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A newly established bovine intestinal epithelial cell line is effective for in vitro screening of potential antiviral immunobiotic microorganisms for cattle

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We evaluated whether a bovine intestinal epithelial (BIE) cell line could serve as a useful in vitro model system for studying antiviral immune responses in bovine intestinal epithelial cells (IECs) and for the primary screening of immunobiotic microorganisms with antiviral protective capabilities. Immunofluorescent analyses revealed that toll-like receptor 3 (TLR3) was expressed in BIE cells, and the results of real-time quantitative PCR showed that these cells respond to stimulation with poly(I:C) by up-regulating pro-inflammatory cytokines and type I interferons. In addition, we demonstrated that BIE cells are useful for the primary screening of immunobiotic lactic acid bacteria strains which are able to beneficially modulate antiviral immune responses triggered by TLR3 activation in bovine IECs. The characterization of BIE cells performed in the present study represents an important step towards the establishment of a valuable bovine in vitro system that could be used for the development of immunomodulatory feed for bovine hosts.

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1. Introduction

Diarrhea is an important cause of morbidity and mortality in young calves, resulting in significant financial losses to cattle producers. In particular, bovine neonatal gastroenteritis is a multifactorial disease that can be caused by a number of pathogens, including bovine rotavirus (BRV), bovine coronavirus (BCV), and bovine viral diarrhea viruses (BVDVs) (Aich et al., 2007; Lee et al., 2008). Although these viruses belong to different families and have distinct physical characteristics, they are all able to infect intestinal epithelial cells (IECs), and induce villous atrophy and diarrhea.

IECs are able to distinguish between the diverse elements present in the intestinal environment through the expression of pattern recognition receptors, such as toll-like receptors (TLRs) (Westendorf et al., 2010). Among TLRs, TLR3 recognizes dsRNA and is therefore important for defense against viral infections. Upon recognition of viral dsRNA, TLR3 transmits signals that activate transcription factors responsible for the induction of type I interferon (IFN; IFN-α/β) and IFN-inducible genes, which play critical roles in antiviral host defense (Matsumoto et al., 2002). Thus, activation of TLR3 signaling is of great importance for defense against BRV, a dsRNA virus, and BCV and BVDV, which replicate via intermediary dsRNA.

Recently, significant progress has been made in understanding the role of TLR3 in innate and adaptive immunity. The majority of studies aimed at dissecting the mechanisms of TLR3 function have been performed principally in mouse and human cell lines (Gauzzi et al., 2010). However, few studies have been conducted on cattle, despite growing interest in the bovine immune system due to the economic importance of cattle as livestock. Therefore, investigating how TLR3 mediates antiviral defenses in bovine IECs is important for understanding the activation and regulation of the intestinal immune system of cattle. Moreover, determining the underlying mechanisms of TLR3 activation and regulation in bovine IECs may aid in the development of effective therapies for the prevention and treatment of viral diseases, such as oral vaccines and functional feed, which specifically target anti-viral immune responses in the bovine gut.

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We have recently established a bovine intestinal epithelial (BIE) cell line originally derived from fetal bovine intestinal epithelial cells (Miyazawa et al., 2010). In the present study, we aimed to characterize the immune response triggered by TLR3 activation in BIE cells in order to evaluate whether this cell line could serve as an in vitro model of bovine IECs for the study of TLR3-mediated antiviral responses. In addition, we attempted to determine whether BIE cells are suitable for the primary screening of immunomodulatory lactic acid bacteria (LAB) with antiviral protective capabilities in cattle.

2. Materials and methods

2.1. BIE cells

The BIE cell line used in this study is a non-transformed intestinal cell line that was previously derived from fetal bovine intestinal epithelial cells (Miyazawa et al., 2010). BIE cells were routinely maintained in Dulbecco’s Modified Eagle medium (DMEM; Gibco, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS) and penicillin-streptomycin. For passaging, BIE cells were treated with a sucrose/EDTA buffer (0.1 M NaH2PO4/12H2O, 0.45 M sucrose, 0.36% EDTA/4Na, and bovine serum albumin (BSA)) for 4 min and then detached using 0.04% trypsin in phosphate-buffered saline (PBS) (Moue et al., 2008). BIE cells were then plated in type I collagen-coated culture dishes (Sumilon, Tokyo, Japan) at a density of 1.5 × 10^4 cells/cm² and cultured at 37 °C in an atmosphere of 5% CO2 in DMEM (10% FBS, 1% streptomycin/penicillin, 100 U/ml streptomycin, high glucose, L-glutamine, and 0.11 mg/ml sodium pyruvate; Gibco).

2.2. Immunocytochemistry

BIE cells were cultured in collagen type I-coated culture dishes (12-well plate; Sumilon) at a cell density of 3 × 10^4 cells/well for 3 days (37 °C, 5% CO2), washed once with cold PBS (2% FCS), and then treated with FACS permeabilization solution (4 °C, 10 min). Following three washes with PBS, the cells were incubated with 5% normal goat serum (Sigma, St. Louis, MO) for 10 min at 4 °C. Cells were then washed with PBS three times and then incubated with anti–TLR3 polyclonal antibody (sc-28999; Santa Cruz, CA, USA) for 16 h at 4 °C. Following three washes with PBS, cells were treated with secondary Alexa Fluor 488-conjugated goat anti-rabbit polyclonal IgG (Invitrogen, Tokyo, Japan) at 1 h at room temperature. BIE cells incubated with rabbit IgG isotype control antibody (20304E; Imgenex, San Diego, CA) and the identical secondary antibody as above were used as controls. After reaction with secondary antibody, cells were washed three times with PBS and then counterstained with SYTOX orange (Invitrogen) for 5 min at room temperature. Cells were washed three times with PBS, rinsed in distilled water, and then mounted on glass slides with PermaFluor (Thermo Fisher, Pittsburgh, PA). Immunofluorescence microscopy was performed using a confocal laser microscope (MRC-1024; Bio-Rad, Richmond, CA).

2.3. Microorganisms

The following LAB strains were used in this study: Lactobacillus gasseri TMC0356, Lactobacillus rhamnosus LGG, L. rhamnosus LA-2, Lactobacillus casei TMC0409, Streptococcus thermophilus TMC1543, Bifidobacterium bifidum 2–2, and B. bifidum 3–9. The lactobacilli and bifidobacteria strains were grown in MRS medium (Difco, Detroit, MI, USA) for 16 h at 37 °C. S. thermophilus cells were grown in Elliker medium (Difco) for 16 h at 37 °C.

2.4. Anti-inflammatory assay in BIE cells

Bacteria were re-suspended in DMEM (10% FBS and 1% SP), enumerated microscopically using a Petroff-Hauser counting chamber, and stored at −80 °C until use. BIE cells were plated at a density of 1.5 × 10^4 cells/well in 24-well type I collagen-coated plates (Sumilon) containing 1 ml DMEM, and were then cultured for 3 days. After changing the medium, lactobacilli or bifidobacteria (5 × 10^7 cells/ml) were added to 1 ml DMEM in each well. After 24 and 48 h of incubation, each well was washed vigorously with medium at least three times to eliminate all of the stimulants. For comparative treatment, BIE cells were stimulated with 200 ng/ml Pam3CSK4 for 24 or 48 h. After the elimination of lactobacilli, bifidobacteria, or Pam3CSK4, BIE cells were stimulated with poly(I:C) at concentrations of 12.5 and 60 ng/ml for 3, 6, 12, and 24 h.

2.5. Quantitative expression analysis by real-time polymerase chain reactions (PCR) in BIE cells

We performed a two-step real-time quantitative PCR to study the expression of mRNAs in BIE cells. Total RNA was isolated from BIE cells using TRIzol reagent (Invitrogen). All cDNAs were synthesized using a Quantitect Reverse Transcription kit (Qiagen, Tokyo, Japan) according to the manufacturer’s recommendations. Real-time quantitative PCR was performed using a 7300 Real-time PCR System (Applied Biosystems, Warrington, UK) using Platinum SYBR Green qPCR SuperMix UDG with ROX (Invitrogen). The primers used in this study were: β-actin forward (TGG ATT GCC GCC TCC AT) and reverse (GCT GAT CCA CAT CTG GAA); IFN-α forward (GCT GCC AGC CAG TTA CAG AAG) and reverse (TGG TGC ACC TCA TGG A); IFN-β forward (CTT GAG TTT GCC CTG GAT CGT); and reverse (GAA CAA GCT GTA GCT CCT GGA); IFN-γ forward (FGA GGA CTT CAA AAA GAT TCA) and reverse (GGC TTT GCC CTG GAT CTG); IL-6 forward (CCA CCC CAG GAC TAC TTC) and reverse (CCA TGC GCT TAA TGA GAG CCT); IL-8 forward (TGC TCT CTT GGC AGC TCC TT) and reverse (TCT TGA CAC TGC AGC TTC AC); and MCP-1 forward (CAC CAG CAA GTG TTC TAA A) and reverse (CAG ATA ACT CTT GTC CCA GGA T). The PCR cycling conditions consisted of 2 min at 50 °C, followed by 2 min at 95 °C, and then 40 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. The reaction mixture contained 5 ml of sample cDNA and 15 ml of master mix, which included the sense and antisense primers. Cytokines, chemokines, and β-actin cDNAs for real-time PCR analysis were amplified using the primers listed above. The PCR products were inserted into the vector pGEM-T Easy DNA (Promega, Madison, WI, USA) using standard techniques. We confirmed the sequence of each insert with the dideoxy chain-termination method using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The constructed plasmid vectors were applied to calculate the relative expression levels of each cDNA in samples by the relative standard curve method. The expression levels of β-actin were used to normalize individual cytokine and chemokine cDNA expression levels in the samples. The relative index of cytokine expression levels from a minimum of three samples stimulated with poly(I:C) and without pre-treatment with LAB. After setting this value as 1, the relative expression of LAB pre-stimulated samples following poly(I:C) stimulation was then calculated.

2.6. Statistical analysis

All data were adjusted to give relative values against the appropriate controls. Statistical analyses were performed using the GLM procedure of SAS statistical software (SAS, 1994). Comparisons
among mean values (N = 4) for relative mRNA expression levels of target cytokines in cells were performed using one-way ANOVA and Fisher’s least significant difference (LSD) test against the controls. For the analyses, $P$ values of <0.05 and <0.01 were considered significant. Comparisons (N = 4) among relative mRNA expression levels of cytokines in BIE cells stimulated with LAB strains for 48 h are shown using two broken lines for $P$ values of <0.05 and <0.01, following one-way ANOVA and LSD multi-comparison test. All presented data represents the results of four independent experiments.

3. Results

3.1. Functional expression of TLR3 in BIE cells

To evaluate the expression of TLR3 protein in BIE cells, we first performed immunohistochemical analysis. As shown in Fig. 1A, BIE cells strongly expressed TLR3 in the cytoplasm, whereas no expression of TLR3 was detected at the cell surface (data not shown).

As delineating cytokine and chemokine responses to TLR3 stimulation is important to understand TLR3-mediated immune responses and pathogenicity in IECs, we next evaluated the response of BIE cells to stimulation with poly(I:C). For these experiments, we treated BIE cells with two doses of TLR3 agonist to establish the most appropriate dose to study TLR3-mediated immune responses. Challenge of BIE cells with both low (12.5 ng/ml) and high (60 ng/ml) concentrations of poly(I:C) significantly increased the expression of type I IFN, IFN-$\gamma$, and pro-inflammatory cytokines and chemokines (Fig. 1B). The most significant changes were observed in the expression of IFN-$\beta$ and the pro-inflammatory cytokines IL-6, IL-8, and MCP-1. We also observed that treatment of cells at the higher dose of poly(I:C) induced earlier up-regulation of IFN-$\beta$, as well as IL-6, IL-8, and MCP-1, when compared with the lower dose. Thus, poly(I:C) at a concentration of 60 ng/ml was selected for the following experiments.

3.2. Effect of LAB on cytokine expression by BIE cells

We evaluated changes in the expression of IFN-$\beta$, IL-6, IL-8, and MCP-1 in BIE cells after treatment with different LAB strains or the TLR2 agonist Pam3CSK4 for 24 and 48 h (Fig. 2). We observed that
3.3. Effect of LAB on the response of BIE cells to poly(I:C) stimulation

We next evaluated the ability of BIE cells to serve as an in vitro system for the selection of LAB strains with the capacity to modulate the response of bovine IECs to poly(I:C) challenge. As IFN-β is an important cytokine for protection against viral infections, we aimed to identify LAB strains with the ability to augment the production of IFN-β by BIE cells stimulated with TLR3 agonist. BIE cells were stimulated with the different LAB strains for 48 h and then challenged with poly(I:C) (Fig. 3). The determination of IFN-β mRNA expression levels at 3 h (Fig. 3A) and 12 h (Fig. 3B) post-stimulation with poly(I:C) revealed that only *L. rhamnosus* LA-2 and *S. thermophilus* TMC1543 were able to increase IFN-β levels after 12 h when compared to controls. Notably, the effect of *L. rhamnosus* LA-2 exposure was superior to that of *S. thermophilus* TMC1543. In addition, we observed that *L. rhamnosus* LA-2 induced the down-regulation of IL-6 after 3 h (Fig. 3A), while *S. thermophilus* TMC1543, *L. casei* TMC0409, and both *B. bifidum* strains had reduced the expression of IL-6 by 12 h (Fig. 3B). In addition, *L. gasseri* TMC0356 down-regulated the expression of IL-8, while *L. casei* TMC0409 reduced IL-8 and MCP-1 mRNAs levels after 12 h. These results were confirmed by comparing the relative mRNA levels of cytokines in BIE cells stimulated with each LAB strain and challenged with poly(I:C) for 12 h using multi-comparison tests (Fig. 3C).

4. Discussion

Epithelial TLR expression is thought to play a key role in host defenses against pathogens. Epithelial cells, more than any other cell type, express TLR3 in numerous organs, including the gastrointestinal tract (Cario and Podolsky, 2000). It was reported that human IECs express low levels of TLR2 and TLR4, whereas TLR3 appears to be abundantly expressed in the normal human small intestine and colon (Cario and Podolsky, 2000). In addition, our laboratory has previously shown that TLR3 is also strongly expressed in swine IECs (Moue et al., 2008). Moreover, TLR3 transcription in bovine colonic epithelium was recently described for the first time (Bridger et al., 2010). In the present study, immunohistochemical...
analyses revealed abundant expression of TLR3 in BIE cells. Therefore, BIE cells, in addition to displaying characteristics of epithelial cells, such as cobblestone morphology, microvilli-like structures, and strong expression of cell-to-cell junctional proteins and cytokeratin (Miyazawa et al., 2010), also express TLR3 and thus resemble the IECs of other species.

We also evaluated the response of BIE cells to stimulation with the TLR3 agonist poly(I:C) and found that the cells up-regulate the expression of cytokines. Fig. 3 shows the effect of lactic acid bacteria (LAB) on the response of bovine intestinal epithelial (BIE) cells to poly(I:C) challenge. Expression of IFN-β, IL-6, IL-8, and MCP-1 mRNA in BIE cells after stimulation with different bacterial strains and poly(I:C). BIE cells were stimulated with Lactobacillus gasseri TMC0356, Lactobacillus rhamnosus LGG, L. rhamnosus LA-2, Lactobacillus casei TMC0409, Streptococcus thermophilus TMC1543, Bifidobacterium bifidum 2-2, or B. bifidum 3-9 for 48 h. Cells were then challenged with poly(I:C) (60 ng/ml) and cytokine levels were evaluated 3 h (A) and 12 h (B) post-challenge. All values are presented as the mean ± standard deviation (error bars). The results represent four independent experiments. *P < 0.01 and **P < 0.05 vs. cells cultured in the absence of bacterial stimulation and challenged with poly(I:C). (C) Comparisons of relative mRNA levels of cytokines in BIE cells stimulated with the indicated LAB strains for 48 h and challenged with poly(I:C) for 12 h. One-way ANOVA and Fisher’s least significant difference multi-comparison test were used. P values of <0.05 and <0.01 are indicated by the two types of broken lines.

Fig. 3. Effect of lactic acid bacteria (LAB) on the response of bovine intestinal epithelial (BIE) cells to poly(I:C) challenge. Expression of IFN-β, IL-6, IL-8, and MCP-1 mRNA in bovine intestinal epithelial (BIE) cells after stimulation with different bacterial strains and poly(I:C). BIE cells were stimulated with Lactobacillus gasseri TMC0356, Lactobacillus rhamnosus LGG, L. rhamnosus LA-2, Lactobacillus casei TMC0409, Streptococcus thermophilus TMC1543, Bifidobacterium bifidum 2-2, or B. bifidum 3-9 for 48 h. Cells were then challenged with poly(I:C) (60 ng/ml) and cytokine levels were evaluated 3 h (A) and 12 h (B) post-challenge. All values are presented as the mean ± standard deviation (error bars). The results represent four independent experiments. *P < 0.01 and **P < 0.05 vs. cells cultured in the absence of bacterial stimulation and challenged with poly(I:C). (C) Comparisons of relative mRNA levels of cytokines in BIE cells stimulated with the indicated LAB strains for 48 h and challenged with poly(I:C) for 12 h. One-way ANOVA and Fisher’s least significant difference multi-comparison test were used. P values of <0.05 and <0.01 are indicated by the two types of broken lines.
expression of type I IFN, IFN-γ, and pro-inflammatory cytokines and chemokines. The observed changes in the expression of cyto-
kines induced by poly(I:C) correlate with the changes reported in
various intestinal viral infections of cattle and other hosts. For
example, increased gene expression of RANTES, IP-10, IL-8, and
MCP-1 were observed in rotavirus-infected HT-29 cells (Rollo
et al., 1999; Xu et al., 2009). In addition, in vitro studies with bovine
intestinal tissues demonstrated that exposure to BRV activated
TLR3-induced up-regulation of NF-κB and IL-6 production (Aich
et al., 2007). These findings, together with our present results, indi-
cate that BIE cells are valuable tools for the in vitro study of im-
une responses triggered by TLR3 expressed on bovine IECs.

To date, a few studies have evaluated the antiviral effects of
probiotic LAB strains in animals. In vivo studies using gnotobiotic
pigs demonstrated that probiotic LAB administration has a signifi-
cant influence on IFN-α, TGF-β, IL-4, and IFN-γ serum levels in-
duced by rotavirus infection (Wen et al., 2009). The antiviral
effects of immunobiotics have also been examined in a few animal
cell lines. For example, Maragkoudakis et al. (2010) reported that
probiotics are able to protect porcine and goat epithelial cells
against rotavirus and transmissible gastroenteritis virus chal-
lenes; however, the immunological mechanisms involved in the
protective effect were not determined. Recent studies have also
demonstrated that probiotics significantly decrease IL-6 produc-
tion by porcine IPEC-J2 cells infected with porcine rotavirus, sug-
gest that probiotics have immunoregulatory effects (Liu et al.,
2010). To our knowledge, no prior studies have investigated the ef-
fects of probiotics in bovine IECs lines. Therefore, we evaluated
whether our bovine in vitro system could be used for the selection
of LAB strains with antiviral immune-enhancing activities.

We first examined if the stimulation of BIE cells with different
LAB strains was able to induce changes in the expression of IFN-
β, IL-6, IL-8, or MCP-1. We found that the different strains had dis-
tinct effects on cytokine production by BIE cells. Notably, L.
rhamnosus GG and S. thermophilus TMC1543 were the only strains
able to increase IFN-β and IL-6 mRNA, respectively, in BIE cells
after 48 h of stimulation. In addition, only L. rhamnosus LA-2 in-
creased MCP-1 mRNA, while this bacterium, L. casei TMC0409,
and both B. bifidum strains up-regulated IL-8. It has been shown
that many of the immunomodulatory effects of probiotic microor-
ganisms are mediated by their ability to activate TLR2 (Tohno et al.,
2005, 2006, 2007; Kitazawa et al., 2008; Alvarez et al., 2009; Fujie
et al., in press). In addition, TLR2 agonists are able to induce IFN-β
transcription (Dietrich et al., 2010). Moreover, it was observed
that certain lactobacilli trigger the expression of IFN-β genes in den-
dritic cells in a TLR2-dependent manner (Weiss et al., 2010). In
our present analyses, we therefore expected to identify strains
capable of increasing IFN-β levels in BIE cells; however, we only
detected a slight increase of this cytokine in BIE cells treated with
L. rhamnosus GG. In addition, we found that the TLR2 agonist
Pam3CSK4 was not able to modify the expression of IFN-β in BIE
cells. Therefore, the information obtained in these experiments
did not allow us to draw any conclusions concerning the antiviral
effects of LAB strains in bovine IECs.

We next studied the effects of LAB strains on the response of BIE
cells to TLR3 stimulation and found that only L. rhamnosus LA-2
and S. thermophilus TMC1543 were able to increase IFN-β levels
by 12 h post-stimulation. The increased production of IFN-β by
BIE cells in response to TLR3 activation induced by L. rhamnosus
LA-2 may have significant in vivo effects in the protection against
enteric viruses. Global gene-expression analyses of bovine intesti-
nal tissues following infection with BRV or BCV indicate that sev-
eral IFN-regulatory and -stimulatory genes are down-regulated,
supporting the conclusion that both viruses may have evolved mechanism(s) to inhibit IFN-mediated immune responses (Aich
et al., 2007). Moreover, it was shown that BVDV significantly inter-
feres with the induction of type I IFN, which impairs not only in-
nate defenses, but also interferes with the establishment of
adaptive immune responses (Peterhans et al., 2003; Lee et al.,
2008). Based on these findings, L. rhamnosus LA-2, which enhances
IFN-β production in BIE cells, may play an important role in the
improvement of innate and specific immune responses against bo-
vine intestinal virus. Thus, our studies with the BIE cell line have
allowed us to identify a LAB strain that is a good candidate for fu-
ture in vivo studies.

As the degree and duration of pro-inflammatory cytokine secre-
tion after TLR3 recognition of dsRNA can become harmful to the
host (Vercammen et al., 2008), we also evaluated the production of
the immunomodulatory cytokines IL-6, IL-8, and MCP-1 in BIE cells.
Our analyses demonstrated that BIE cells pretreated with L. casei
TMC0409 produce lower levels of the three pro-inflammatory
cytokines when compared with control cells 12 h post-stimulation
with poly(I:C). It was reported that TLR3 mediates harmful inflam-
matory responses in the intestine, thus contributing to the patho-
genesis of viral infections (Zhou et al., 2007). Therefore, the lower
production of pro-inflammatory cytokines following exposure to L.
casei TMC0409 may allow for the efficient regulation of inflamma-
ty responses and avoidance of tissue injury, offering a different
protection mechanism against bovine viral infection.

In conclusion, the in vitro system described in this study was use-
ful for the primary screening of two types of immunomodulatory
LAB strains that would be able to protect against viral intestinal dis-
eases in cattle: strains capable of increasing antiviral defenses and
strains with anti-inflammatory capacities. To define the characteris-
tic immunomodulatory abilities of individual LAB strains, their
influence on cytokine production after challenge of BIE cells with
TLR3 agonist can be studied using statistical multi-comparison tests,
as shown in Fig. 3C. We also demonstrated that TLR3 is expressed
in BIE cells and that these cells respond to stimulation with the TLR3
agonist poly(I:C). Characterization of the inflammatory immune re-
response triggered by TLR3 activation in BIE cells showed that this
in vitro system can be used for the study of TLR3-mediated immune
responses in bovine IECs. In addition, our findings indicate that BIE
cells are useful for the primary screening of immunobiotic LAB
strains which are able to beneficially modulate the antiviral immune
response triggered by TLR3 activation in bovine IECs. Although it is
difficult at present to predict if the observed in vitro changes in gene
expression are biologically relevant in vivo, given the diverse factors
capable of influencing intestinal immune responses, we propose
that BIE cells can serve as a useful in vitro tool to identify a small
number of potentially immunobiotic strains which can then be sub-
jected to appropriate in vivo screening tests. The present charac-
terization of BIE cells represents an important step towards the
establishment of a valuable bovine in vitro system that could be used
for the development of immunomodulatory feed for bovine hosts.

5. Conflict of interest statement

The authors declare no financial or commercial conflicts of interest.

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