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In-vitro Validation of Cytokine Neutralizing Antibodies by testing with Ovine Mononuclear Splenocytes

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Summary

Cytokines have gained increasing attention as therapeutic targets in inflammation-related disorders and inflammatory conditions have been investigated in sheep. Monoclonal antibodies (mAbs) specific for the ovine pro-inflammatory cytokines, interleukin (IL)-1 β and IL-6 could be used to study the effects of blocking pro-inflammatory cytokines in sheep. Ovine-specific IL-1 β and IL-6 proteins and mAbs specific for these molecules were produced and the ability of the mAbs to neutralize the proteins was tested in cultures of ovine splenic mononuclear cells. Expression of nuclear factor (NF)- κ B and signal transducer and activator of transcription (STAT)-3 was evaluated by western blotting and densitometric quantification. Treatment with purified IL-1 β and IL-6 proteins increased NF- κ B ($P < 0.001$) and STAT-3 ($P < 0.01$) expression, (respectively, in cell culture. Treatment with these proteins that were pre-incubated with IL-1 β and IL-6 mAbs attenuated ($P < 0.01$) these effects. These results confirm the bioactivity of ovine IL-1 β and IL-6 proteins and neutralizing capacity of anti-ovine-IL-1 β and -IL-6 mAbs *in vitro*. These mAbs could be used to investigate anti-inflammatory strategies for attenuation of the effects of these pro-inflammatory cytokines in sheep.

Keywords

monoclonal antibody; mononuclear cells; pro-inflammatory cytokines; sheep

Introduction

Cytokines are a group of proteins that are secreted by cells of the immune system, glial cells of the nervous system, trophoblasts and endothelial cells (Pantoni *et al.*, 1998; Thaxton *et al.*, 2009; Thaxton and Sharma, 2010; Lai *et al.*, 2011). Cytokines are signalling molecules, binding with high affinity to cell surface receptors that trigger intracellular signalling cascades that regulate immunity and inflammation in target cells. Injury increases vascular

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Conflict of Interest

The authors declare no conflict of interest.

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and local cytokine expression, which can exacerbate tissue damage (Stanimirovic *et al.*, 1997; Rothwell, 1999; Ransohoff *et al.*, 2003). Interleukin (IL)-1 β and IL-6 are two of the most widely studied pro-inflammatory cytokines known to cause tissue damage (Dinarello, 2000; Denker *et al.*, 2007).

Pro-inflammatory cytokines have become increasingly important therapeutic targets in numerous immune and inflammatory disorders (Vilcek and Feldmann, 2004). Therefore, reducing the biological effects of pro-inflammatory cytokines with neutralizing antibodies (Dinarello, 2000) has recently been posited as a therapeutic intervention and has been successful in disorders such as rheumatoid arthritis (Alten *et al.*, 2008) and neuronal damage in traumatic brain injury (Lu *et al.*, 2005).

Sheep have been used widely to study a variety of disorders related to human disease (Petersson *et al.*, 2002; Petersson *et al.*, 2004; Soehnlein *et al.*, 2010), including inflammatory conditions in both adult and fetal sheep (Hagberg *et al.*, 2002; Mallard *et al.*, 2003; Collins *et al.*, 2010; Hillman *et al.*, 2010; Lange *et al.*, 2010; Shah *et al.*, 2010; Soehnlein *et al.*, 2010; Rehberg *et al.*, 2011). Monoclonal antibodies (mAbs) specific for ovine IL-1 β and IL-6 have been generated (Rothel *et al.*, 1997; McWaters *et al.*, 2000). Additional characterization of their neutralizing properties (Rothel *et al.*, 1997) could result in the potential use of these mAbs to examine the effects of blocking pro-inflammatory cytokines in sheep.

To begin to establish the feasibility of using these neutralizing mAbs to examine their anti-inflammatory characteristics in sheep, we generated and further purified ovine IL-1 β and IL-6 proteins and their respective mAbs using previously established protocols (Wood *et al.*, 1990; Rothel *et al.*, 1997; McWaters *et al.*, 2000). Although these mAbs exhibit high sensitivity and specificity to their corresponding cytokine proteins, their neutralizing characteristics have only been characterized partially for IL-1 β mAbs (Rothel *et al.*, 1997), but not for IL-6 mAbs (McWaters *et al.*, 2000). Therefore, the aims of the present study were to investigate the biological effects of ovine IL-1 β and IL-6 proteins and to characterize the neutralizing properties of their respective specific mAbs in cultures of ovine mononuclear spleen cells.

Materials and Methods

Cytokines and Antibodies

Recombinant ovine IL-1 β and IL-6 proteins encoded by pGEX-2T and pQE30 vectors, respectively, were produced as described by Seow *et al.* (1994) with modifications as follows. IL-6 and IL-1 β were further purified on DEAE-CIM anion-exchange monolithic resins (BIASeparations, Villach, Austria). Most of the contaminants were bound tightly on the DEAE column, whereas the IL-6 and IL-1 β proteins were found and collected in the unbound fraction. An additional separation of high molecular weight contaminants was performed on a TSKgel G3000SW size exclusion chromatographic column (Tosoh Bioscience, King of Prussia, Pennsylvania, USA). Pure IL-6 and IL-1 β proteins were obtained after these two chromatographic procedures.

Anti-ovine IL-1 β and IL-6 murine mAbs were produced as described previously (Wood *et al.*, 1990; Rothel *et al.*, 1997; McWaters *et al.*, 2000) with modifications as follows. The immunoglobulin (Ig) G of the mAbs was purified from cell culture supernatants by affinity chromatography on protein G sepharose (GE Healthcare Bio-Sciences Corp., Piscataway, New Jersey, USA). Bound antibodies were eluted by 0.1 M glycine-HCl (pH 2.3), neutralized, buffer exchanged and concentrated on Millipore ultrafiltration devices with 30 kDa cut-off membranes. Anti-ovine IL-1 β and IL-6 mAbs have been reported to be IgG1

isotypes (Rothel *et al.*, 1997; McWaters *et al.*, 2000). The original clone identification for anti-ovine IL-1 β was designated as AH.IL1.1 (Rothel *et al.*, 1997) and for anti-ovine IL-6 mAbs designated as 4B6 (McWaters *et al.*, 2000). The pGEX-2T and pQE30 vectors and mouse hybridoma cells were generously supplied by the Commonwealth Scientific and Industrial Research Organization (CSIRO), Livestock Industries, Victoria, Australia.

Ovine Splenic Mononuclear Cells

Six individual spleens from healthy yearling sheep were obtained from a US Department of Agriculture-approved facility. The spleens were placed in 500 ml of cold 0.9% phosphate buffered saline (PBS; Bio-Rad Laboratories, Hercules, California, USA) before processing. The outer splenic membrane was removed and the inner pulp homogenized with 200–300 ml of cold PBS. The homogenized splenic tissue was filtered through successively smaller mesh filters (120 to 20 μ m; Bio-Rad). Equivalent amounts of PBS were added to the filtered cells and these were centrifuged at 650 g at 4°C for 5 min. After removing the supernatant, the pellet was resuspended in four equal volumes of red blood cell (RBC) lysis buffer (Sigma, St Louis, Missouri, USA), the cell suspension was vortexed for 30 sec and incubated at room temperature for another 2 min. Thereafter, the RBC lysis buffer was neutralized with an equal volume of cold PBS, the cells were centrifuged again and the pellet obtained as described above. After an additional wash with four equal volumes of PBS, the pellet was collected and resuspended in 10 ml of Roswell Park Memorial Institute (RPMI) medium (Sigma). Cell viability was assessed using trypan blue dye and the number of cells was determined in a counting chamber. After isolation the cell viability was 68%.

Ovine Splenic Mononuclear Cell Assays

The same volume of isolated ovine splenic mononuclear cells was placed into 60 mm Petri dishes containing 20 ml of RPMI at a density of 1×10^7 cells per dish. In order to have sufficient mAbs to bind to the respective cytokine proteins, the concentrations of mAbs used were several orders of magnitude higher than the protein concentrations. In view of the fact that McWaters *et al.* (2000) used 500 ng and 1,000 ng of anti-IL-6 mAb in enzyme-linked immunosorbent assay (ELISA) and flow cytometry assays, respectively, and Rothel *et al.* (1997) used 1,000 ng/ml of ovine IL-1 protein to screen the antibodies by ELISA, we added 100 ng/ml of IL-1 β or IL-6 protein, 1,000 ng/ml of anti-IL-1 β or anti-IL-6 antibody alone, and 100 ng/ml of IL-1 β or IL-6 protein, which had been pre-incubated at 4°C for 1 h with 1,000 ng/ml anti-IL-1 β or anti-IL-6 antibodies, respectively, to the mononuclear cells. In addition to PBS (100 μ l), mononuclear cells were treated with 1,000 ng/ml of non-specific mouse anti-sheep IgG (isotype IgG1, AbD Serotec, Raleigh, North Carolina, USA) to serve as an additional control. For the additional control experiments, ovine mononuclear cells derived from one spleen were treated in five independent experiments with PBS, IL-1 β protein, non-specific mouse anti-sheep IgG antibodies alone and IL-1 β protein, which had been pre-incubated with the non-specific mouse anti-sheep IgG antibodies, respectively.

After 30 min of incubation at 37°C in a humidified incubator, the cell suspensions were transferred into 50 ml falcon tubes and centrifuged at 250 g at 4°C for 5 min. The cell pellets were washed with 10 ml of cold PBS and resuspended in 1 ml of buffer F (10 mM Tris-HCl pH 7.05, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 5 μ M ZnCl₂, 0.1 mM NaVO₄, 1% Triton-X 100) to which a proteinase cocktail inhibitor and phosphatase inhibitor (Roche, Tucson, Arizona, USA) were added. The cell suspension was incubated on ice for 10 min, vortexed for 45 sec and centrifuged at 16,000 g at 4°C for 10 min. The protein concentrations of each fraction were determined using a bicinchoninic acid protein assay (BCA, Pierce, Rockford, Illinois, USA) with bovine serum albumin as a standard.

Western Blotting

Aliquots adjusted for equal loading of 10 µg of protein in 20 µl of solution were loaded onto sodium dodecyl sulphate (SDS) polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membranes (0.2 µm; Bio-Rad) using a semi-dry technique. Ten percent polyacrylamide gels were used for detection of IL-1β, IL-6, nuclear factor (NF)-κB and signal transducer and activator of transcription (STAT)-3 detection. Membranes were blocked with 5% non-fat milk in Tris-buffered saline with Tween (TBST) for 1 h at room temperature, washed in TBST three times for 10 min per wash, and incubated overnight at 4°C with the appropriate primary antibody solutions. The membranes were probed with anti-IL-1β (rabbit polyclonal antibody; Lifespan Biosciences, Seattle, Washington, USA), anti-IL-6 (mouse mAb; Millipore, Billerica, Massachusetts, USA), anti-NF-κB (rabbit polyclonal antibody; Abcam, Cambridge, Massachusetts, USA), and anti-STAT-3 antibodies (rabbit polyclonal antibody; Cell Signalling, Danvers, Massachusetts, USA) at dilutions of 1 in 10,000, 1 in 5,000, 1 in 500 and 1 in 2,000, respectively. Rabbit polyclonal anti-NF-κB and anti-STAT-3 antibodies detect the p65 subunit of NF-κB and total STAT-3 protein expression, respectively. These specific antibodies were selected because of their ability to detect these proteins in ovine mononuclear cells.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as an internal loading control to ensure that equal amounts of protein were applied to each lane. GAPDH was probed with mouse monoclonal anti-GAPDH at a dilution of 1 in 5,000 (Imgenex, San Diego, California, USA). The blots were washed in TBST three times for 10 min per wash and incubated for 1 h at room temperature with goat anti-rabbit secondary antibody (Alpha Diagnostic, San Antonio, Texas, USA) at dilutions of 1 in 10,000, 1 in 2,000 and 1 in 5,000 for IL-1β, NF-κB and STAT-3, respectively, and with goat anti-mouse secondary antibody (Invitrogen, Grand Island, New York, USA) at dilutions of 1 in 5,000 and 1 in 10,000 for IL-6 and GAPDH, respectively. After washing three times with TBST (10 min per wash), the blots were 'developed' using enhanced chemiluminescence (ECL)-plus western immunoblotting detection reagents (GE Healthcare) and exposed to Hyperfilm ECL (GE Healthcare).

Densitometry

Gel-Pro Analyzer software (Media Cybernetics, Bethesda, Maryland, USA) was used to analyze band intensities. Densitometry values for each experiment from each mononuclear cell preparation were derived from one or two blots and expressed as a ratio to the PBS-treated control samples

Statistical Analysis

One-way analysis of variance (ANOVA) was used to compare values among ovine splenic mononuclear cells that had been exposed to the four different treatment conditions: PBS, IL-1β or IL-6 protein, anti-IL-1β or anti-IL-6 mAbs or mouse anti-sheep IgG1 each alone, and IL-1β or IL-6 or IL-1β proteins that were pre-incubated with IL-1β or IL-6 mAbs or mouse anti-sheep IgG1, respectively. If a significant difference was found by ANOVA, the Fischer's least-significant difference (LSD) test was used to detect specific differences among the study conditions. Values are expressed as means ± SD. $P < 0.05$ was considered statistically significant.

Results

Purity of Ovine IL-1β and IL-6 Proteins

Previous findings that the purity of IL-1β and IL-6 proteins was > 95% (McWaters *et al.*, 2000) were confirmed, but additional purification procedures described above were also

performed. Figs. 1A and B illustrate the western blots for IL-1 β and IL-6, respectively, before and after the additional purification procedures. Fig. 1A shows that the IL-1 β protein contained a 37 kDa contaminant before, but not after purification, and Fig. 1B shows that the IL-6 protein contained a 50 kDa contaminant before, but not after, purification. Western blots for IL-1 β and IL-6 also show that the respective antibodies for both IL-1 β and IL-6 proteins recognize clear double bands, suggesting the presence of molecular variants for both proteins. There were no other reactive bands observed on either western blot other than those shown in Figs. 1A or 1B, suggesting the high grade of purity of these proteins.

Effects of Ovine IL-1 β and IL-6 Protein Stimulation and Inhibition of Protein Stimulation by IL-1 β and IL-6 mAbs on NF- κ B and STAT-3 Expression

Incubation of splenic mononuclear cells with ovine IL-1 β increased the expression of NF- κ B by 56% compared with the PBS control values (Fig. 2A, solid bar, $P < 0.001$; Table 1). Pre-incubation of ovine IL-1 β protein with the purified anti-IL-1 β mAb attenuated the effects of the IL-1 β protein stimulation on NF- κ B expression in the mononuclear cells (Fig. 2A, $P < 0.001$ solid versus grey bar; Table 1). In contrast, pre-incubation of ovine IL-1 β protein with non-specific mouse anti-sheep IgG1 antibody did not attenuate the stimulatory effects of IL-1 β protein on the NF- κ B protein expression protein in mononuclear cells (Fig. 2B, $P > 0.05$, solid bar versus grey bar). In addition, western blotting analysis revealed that incubation with the anti-IL-1 β and non-specific mouse anti-sheep IgG1 alone at 1,000 ng/ml also did not affect the expression of NF- κ B compared with the PBS control (Fig. 2A, hatched bar versus PBS, P value is non-significant and Table 1, and Fig. 2B hatched bar versus PBS, P value is non-significant).

Similarly, incubation of mononuclear cells with ovine IL-6 increased the expression of STAT-3 by 34% compared with PBS control values (Fig. 3 solid bar; $P < 0.01$ and Table 1). Pre-incubation of the ovine IL-6 protein with the purified anti-IL-6 mAbs attenuated the stimulatory effects of the IL-6 protein on STAT-3 expression (Fig. 3, grey bars versus solid bars, $P < 0.01$). Western blotting revealed that incubation with the anti-IL-6 mAbs alone did not increase the expression of STAT-3 compared with the PBS control (Fig. 3 hatched bars, P is non-significant and Table 1). Expression of GAPDH protein confirmed equal protein loading in each lane on western blots (Figs. 2A and 3).

Discussion

There is increasing evidence that inflammatory cytokines are involved in the pathogenesis of a range of human perinatal disorders. The roles of IL-1 β and IL-6 in perinatal disease are not entirely clear, although their increased expression in the central nervous system (CNS) correlates with the severity of brain injury and neurological deficits in infants (Mustafa *et al.*, 1989; Aly *et al.*, 2006). However, extensive investigation into the role of these cytokines in perinatal brain damage has been conducted mainly in small animal models. Despite extensive study utilizing different methods, the precise contribution of IL-1 β and IL-6 in the pathogenesis of brain damage in the premature infant has not been firmly established. Adult and fetal sheep have also been used as models to study clinically relevant conditions in a variety of organs including brain (Hagberg *et al.*, 2002; Mallard *et al.*, 2003; Collins *et al.*, 2010; Hillman *et al.*, 2010; Lange *et al.*, 2010; Shah *et al.*, 2010; Soehnlein *et al.*, 2010; Rehberg *et al.*, 2011), and thus, neutralizing antibodies to ovine cytokines could provide a novel strategy to understanding the possible roles of IL-1 β and IL-6 in the pathophysiology of perinatal injury (Hagberg *et al.*, 1996).

Generation of mAbs specific for ovine IL-1 β and IL-6 and their functional characterization is essential for determining the potential use of such mAbs in pathophysiological studies. Several factors need be considered when determining the quality of antibodies, including

their specificity, sensitivity and functional efficacy. The sensitivity and specificity of anti-ovine IL-1 β and IL-6 mAbs were characterized previously by ELISA and western blotting (Rothel *et al.*, 1997; McWaters *et al.*, 2000). The mAb to ovine IL-1 β does not cross-react with recombinant ovine IL-1 α , IL-2, IL-4, IL-8, tumour necrosis factor (TNF)- α , interferon (IFN)- γ or recombinant human IL-1 β , reflecting the sensitivity and specificity of the antibody (Rothel *et al.*, 1997). The mAb to ovine IL-6 does not cross-react with recombinant ovine IL-1 β , IL-2, IL-8, TNF- α , granulocyte macrophage colony-stimulating factor (GM-CSF) or recombinant bovine IFN- γ (McWaters *et al.*, 2000). In the present study, we further documented that specific anti-IL-1 β and anti-IL-6 mAbs neutralize the stimulatory effects of ovine IL-1 β and IL-6 proteins in isolated splenic mononuclear cells. It is well known that IL-1 β and IL-6 mediate their actions mainly by binding to their receptors and increasing expression of the NF- κ B and STAT-3 transcription factors through the TNF and Janus kinase (JAK)/STAT signalling pathways, respectively (Kitamura *et al.*, 2005; Albrecht *et al.*, 2007; Carey *et al.*, 2008; Duncan *et al.*, 2009). Compared with control experiments using the non-specific mouse anti-sheep IgG1, treatment with specific IL-1 β antibody attenuated the increased NF- κ B protein expression after exposure to ovine IL-1 β protein, which supports the contention that IL-1 β mAb is a neutralizing antibody. Our findings are consistent with previous work in which pre-incubation of ovine IL-1 β protein with its specific mAb reduced the biological activity of ovine IL-1 β in a thymocyte-based co-stimulation assay (Rothel *et al.*, 1997). It is also important to note that the present study is the first to demonstrate the ability of specific ovine IL-6 mAb to neutralize the stimulatory effects of ovine IL-6 protein, because pre-incubation of the protein with its specific mAb inhibited IL-6 protein stimulatory effects on the STAT-3 expression.

In summary, the present study has described two downstream transcription factors, NF- κ B and STAT-3, whose expression is induced by signalling pathways in response to ovine IL-1 β and IL-6 proteins, respectively. Up-regulation of protein expression of these transcription factors in response to cytokines is reduced by the respective mAbs specific for these cytokines. In view of the fact that IL-1 β and IL-6 mAbs have high sensitivity and specificity for the corresponding ovine cytokines, both specific mAbs could be used in studies that investigate the mechanisms of pro-inflammatory cytokines in inflammation-related disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge the supply of the ovine IL-1 β pGEX-2T and ovine IL-6 pQE30 vectors, with which we produced the IL-1 β and IL-6 specific proteins, and the mouse hybridoma cell lines, from which we produced the mAbs against ovine IL-1 β and IL-6, from CSIRO, Livestock Industries, Victoria, Australia. We also would like to acknowledge the excellent editorial assistance of Dr. C. A. Hill. The study was supported by grants HD-057100, 3R01HD057100-02S1 and R1-INBRE P20RR016457-11.

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Fig. 1.

Western blots for (A) IL-1 β and (B) IL-6 before and after the additional purification procedure. (A) The IL-1 β protein contained a 37 kDa contaminant before, but not after purification. (B) The IL-6 protein contained a 50 kDa contaminant before, but not after purification.

Fig. 2.

(A) Representative western blots and bar graphs of NF- κ B protein expression by splenic mononuclear cells. NF- κ B expression is plotted as a ratio to PBS control (open bar). Treatment with ovine IL-1 β protein increased NF- κ B (solid bar, $n = 6$, $P < 0.001$) expression. Anti-IL-1 β mAb (hatched bar, $n = 6$) alone did not influence NF- κ B protein expression. Pre-incubation of ovine IL-1 β with its mAb (grey bar, $n = 6$) reduced NF- κ B ($P < 0.001$) expression. GAPDH was used as a loading control. (B) Representative western blots and bar graphs of control experiment using anti-ovine IgG1 antibody. NF- κ B expression is plotted as a ratio to PBS control (open bar). Treatment with ovine IL-1 β increased NF- κ B (solid bar, $P < 0.001$) expression. Anti-ovine IgG1 antibody (hatched bar) alone did not influence NF- κ B protein expression. Pre-incubation ovine IL-1 β with anti-ovine IgG1 antibody (grey bar) did not attenuate the ovine IL-1 β protein stimulated increase in NF- κ B expression ($P > 0.05$). GAPDH was as a loading control.

Fig. 3.

Representative western blots and bar graphs of STAT-3 protein expression by splenic mononuclear cells. STAT-3 expression is plotted as a ratio to PBS control (open bar). Treatment with ovine IL-6 increased STAT-3 (solid bar, $n = 5$, $P < 0.01$) expression. Anti-IL-6 mAb (hatched bar, $n = 5$) alone did not influence STAT-3 protein expression. Pre-incubation of ovine IL-6 with its mAb (grey bar, $n = 5$) reduced STAT-3 expression (ANOVA, $P < 0.01$). GAPDH was used as a loading control.

Table 1

Densitometry analysis for NF-κB and STAT-3 protein expression

		NF-κB			STAT-3		
Treatment	PBS	IL-1β protein	IL-1β Ab	IL-1β protein + IL-1β Ab	PBS	IL-6 protein	IL-6 protein + IL-6 Ab
Ratio to PBS	1.00	1.56 ± 0.26 [*]	1.07 ± 0.13	1.03 ± 0.28 [†]	1.00	1.34 ± 0.25 [*]	1.03 ± 0.14 [†]

PBS, phosphate buffered saline; Ab, antibody; values are means ± SD; NF-κB, *n* = 6, STAT-3, *n* = 5;

^{*} *P* < 0.05 versus PBS,

[†] *P* < 0.05 versus IL-1β or IL-6 protein treated