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BOVINE S PROTEIN (VITRONECTIN) INCREASES PHAGOCYTOSIS OF *STREPTOCOCCUS DYSGALACTIAE*

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ABSTRACT

The effects of bovine S protein (vitronectin) on phagocytosis of *Streptococcus dysgalactiae* strains isolated from cattle with mastitis were investigated. Phagocytized streptococci were determined by a fluorometric microassay using glass adherent polymorphonuclear neutrophils (PMN). Preincubation of *S. dysgalactiae* with bovine S protein significantly increased their phagocytosis by PMN. Bovine S protein had no effect on phagocytic killing of non-S protein binding *S. pyogenes* cultures. Enzymatic digestion of the bovine S protein binding sites on *S. dysgalactiae* with pronase resulted in a significative reduction of the effects of S protein on phagocytosis. It could thus be concluded that in addition to its role as a promoter of cellular adhesion and complement inhibitor, bovine S protein may also influence the phagocytosis of *S. dysgalactiae* during inflammatory processes.

Key words: *Streptococcus dysgalactiae*, bovine S protein, phagocytosis, bovine mastitis

INTRODUCTION

Streptococcus dysgalactiae, *S. agalactiae* and *S. uberis* are three streptococcal species frequently reported to cause bovine mastitis (9). Although the causative organisms of bovine mastitis have been identified, the exact mechanisms of the initiation and development of infection have not yet been fully elucidated.

Selective adherence of bacteria to bovine epithelium could be a prerequisite for the organism to be a successful parasite in the mammary gland (19). Also, binding of host plasma proteins to gram-positive cocci might play an important role in pathogenicity (20,15,18). The binding of bovine complement S protein (vitronectin) to *Streptococcus dysgalactiae* isolates from cattle with mastitis and its role in adherence to bovine epithelial cells were observed (4). Vitronectin is a multifunctional protein that has important role in complement-dependent cell lysis (7), in the coagulation system (12), and in cellular adhesion (2). The bovine S protein exists in plasma with molecular weight of 76,000 and 65,000 (4) and its biological properties were indicated by the ability to spread cultured endothelial cells, as described for human S protein (11). The purpose of this study was to determine the effects of bovine S protein binding by *S. dysgalactiae* on its phagocytosis by bovine polymorphonuclear neutrophils (PMN).

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MATERIALS AND METHODS

Streptococci. Four clinical isolates of *S. dysgalactiae* recovered from cattle with mastitis were used. In addition, two cultures of *S. pyogenes* (lacking the capacity to bind bovine S protein) served as controls. Each strain was inoculated in 1-litre Erlenmeyer flasks containing 100 ml of Todd-Hewitt broth (GIBCO/BRL, GmbH, Eggstein, Federal Republic of Germany). After incubation on a rotary shaker for 18 h at 37°C and 60 rpm, the bacteria were harvested by centrifugation for 20 min at 15,000 xg.

For proteolytic treatments, two *S. dysgalactiae* strains (S.dys 8 and S.dys 12) with the highest S protein binding activities were used. One ml samples of suspensions containing 10^8 streptococcal cells/ml were incubated with increasing (2,5 to 250µg) concentrations of pronase (E. Merck AG, Darmstadt, Germany) as described (3).

Purification of bovine S protein. S protein was purified from bovine plasma as previously described (4). The purity of the protein was tested by sodium dodecyl sulfate polyacrylamide gel electrophoresis (8) and western blot analysis (16) with anti-bovine S protein antibodies. Antibodies against bovine S protein were raised in rabbits.

Radioiodination and binding assays. S protein was radiolabeled with ^{125}I (Carrier Free, Amersham Buchler, Braunschweig) using the chloramine-T method (6). The specific activity of each of the 3 preparations was approximately 1.4 mci/mg of protein. Radiolabelled S protein was analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (8). The binding assays were performed as previously described (4), using 2 x 10^8 streptococci and 10 mg of the respective ^{125}I-labelled protein in 0.15 M phosphate-buffered saline solution (PBSS, pH 7.5) containing 0.05% Tween 20 (PBSS-Tween). After 1 h at 25°C, the streptococci were centrifuged for 3 min at 10,000 xg and washed with ice-cold PBSS-Tween. The radioactivity in the sediment was measured in a γ-spectrometer (Packard Instrument Co., Inc., Rockville, MD). The radioactivity uptake was expressed as percentage of the total activity remaining in the pellet.

Preparation of polymorphonuclear neutrophils (PMN). PMN were prepared from ethylenediaminetetraacetic acid (EDTA) treated bovine blood using ficoll-hypaque gradients (Histopaque, Sigma, Munich, Federal Republic of Germany). Remaining erythrocytes were lysed with 0.162 M ammonium chloride (pH 7.2) and the sedimented PMN washed twice in Hanks balanced salt solution (HBSS), containing 0.1% bovine serum albumin. Cell viability was higher than 95% as determined by trypan blue dye exclusion test. The PMN suspensions were adjusted to 5 x 10^6 cells/ml using a hemocytometer.

Phagocytic assays. The method of Rainard (14) for the phagocytic assays was used essentially as previously described (17). Streptococcal suspensions (10^8 streptococci/ml in HBSS) were incubated with 3 to 300 µg of bovine S protein for 30 min at 37°C. Subsequently, the streptococci were washed in HBSS, opsonized with free S protein bovine serum at a final concentration of 10% and used in phagocytic assays. Phagocytosis was observed on glass-adherent polymorphonuclear neutrophils. One hundred ml of PMN suspensions were dripped on microscopic slides and, after incubation for 1 h at 37°C under 5% CO_2, the preparations were washed with HBSS. Streptococcal suspension (100 µl) were added and after incubation for 1 h at 37°C under CO_2, the preparations were washed twice with HBSS and stained with an acridine orange (Sigma) solution (20 µg/ml in HBSS) for 1 min at room temperature. The slides were washed with HBSS, mounted with cover slips, sealed with paraffin and examined with a fluorescence microscopic. Viable streptococcal cells appeared in green and intracellular killed streptococci appeared in red (10). The phagocytized streptococci, in at least 50 randomly selected PMN, were counted. In controls either PMN or streptococci were replaced by HBSS.

RESULTS AND DISCUSSION

The purified bovine S protein migrated as a doublet, with molecular weight of 76,000 and 65,000, in the sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fig.1). The purified preparation had a characteristic doublet when analysed by immunoblotting (Fig. 2), but did not cross-react with antibodies against human or bovine fibronectin (4) . The functional properties of S protein were demonstrated by its ability to facilitate the spreading of cultured endothelial cells (11), to inhibit complement-dependent lysis and to neutralize the anticoagulant activity of heparin in the thrombin-antithrombin III reaction (12, 13).

The four strains of *S. dysgalactiae* bound ^{125}I-labeled bovine S protein with a mean value of 45% and those of *S. pyogenes* did not interact with this protein. Therefore *S. pyogenes* served as a negative control in the phagocytic assays.

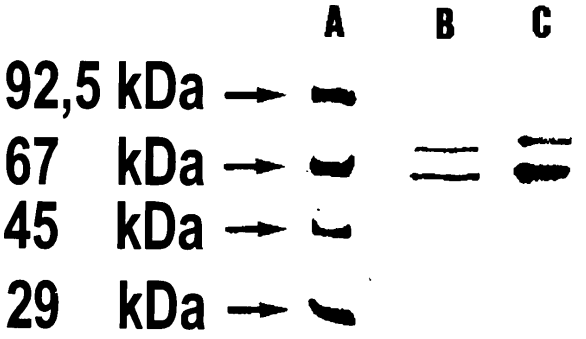

Figure 1 - SDS-Polyacrylamide gel electrophoresis of purified bovine S protein. Lanes: **A**, size markers (phosphorylase B, 92,5 kDa; bovine serum albumin, 67 kDa; ovalbumin, 45 kDa; carbonyc anhydrase, 29 kDa); **B**, human S protein; **C**, purified bovine S protein.

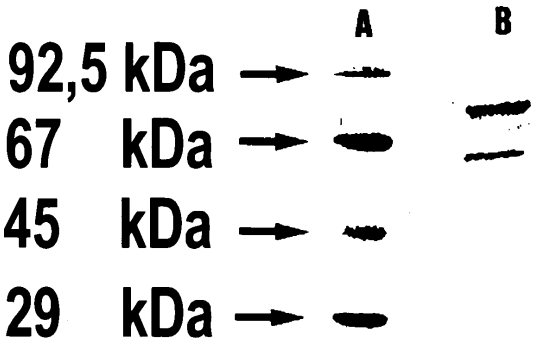

Figure 2 - Western blot of purified bovine S protein using anti-bovine S protein antibodies. Lanes: **A**, size markers (phosphorylase B, 92,5 kDa; bovine serum albumin, 67 kDa; ovalbumin, 45 kDa; carbonyc anhydrase, 29 kDa); **B**, purified bovine S protein.

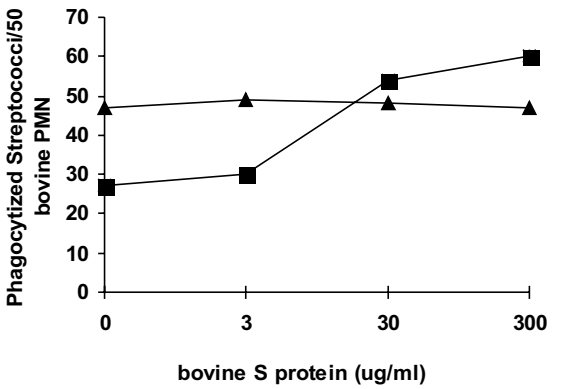

Figure 3 - Phagocytosis of *Streptococcus dysgalactiae* (■) and *Streptococcus pyogenes* (▲) by bovine polymorphonuclear neutrofils after preincubation of streptococci with increasing concentrations (3-300 µg/ml) of bovine S protein. Results represent means obtained with four cultures of *S. dysgalactiae* and two cultures of *S. pyogenes*.

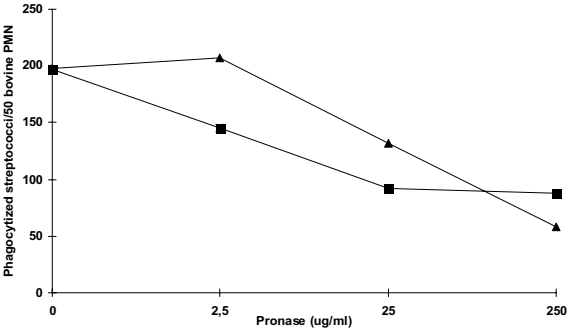

Figure 4 - Phagocytosis of *Streptococcus dysgalactiae*, strains S.dys 8 (■) and S.dys 12 (▲), by bovine polymorphonuclear neutrofils after treatment with increasing concentrations (2.5-250µg/ml) of pronase, in the presence of bovine S protein (100µg/ml). Each point represents mean of duplicates.

In the phagocytic assays, prior incubation of *S. dysgalactiae* strains with bovine S protein significantly increased their phagocytosis by bovine polymorphonuclear neutrophils. A phagocytic increase of almost 120% was observed when 300 µg/ 10^8 streptococcal cells were applied (Fig. 3). Preincubation of *S. pyogenes* strains with the same concentration of bovine S protein did not affect their phagocytosis. To further elucidate the involvement of bovine S protein binding sites in the phagocytic killing of *S. dysgalactiae*, streptococci were treated with proteolytic enzymes prior to phagocytosis assays. After enzymatic digestion of the streptococcal binding sites for S protein with pronase, the effects of bovine S protein on *S. dysgalactiae* phagocytosis were reduced (Fig. 4).

Intramammary infections of dairy cows caused by *S. dysgalactiae* have been characterized by persistence of the infection and by a poor response to conventional control measures. Significant differences in intramammary infectivity and binding capacities among certain strains of *S. dysgalactiae* were reported (5, 4). Previous reports (4, 1) demonstrated that *S. dysgalactiae* strains could adhere and invade bovine epithelial cells, induced cellular damage, and were capable of persisting inside bovine mammary epithelial cells.

Phagocytosis by polymorphonuclear neutrofils is one of the first defense lines against invading streptococci and the specific interaction of streptococci with fibrinogen plays an important role as an antiphagocytic factor (20, 17). In adherence

experiments, bovine S protein enhanced streptococcal adherence to bovine epithelial cells (4). However, no studies about the influence of S protein in the phagocytosis of *S. dysgalactiae* were described. This study presents evidence that binding of bovine S protein by *S. dysgalactiae* increased phagocytosis by PMN. The involvement of the specific S protein binding structures of S. *dysgalactiae* in the phagocytosis could be confirmed by assays using proteolyzed streptococci, a procedure known to destroy S protein binding activities of *S. dysgalactiae* (4). The results of the present study indicate that in addition to its role as a promoter of cellular adhesion and complement inhibitor (7, 2), S protein may also influence the phagocytosis during inflammatory processes. These data will be helpful in the elucidation and understanding of the pathogenesis of *S. dysgalactiae* bovine mastitis and the exact relationship between bovine S protein binding, adherence and infectivity .

RESUMO

Aumento na fagocitose de *Streptococcus dysgalactiae* pela ação da proteína S bovina (vitronectina)

Foram investigados os efeitos da proteína S bovina (vitronectina) na fagocitose de amostras de *Streptococcus dysgalactiae* isoladas de bovinos com mastite. A determinação do número de estreptococos fagocitados foi realizada pelo método fluorométrico utilizando neutrófilos polimorfonucleares (NPM) aderidos em lâminas de vidro. A pré-incubação do *S. dysgalactiae* com a proteína S bovina aumentou significativamente a sua fagocitose por NPM. A proteína S bovina não causou efeito na fagocitose de culturas de *S. pyogenes*, já que não apresentam sítios de ligação para esta proteína. A digestão enzimática com pronase dos sítios de ligação *S. dysgalactiae* para a proteína S bovina resultou numa significativa redução do efeito da proteína S na fagocitose. Pode-se concluir que além do papel como promotor da adesão celular e inibidor do complemento, a proteína S bovina pode também influir na fagocitose do *S. dysgalactiae* durante os processos inflamatórios.

Palavras-chave: *Streptococcus dysgalactiae*, proteína S bovina, fagocitose, mastite bovina.

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