosan activated serum (10%)	331.29 ± 59.04*

The results are presented as percentage averages ± SD for three independent experiments; * – P<0.05.

Binding of conglutinin to microorganisms

Binding of conglutinin to microbial surfaces was assessed using immunoblotting. Conglutinin was shown to bind strongly to yeast cells and zymosan particles, while weak binding was observed in the case of all the bacterial strains examined (Fig. 1). The distinct 49-kDa band that appeared in the immunoblot in the lane with *S. aureus* was the result of a non-specific reaction between protein A (cell wall protein of *S. aureus*) and the antibodies.

Effect of conglutinin on the internalization of zymosan particles and *E. coli* by bovine granulocytes

The conglutinin concentrations used in the experiment were chosen based on spectrophotometric measurement of zymosan and *E. coli* suspensions incubated with various concentrations of the protein. In the case of zymosan, changes in optical density were observed at a concentration of only 0.1 μg/ml (measured at 600 nm) (Table 2). In the samples with 2 and 5 μg/ml concentrations of conglutinin, agglutination of zymosan particles could be seen with the naked eye (Fig. 2), so these concentrations were considered to be too high and were ruled out. None of the concen-

Table 2. Effect of conglutinin on optical density of zymosan suspensions. FITC-labelled zymosan suspended in HBSS was incubated with a range of conglutinin concentrations at 37°C for 1 h and then each tube was measured spectrophotometrically at 600 nm.

Concentration of conglutinin (μg/ml)	0	0.1	0.2	0.5	1	2	5
Absorbance (600 nm)	0.490	0.476	0.435	0.417	0.373	0.290	0.175

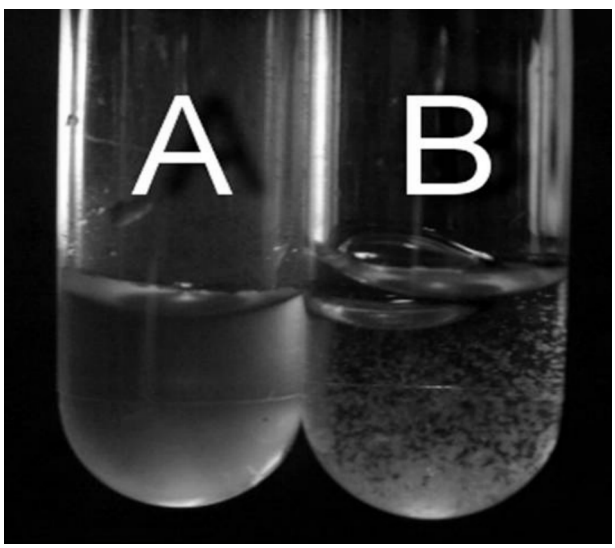

Fig. 2. FITC-labelled zymosan suspension in HBSS incubated for 1 h at 37°C in the absence (A) or in the presence (B) of conglutinin at a concentration of 5 μg/ml.

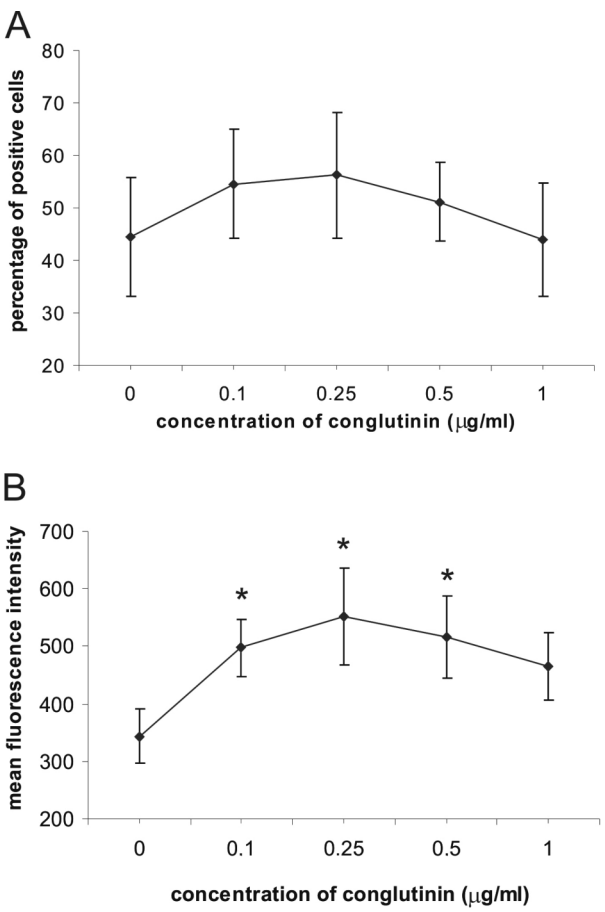

Fig. 3. The effect of conglutinin on the ingestion of zymosan particles by bovine granulocytes shown as the percentage of positive cells (A) and as the mean fluorescence intensity of positive cells (B). Leukocytes were incubated with FITC-labelled zymosan particles in the presence or absence of conglutinin for 25 min at 37°C. Results are expressed as the mean of three independent experiments ± SD; * – $P<0.05$.

trations applied (0.1 – 5 μg/ml) induced changes in the optical density of the bacterial suspensions (data not shown).

In the samples in which zymosan had been incubated with conglutinin at concentrations of 0.1, 0.25 and 0.5 μg/ml, the number of positive granulocytes increased, although the results obtained were statistically insignificant (Fig. 3A). Conglutinin concentrations of 0.1-0.5 μg/ml induced statistically significant increases in the MFI of granulocytes incubated with FITC-labelled zymosan particles. The maximum value – 551.98 ± 84.7, compared to 343.93 ± 47.8 for the control was obtained when the conglutinin concentration was 0.25 μg/ml (Fig. 3B).

In the case of internalization of *E. coli*, conglutinin at concentrations of 0.1-5 μg/ml was not observed to induce statistically significant changes in either the percentage of positive granulocytes or the MFI of the cells (data not shown).

The results of the experiment are additionally shown as representative cytograms and histograms (Fig. 4). The cells examined, enclosed within gates', are granulocytes. The X-axis values (FCS) correspond to cell size, and the Y-axis values (SSC) indicate their granularity. There was an overall increase in the MFI of positive cells among granulocytes incubated with fluorescent zymosan particles, compared with the controls (cells incubated with zymosan in the absence of conglutinin). No such increase was observed in the samples in which leukocytes were incubated with *E. coli*. Moreover, in the zymosan samples the granulocyte profile observed in the dot plots shifted towards the increasing values on the X-axis. This means that some of the cells increased in size, probably due to internalization of rather large zymosan particles.

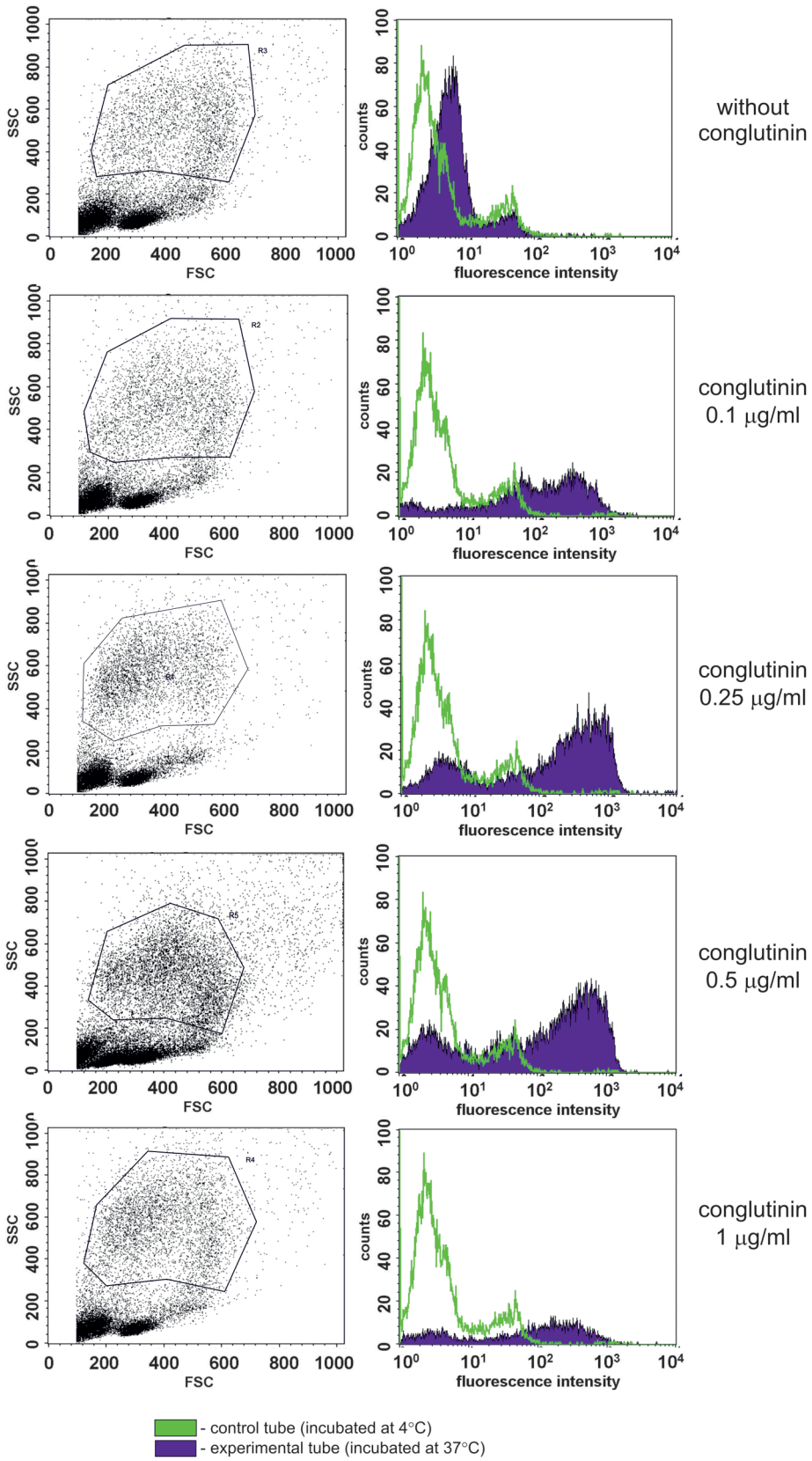

Fig. 4. Representative fluorescence-activated cell sorting (FASC) profiles of granulocytes exposed to FITC-labelled zymosan particles (A) or *E. coli* (B). Bovine peripheral leukocytes were incubated with zymosan or *E. coli* for 25 min in the absence or presence of conglutinin. At left, the graphs depict cell size [forward scatter (FSC), x-axis] vs. cell granularity [side scatter (SSC), y-axis]. Analyses of granulocytes were performed on cells within the indicated gate. At right, the graphs depict the level of FITC fluorescence (x-axis) vs. the number of cells (y-axis).

Discussion

In the present study we investigated the effects of conglutinin on two stages of phagocytosis – chemotaxis and ingestion. We also examined the binding of conglutinin to different species of bacteria and yeasts isolated from cattle, as well as to standard strains of *E. coli* O8 and *K. pneumoniae* O5.

The experiment showed that conglutinin at concentrations of 1-10 μg/ml did not have chemotactic properties for bovine peripheral blood leukocytes. The results can only be related to experiments using other collectins, which have shown that the effect of collectin family proteins on leukocyte migration depends on the subpopulation of leukocytes and on the concentration of the lectins. Schagat et al. (2003) found that human SP-A at concentrations ranging from 0.5 to 25 μg/ml did not stimulate chemotaxis of rat peripheral neutrophils or inflammatory bronchoalveolar lavage (BAL) neutrophils isolated from LPS-treated lungs. However, SP-A added to chemoattractants (fMLP, MIP-2, ZAS) affected the chemotaxis of both peripheral and inflammatory lung neutrophils towards these chemoattractants. The effect was dependent on the activation state of the leukocytes; SP-A significantly inhibited peripheral neutrophil chemotaxis towards MIP-2 (50 ng/ml) and fMLP (10 nM), while it significantly enhanced inflammatory cell chemotaxis towards each chemoattractant tested. SP-A (25 μg/ml) also inhibits chemotaxis of human peripheral neutrophils towards IL-8. In contrast to SP-A, rat MBL (25 μg/ml) and human C1q complement fragment (25 μg/ml) do not significantly affect migration of rat neutrophils (Schagat et al. 2003). Human SP-D has been found to be chemotactic for neutrophils, with the maximum effect at 5 ng/ml, decreasing with higher concentrations (von Bredow et al. 2006). Madan et al. (1997) showed that human lung surfactant proteins A and D act as chemoattractants for human neutrophils. A maximum increase in cell migration occurred at a concentration of 5 ng/ml for SP-D and at 0.5 μg/ml for SP-A.

When collectins bind microorganisms they prevent them from colonizing and spreading, and at the same time they enable more extensive stimulation of cellular receptors.

In this study we observed that conglutinin in the presence of Ca^{2+} ions binds strongly to *S. cerevisiae* and *C. albicans* cells and to particles of zymosan isolated from yeast cell wall, causing them to aggregate. Zymosan is a protein-and-sugar complex rich in mannan – a sugar composed of many subunits of mannose, which conglutinin exhibits affinity for. Mannan also occurs on the surface of some bacteria, e.g. *E. coli* O8 and *K. pneumoniae* O5. However, conglutinin binds weakly to these bacteria and does not induce aggregation of their cells. Hence two factors must determine the binding of conglutinin to microorganisms that have mannan on their surfaces: the chemical structure of the mannan and the degree of its cell surface exposure. Yeast mannan is a branched polysaccharide containing α-1,2-, α-1,3-, α-1,6- and β-1,2 bonds (Kobayashi et al. 2003), while the mannan which is part of the LPS of *E. coli* O8 or *K. pneumoniae* O5 is linear with α-1,2, or more rarely, α-1,3 bonds. Moreover, surface mannan in bacteria can be masked by a capsular antigen, which often appears in the case of *E. coli* and *K. pneumoniae* (Ovodov 2006).

The experiment aimed at assessing the effect of conglutinin on ingestion of bacteria or zymosan by bovine granulocytes showed that conglutinin induces aggregation of zymosan particles, but not the *E. coli* O8 used in the experiment. The results are presented in two ways – as the percentage of positive cells, i.e. those which ingested zymosan or bacteria labelled with fluorochrome, and their mean fluorescence intensity. Conglutinin (0.1-5 μg/ml) was not observed to affect ingestion of bacteria, but at concentrations of 0.1 – 1 μg/ml the protein stimulated ingestion of FITC-labelled zymosan particles, expressed as MFI of positive granulocytes. This was most likely the result of phagocytes ingesting zymosan particles agglutinated by conglutinin, whose combined fluorescence intensity was higher than the fluorescence of a single zymosan particle. In a similar study Madan et al. (1997) observed a stimulatory influence of the proteins SP-A and SP-D (0.5-1 μg/ml) on ingestion of *A. fumigatus* conidia. The increase in the mean fluorescence intensity of positive leukocytes was probably caused by aggregation of the conidia, which was induced by binding of SP-A and SP-D to spore surface glycoproteins such as galactomannan. Brouwer et al. (2008) observed an increase in phagocytosis (expressed as MIF multiplied by the percentage of FITC-positive neutrophils) of zymosan opsonized with MBL-sufficient sera, compared to MBL-deficient sera.

In addition, the role of conglutinin in phagocytosis has been shown in immunohistochemical experiments by Holmskov et al. (1992), in which the protein was detected in macrophages from the liver, lungs, thymus and spleen of cattle. Conglutinin was detected in the phagolisosomes of alveolar microphages.

In conclusion, the effect of conglutinin on ingestion by bovine granulocytes depends on the degree to which conglutinin binds to microorganisms and induces their aggregation. Conglutinin (at concentrations of 1-10 μg/ml) does not, however, affect migration of peripheral blood leukocytes.

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