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The In Vitro Anti-pathogenic Activity of Immunoglobulin Concentrates Extracted from Ovine Blood

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Abstract An immunoglobulin-rich fraction has been prepared from ovine blood in our laboratory. We have investigated its antibacterial activity and binding activity to pathogenic whole cell antigens, lipopolysaccharide (LPS) and staphylococcal enterotoxin B. Ovine immunoglobulin concentrate (OIC) comprised about 73±2% of IgG and 11±1% of IgM on a protein basis. It inhibited the growth of all 13 strains of pathogens tested, but the inhibitory activity varied according to bacterial strain. The inhibitory activity of OIC was attributed to the high contents of undenatured immunoglobulin present because its inhibitory activity was destroyed by pepsin digestion and heat treatment (65°C for 30 min). OIC bound to all the Gram-positive and Gram-negative pathogens, regardless of cell wall structure. The highest magnitude of crossreactivity to whole cell antigens was against *Staphylococcus epidermidis* and *Shigella soneii* strains ($p<0.001$). The binding activity of OIC to LPS obtained from *Escherichia coli* O111:B4 and *Salmonella enterica* serotype *typhimurium* was assessed by enzyme-linked immunosorbent assay and lymphoblast K-562 proliferation assay. OIC bound to LPS with a binding activity that was dependent on OIC concentration and saturable, showing typical hyperbolic curves. For toxin-binding activity, an OIC concentration-dependent trend like that for LPS-binding activity was also observed. This preliminary evidence suggests that the OIC used in this study could be a promising supplement for protecting against pathogenic bacteria.

Keywords Ovine immunoglobulin concentrate · Inhibitory activity · Crossreactivity · Lipopolysaccharide (LPS) · Staphylococcal enterotoxin B (SEB)

Introduction

Spray-dried animal plasma (SDAP) has been shown to improve intestinal growth, feed intake, and body weight gain, and to mitigate against intestinal diseases of domestic

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animals such as pigs, calves, broilers, and turkeys [1–7]. These effects are more pronounced when animals are challenged with pathogenic bacteria or toxins [1, 8–12].

Although the mechanism of action of SDAP is not completely understood, it may include improvement of immunocompetence, and prevention of pathogen infectivity and attachment to mucosa by the immunoglobulin and glycoproteins present in SDAP [13, 14]. Among animal plasma proteins, the immunoglobulins are known to have a bacteriostatic action on some microorganisms, particularly those of enteric origin and to bind specifically with bacterial toxin or lipopolysaccharide (LPS). This is implicated in a majority of the pathophysiological responses to infection by Gram-negative bacteria [15–17]. Serum immunoglobulin concentrate supplementation has also been demonstrated to improve viral gastroenteritis in children [18].

Considerable quantities of animal blood are available commercially. In New Zealand alone, approximately 40 million liters of ovine blood is produced annually as a by-product of the meat industry and is used for low-value products such as fertilizer or is discarded [19]. The characterization and application of ovine blood components including immunoglobulin have not been well documented. An immunoglobulin-rich fraction has been prepared from ovine blood in our laboratory. The aim of this study was to assess the anti-pathogenic activities of this fraction by in vitro trials.

Materials and Methods

Bacterial Strains, Cell Line, and Culture Conditions

The bacterial strains used in this study were obtained from the New Zealand Reference Culture Collection (Institute of Environmental Science & Research Limited, Porirua, New Zealand). Pathogenic bacteria were grown in tryptic soy broth (TSB; Difco, MI, USA) at 37°C for 18 h (Table 1). Prior to use in the experiments, all bacteria were subcultured at least three times. For long-term storage, the stock culture was stored at −80°C in fresh TSB broth containing 20% glycerol. Lymphoblast K-562 was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco BRL, Life Technologies Inc., CA, USA), supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL), penicillin G (100 IU/ml), and streptomycin (100 μg/ml), at 37°C in an atmosphere containing 5% CO_2.

Preparation and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis of Ovine Immunoglobulin Concentrates

Ovine immunoglobulin concentrate (OIC) was prepared according to procedures previously developed in our laboratory based on the standard precipitation technique [20]. Fresh ovine blood was mixed with sodium citrate (1%) and centrifuged (Sorvall Instruments, Du Pont, USA) at 11,000×g for 20 min at room temperature for separation of plasma. Calcium chloride (0.5%) was then added to the plasma from which the serum was separated and adjusted to pH 6.4. Ammonium sulfate was added to serum to reach 1.7 M and stirred for 1 h at room temperature, followed by centrifugation at 11,000×g for 20 min. The precipitate (OIC) was dissolved in distilled water, adjusted to pH 9, and then filtered through a 12-kD membrane using ultrafiltration (Model X702, XTravert, PDL Electronics Ltd, New Zealand). The final product was freeze-dried.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis based on the method of Laemmli [21] was used to investigate the composition of OIC. The electrophoresis was

Table 1 Inhibitory activity of ovine immunoglobulin concentrates against pathogens.

No.	Bacterium[a]	Inhibition[b]
Gram positive		
1	*Bacillus cereus* NCTC 11145	++
2	*Enterobacter aerogenes* NCTC 10006	++
3	*Listeria monocytogenes* CDC KC36	+
4	*Staphylococcus aureus* NCTC 4163	+
5	*Staphylococcus aureus* NCTC 8530	+
6	*Staphylococcus aureus* ATCC 6538	+
7	*Staphylococcus epidermidis* ATCC 49134	++
Gram negative		
8	*Escherichia coli* NCTC 10863	+++
9	*Escherichia coli* ATCC 25922	++
10	*Escherichia coli* O157:H7 CDC strain G5244	++
11	*Pseudomonas aeruginosa* ATCC 25668	+++
12	*Salmonella typhimurium* CDC AMO 3398	++
13	*Shigella soneii* ATCC 29029	+

NCTC National Collection of Type Cultures (England), *CDC* Centers for Disease Control and Prevention (USA), *ATCC* American Type-Culture Collection (USA)

[a] All the bacteria were incubated in TSB at 37°C.

[b] +: less than 10%; ++: more than 10% and less than 30%; +++: more than 30%

performed at 25 mA (constant) for 1 h and the gel (9% polyacrylamide) was stained with Coomassie brilliant blue R250 (Bio-Rad, CA, USA). OIC bands were compared with the relative mobility of precision-plus protein standards (Bio-Rad), sheep serum IgG (Sigma, MO, USA) and bovine serum albumin (BSA; Sigma).

Measurement of IgG and IgM

The content of IgG and IgM in OIC was analyzed by direct enzyme-linked immunosorbent assay (ELISA). Sheep IgG and IgM from Auspep Pty Ltd (Parkville, Victoria, Australia) were used as reference antigens for construction of the standard curve. The protein content in samples was determined by BCA protein assay kit (Pierce, IL, USA). Flat-bottomed Immunopure Polysorp 96-well plates (Nunc, Roskilde, Denmark) were coated with reference antigens or OIC in phosphate-buffered saline (PBS) buffer (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4). The plates were incubated overnight at 4°C, washed four times with PBST (PBS containing 0.05% Tween-20) and then the remaining binding sites were blocked with 3% BSA in PBS for 1 h at 37°C. The plates were then washed five times with PBST. The 96-well plates were incubated with HRP-conjugated chicken anti-sheep IgG (Auspep Pty Ltd) or HRP-conjugated rabbit anti-sheep IgM (Auspep Pty Ltd) at a 1:1,000 dilution in PBST for 2 h at 37°C. The plates were then washed five times and developed using *O*-phenylene diamine (Sigma) for 20 min. The reactions were stopped by the addition of 2 N H_2SO_4 and absorbance was measured at 450 nm using an ELISA plate reader (Perkin Elmer, MA, USA).

Pathogen Growth Inhibition

The OIC solution was made up at concentrations of 10% (*v*/*w*) or 20% (*v*/*w*) in buffered peptone water (BPW; Difco, pH 7). The BSA solution (10%) was made up in BPW as a

positive control. To determine the effect of heat on the antibacterial property of OIC, the OIC solution (10%) was heated at 65°C for 30 min. For digestion of OIC by pepsin, the OIC solution which had been adjusted to pH 2.0 was incubated in the presence of 0.25 mg/ml pepsin (E.C. No. 3.4.23.1; Sigma) at 37°C for 1 h and the pH readjusted to 7.0. Pathogen cultures suitably diluted to contain a few thousand colony-forming units (0.1 ml) were added to 0.9 ml of OIC solution. After an initial count (0 h) of viable bacteria in the reaction mixture, the mixtures were incubated at 37°C for 3 h and then recounted with tryptic soy agar. The inoculation count of cells and incubation time were determined through preliminary experiments. To establish the extent of proliferation of pathogens in the absence of OIC, 0.9 ml BPW was inoculated with pathogen, incubated, and plated out in an identical manner. All of the OIC solutions were filtered through a series of Millipore membrane filters (0.8 and 0.45 μm) before being subjected to the antibacterial test. All measurements were conducted in triplicate and the percentage pathogen growth was determined as follows:

$$\%\ \text{growth} = \frac{\text{Log}_{10}(N_m/N_0)}{\text{Log}_{10}(N_c/N_0)} \times 100$$

N_0	Inoculation number of pathogens per 1 ml reaction solution.
N_c and N_m	Number of pathogens per 1 ml of BPW control without OIC (N_c) and number of reaction mixture (N_m) after 3 h incubation.

Crossreactivity

Crossreactivity of OIC was determined following a slight modification of the method described by Tomita et al. [15]. Antigen preparations were concentrated by centrifuging (3,000×g, 5 min) pathogens which had been grown at 37°C for 18 h and resuspended in PBS (pH 7.4). The cell washing procedure was repeated three times with PBS and the absorbance of the final suspension adjusted to 1.0 at 610 nm. Bacterial antigens were bound by incubating 100 μl of the antigen suspensions overnight at 4°C in a flat-bottomed 96-well plate. Unbound antigen was removed by washing four times with PBST, and then the remaining binding sites were blocked with 3% BSA in PBS for 1 h at 37°C. The OIC solution (10 mg/ml) was added at 100 μl per well and the plate incubated for 2 h at 37°C. Unbound OIC was removed by washing five times with PBST. The positive control was bound with the OIC solution only, and without bacterial antigen suspension. Bound OIC was measured by incubating the wells with donkey anti-sheep IgG-peroxidase antibody (Sigma, diluted 10,000-fold with PBST) for 2 h at 37°C and bound peroxidase activity was determined as described earlier. The degree of crossreactivity was expressed as [(mean A_{450} of test antigen)/(mean A_{450} of OIC control)]×100.

LPS and Toxin-binding Activity

The binding activity of OIC to LPS was investigated by a slight modification of the method of Yu and Kanost [22]. Wells of a flat-bottom 96-well plate were coated overnight at 4°C with 10 μg and 0.1 μg of LPS of *Escherichia coli* O111:B4 and *Salmonella enterica* serotype *typhimurium* (Sigma), respectively. For toxin-binding activity, 1 and 0.01 μg of staphylococcal enterotoxin B (SEB, Sigma) was used instead of LPS. The concentrations of LPS,

toxin, and OIC were determined through preliminary experiments. The plates were washed four times with PBST and blocked with 3% BSA in PBS for 1 h at 37°C, followed by washing four times with PBST. OIC solutions at different concentrations prepared in PBS were added at 100 μl per well and binding was allowed for 2 h at 37°C before washing five times with PBST. Bound OIC solutions were measured by incubating the wells with donkey anti-sheep IgG-peroxidase antibody (Sigma, diluted 10,000-fold with PBST) as described earlier.

The binding activity of OIC to LPS was also determined based on a culture system using a lymphoblast K-562 cell line. Lymphoblast K-562 cells were plated in 96-well microplates at a cell density of 1.0×10^4 cells per well and then incubated for 24 h. Various concentrations of OIC solution (50 μl) were combined with 50 μl LPS solution (160 μg/ml) of *E. coli* O111:B4 or *S. enterica* serotype *typhimurium* (Sigma), respectively, and incubated at 37°C for 30 min. The mixed solutions were added at 100 μl into 100 μl of 24 h incubated lymphoblast K-562 and then incubated at 37°C for 72 h. After incubation for 72 h, cell viability was observed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [23]. Briefly, 50 μl of an MTT solution (2 g/l stock) was overlaid to each well. After 4 h of incubation, the culture medium in each well was replaced with 150 μl of dimethylsulfoxide. Absorbance was measured at 540 nm using an ELISA plate reader.

Statistics

The data were calculated as means±SEM and analyzed using the GLM procedure of the SAS package (SAS Institute, Cary, NC, USA). The statistical significance of differences among means was determined using Duncan's multiple range [24].

Results

Composition of OIC

The ELISA results showed that IgG and IgM comprised about 73±2% and 11±1% of OIC on a protein basis, respectively. As shown in Fig. 1, the OIC purification procedure used in

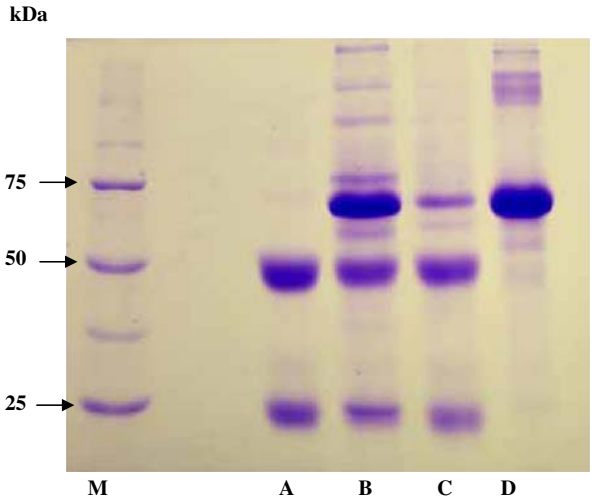

Fig. 1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of an ovine immunoglobulin concentrate purified from ovine blood. *M* Precision-plus protein standard (Bio-Rad), *A* sheep serum IgG (Sigma), *B* ovine serum, *C* ovine immunoglobulin concentrate purified in this study, *D* bovine serum albumin (Sigma)

the present study was capable of isolating high concentrations of immunoglobulin fractions and removing the majority of the albumin. Heavy chain fragments of immunoglobulin migrated to an area of about 50 kDa and light chain fragments migrated as discrete bands to an area corresponding to around 25 kDa on the gel.

Pathogen Growth Inhibition

The inhibitory activity of OIC (percent decrease in growth of bacteria relative to control) against 13 strains of pathogens is shown in Table 1. OIC was able to inhibit the growth of all pathogens tested but the inhibitory activity varied according to the strain of pathogen (Table 1). Among strains tested, the *Pseudomonas aeruginosa* ATCC 25668 and *E. coli* NCTC 10863 strains, the growth of which was the most reduced (more than 30%), were selected for further study. As shown in Fig. 2, the control sample containing BSA instead of OIC did not affect growth in the number of pathogens. OIC was sensitive to temperature, with activity disappearing with heating at 65°C for 30 min. When OIC was digested with pepsin, the inhibitory activity was lost. OIC at concentrations of 10% and 20% significantly reduced the growth of the two pathogens ($p<0.01$) compared with the control; however, there were no significant differences between the results for the two concentrations of OIC.

Crossreactivity

The binding activity of OIC to different strains of pathogens was assessed using a crossreactivity assay. Regardless of cell wall structural differences, OIC bound to all of the Gram-negative and Gram-positive bacteria, with the highest magnitude of crossreactivity being against *Staphylococcus epidermidis* and *Shigella soneii* strains ($p<0.001$; Fig. 3). Crossreactivity differed according to strain, but binding activity against the same species, for example the three strains of *E. coli* (pathogens numbers 8, 9, and 10), showed a similar tendency.

LPS and Toxin-binding Activity

Figure 4 shows that the binding activity of OIC to two kinds of LPS (*E. coli* and *S. typhimurium*) was concentration dependent and saturable, showing typical hyperbolic curves.

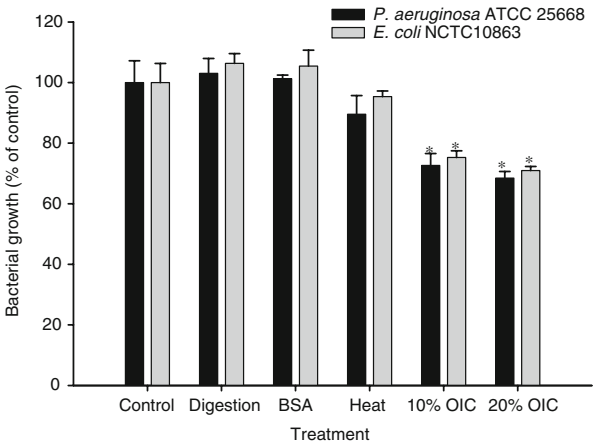

Fig. 2 Growth inhibition of *E. coli* NCTC 10863 and *P. aeruginosa* ATCC 25668 by variously treated samples. *Digestion* Sample digested by pepsin, *BSA* bovine serum albumin, *Heat* sample heated at 65°C for 30 min. *Growth rate was significantly different between treatments ($p<0.01$)

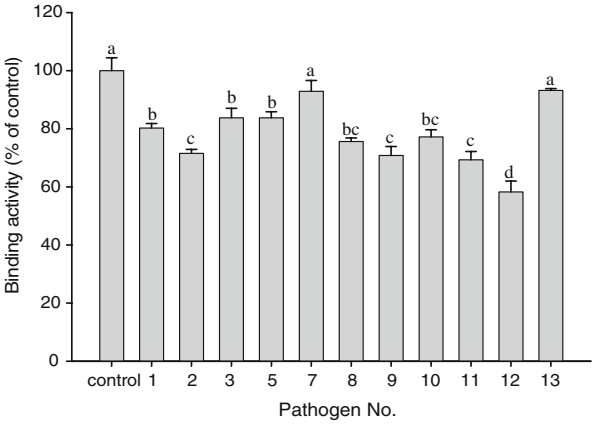

Fig. 3 Crossreactivity of an ovine immunoglobulin concentrate against whole cell antigens. Values are expressed as the mean percentage of crossreactivity compared with control and *bars* indicate the standard error. Means without a common letter differ ($p<0.001$). Pathogen numbers and strains are as described in Table 1

Such curves can be readily analyzed using double reciprocal plots (in the same manner as Michaelis–Menten plots for enzyme activity). Because such plots become error-prone when binding is low, data were plotted for OIC concentrations of 25 to 400 μg/ml (data not shown). These plots were linear (r^2=0.999 and 0.998 for 10 and 0.1 μg of *E. coli* LPS, respectively, and r^2=0.999 for *S. typhimurium* LPS) and enabled the determination of pseudo binding constants (i.e., the concentrations at which the LPS was 50% saturated with OIC) of 66 and 86 μg/ml for 10 and 0.1 μg of *E. coli* LPS, respectively, and 80 and 77 μg/ml for the same concentrations of *S. typhimurium* LPS.

Absorbance values for the higher LPS concentration (10 μg per well) were greater with the addition of increasing amounts of OIC, compared to coating with a lower LPS concentration (0.1 μg per well).

The binding activity of OIC to LPS was also investigated using the lymphoblast K-562 cell line. The effects of OIC on the lymphoblasts which were inhibited by 40 μg/ml of LPS are shown in Fig. 5. When two kinds of LPS were incubated with OIC before being added to the lymphoblast K-562 cells, OIC was found to interact with LPS so that OIC suppressed the inhibition by LPS of the growth of lymphoblast K-562 cells. Cell growth, as indicated by absorbance values, was markedly increased by the addition of 200 μg/ml OIC and at

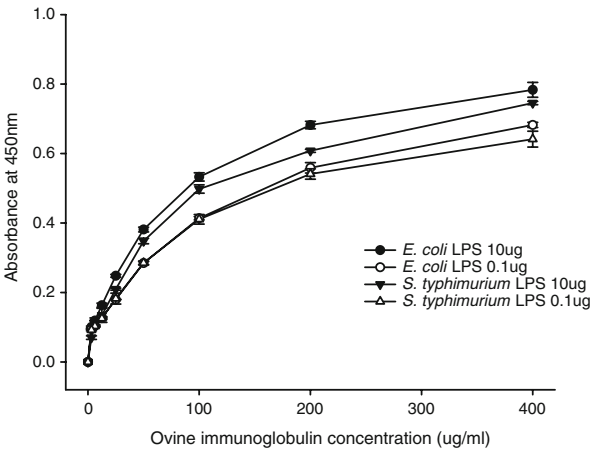

Fig. 4 The binding activity of an ovine immunoglobulin concentrate to two kinds of LPS obtained from *E. coli* O111:B4 and *S. enterica* serotype *typhimurium*. Values are expressed as mean optical density at 450 nm and *bars* indicate the standard error

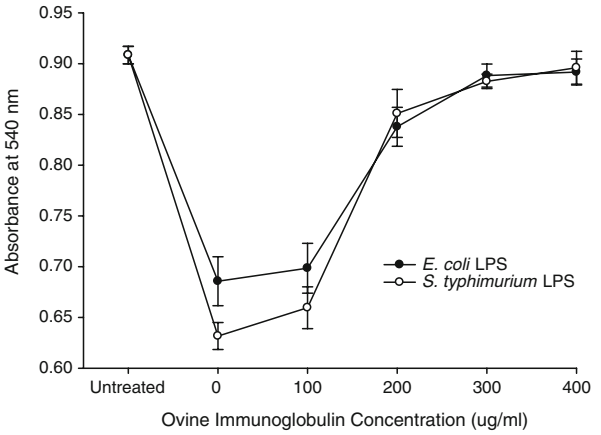

Fig. 5 Effect of an ovine immunoglobulin concentrate on the proliferation of lymphoblast K-562 cells treated with LPS. Values are expressed as means of absorbance at 540 nm and *bars* indicate the standard error

concentrations (300 and 400 μg/ml) above this, increased towards similar values to the control. The lack of an effect of OIC at 100 μg/ml may indicate a titration effect (i.e., there was insufficient OIC to bind to all the LPS), whereas the increases above 200 μg/ml are likely to be due to binding equilibrium.

For toxin-binding activity, the same trend as the LPS-binding pattern was observed. Absorbance values increased with the addition of increasing concentrations of OIC, showing concentration dependence and saturation (Fig. 6). Again, double reciprocal plots were highly linear (r^2=0.997 and 0.998) and pseudo binding constants were 12 and 5 μg/ml for 0.1 and 0.001 μg of SEB, respectively.

Discussion

Animal plasma proteins are natural sources of concentrated immunoglobulins, especially IgG. The availability of both protein sources is substantial and may provide a new alternative supplement for animal feeds or for functional foods for humans [25, 26]. The beneficial

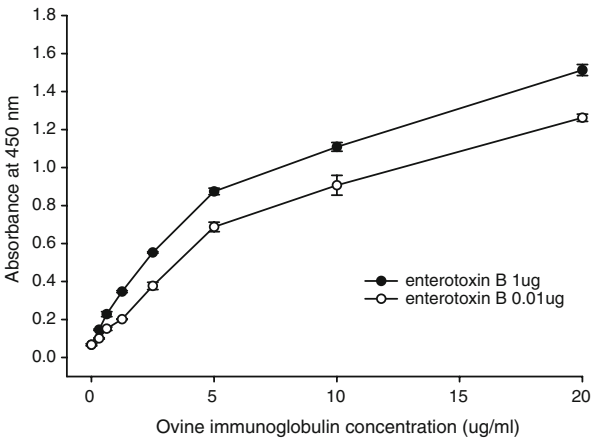

Fig. 6 The binding activity of an ovine immunoglobulin concentrate to staphylococcal enterotoxin B. Values are expressed as mean optical density at 450 nm and *bars* indicate the standard error

effects on growth performance resulting from the inclusion of SDAP-containing IgG in diets for early weaned animals have been reported in various studies [6, 7, 10, 14, 27, 28].

Among plasma proteins, albumin is the most abundant, normally comprising 50–70% of total protein and the other three abundant proteins are the alpha, beta, and gamma globulins. The gamma globulins (also called immunoglobulins or antibodies) consist of two identical light chains (polypeptides of about Mw 25,000) and two identical heavy chains (larger polypeptides of Mw 50,000 or more) [29].

In the present study, OIC was efficiently isolated from ovine blood at around 84% of total protein (including about 73% IgG) through a purification procedure. By comparison, average IgG concentrations of commercial porcine and bovine IgG-rich fractions used in animal experiments reported within the literature are generally less than 60% [5]. In view of the high immunoglobulin contents, the presently studied OIC is a potentially valuable supplement for providing concentrated immunoglobulins. The ability of OIC to exhibit inhibitory activity against a wide range of pathogens is likely attributable to the high content of immunoglobulin present. This is supported by the observation that heating induced the loss of inhibitory activity of OIC, consistent with denaturation of the immunoglobulin proteins. Ovine blood is known to contain various kinds of small antibacterial peptides like cathelicidins [19], and OIC could also include some of these; however, they would not be expected to show heat denaturation. The loss of antibacterial activity when OIC was digested with pepsin also shows that the protein structures in OIC are responsible for the observed antibacterial activity.

Pathogen infections, such as those resulting from the enterotoxigenic *E. coli* and *Staphylococcus aureus* strains tested in this study, are a serious problem for young animals with relatively undeveloped gastrointestinal tracts and immature mucosal immune systems. The mode of action by which SDAP improves the status of young animals challenged with these pathogens is not completely understood, but it has been suggested that the positive responses might result from specific immunoglobulin proteins present in the plasma [9–11, 17, 30].

In the present study, OIC displayed binding activity to the surface of pathogens as well as direct growth inhibition, but we did not find a correlation between crossreactivity and antibacterial activity. The immunoglobulins present in SDAP are reported to bind to potential antigens and prevent their attachment to the lumenal mucosa of the intestine [9]. OIC also bound to LPS which presents on the surface of Gram-negative bacteria and is a potential target for the binding of pattern recognition receptors [22]. Bacterial LPS can enter the circulation via the gastrointestinal tract under certain conditions like gut epithelium damage and can cause serious physiological effects in humans and animals including fever and induction of an acute phase response [31]. Bacterial LPS present in the digestive tract also decreases daily gain, feed intake, and feed efficiency in domestic animals [32, 33], but the addition of SDAP to animal feeds is capable of protecting against LPS-induced effects and has been reported to reduce inflammatory cytokine expression in tissues exposed to LPS [16].

The present work also investigated the effects of OIC on lymphoblast cells treated with 40 μg/ml of LPS demonstrating that OIC can bind to LPS and suppress the inhibitory action of LPS on cell growth. OIC has antibacterial and protective functions which may play a critical role in protection from Gram-negative bacteria diseases.

OIC may bind to the enterotoxin SEB. SEB causes a variety of diseases in animals and humans ranging from food poisoning to shock [17, 34]. Other studies have shown that SDAP or plasma immunoglobulin concentrates reduce diarrhoea in weaned rats challenged with SEB [17, 35].

Effective protection factors against pathogenic bacteria should have the ability to prevent the adherence of the bacteria to the intestinal epithelium and to inhibit the action of toxins of non-invasive pathogens such as enterotoxigenic *E. coli*. OIC is a promising candidate for protecting the gut against pathogenic bacteria, but more work is required especially to determine optimal dosage rates for specific pathogens.

To our knowledge, this is the first demonstration in vitro of the anti-pathogenic activity of ovine serum immunoglobulins. More studies are required to investigate the degradation of OIC in the digestive tract and effects in vivo. OIC may be a viable alternative to in-feed antibiotics.

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