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Low numbers of intestinal Shiga toxin-producing *E. coli* correlate with a poor prognosis in sheep infected with bovine leukemia virus

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Healthy ruminants carry intestinal Shiga toxin (Stx)-producing *Escherichia coli* (STEC). Stx has antiviral activities *in vitro* and STEC numbers correlate with reduced early viremia in sheep experimentally infected with bovine leukemia virus (BLV). This study assessed the impact of intestinal STEC on BLV-induced disease for one year post-BLV-challenge. High STEC scores (CFU/g feces × frequency of STEC-positive samples) correlated with good health, whereas poor weight gain, distress, and tumor development occurred only among animals with low STEC scores. STEC carriage was associated with increased percentages of B cells in peripheral blood.

Keywords: bovine leukemia virus, sheep, Shiga toxin-producing *Escherichia coli*

Introduction

Some serotypes of Shiga toxin (Stx)-producing *Escherichia coli* (STEC) such as O157：H7 can cause severe illness in humans in which toxin(s) cause systemic damage [4,11]. However, healthy ruminants carry intestinal STEC [1-3] with high prevalence. It is not known what, if any, are the benefits of Stx genes or proteins for the bacteria or their ruminant hosts. Stxs belong to a family of ribosome-inactivating proteins (RIPs) prevalent among plants [7]. RIPs are important in the innate plant defense against virus infection [19], and are active *in vitro* against animal cells harboring retroviruses [10,17]. Stxs are not detrimental to normal bovine cells, but inhibit expression and replication of bovine leukemia virus (BLV), bovine immunodeficiency virus, and equine infectious anemia virus, in cell culture [8,10]. We hypothesize that intestinal STEC have an antiviral effect in ruminants and compared viral loads with intestinal STEC in sheep experimentally infected with BLV. In contrast to cattle (that may take 10 years to manifest disease symptoms), sheep are a good experimental model because they exhibit rapid progression of BLV disease with clinical symptoms in 6 ~ 12 months [6,14]. Previously, we showed that early BLV viremia is reduced in sheep carrying intestinal STEC at 10^4 CFU/g feces [9]. Here we examined the impact of intestinal STEC in the late stages (12 to 14 months) of disease.

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Materials and Methods

Experimental animals

All animal procedures were approved by the University of Idaho Animal Care and Use Committee. Twenty white-face Suffolk wethers were divided into four groups with 5 animals, as described previously [9], and fed a maintenance diet of alfalfa hey *ad libitum*. Animals were weighed and bled post-BLV challenge weekly for the first 9 weeks, monthly until 6 months, and then quarterly. Beginning at 4 months post challenge, general health was assessed bi-weekly by two observers (blind to group assignation). Animals consistently exhibiting at least 2 of 3 symptoms of distress (apathy, poor posture, or an uncertain "shuffling" gait) were considered in poor health.

STEC treatment and enumeration

Sheep can sporadically carry naturally acquired STEC. Thus, although some sheep were treated with oral STEC, all sheep carried naturally occurring STEC that were not distinguishable from the dosed strains by our culture procedure. Nonetheless, all sheep were given oral doses of 5.0×10^{10} CFU of either STEC or *stx*-negative *E. coli* K-12 (K-12) twice per week from 2 weeks pre- to 16 weeks

post-BLV challenge. Group 1 received 5 wild-type ovine STEC of different serotypes; group 2 received K-12 prior to BLV challenge, and then STEC beginning at 1 day post BLV challenge; groups 3 and 4 never received STEC. Fecal STEC numbers were determined as described previously [9] by isolation of CFU on hydrophobic-grid filters [20] and colony hybridization with *stx*-specific DNA probes [13] by a modified procedure of Nizetic *et al*. [16]. Carriage of STEC over time was compared among individual animals using an STEC score = (the average logarithms of STEC CFU/g feces × the proportion of STEC-positive samples). STEC measurements from June to September (3 × before and 3 × post BLV) exceeded 10^7 CFU/g in some sheep, but subsequent positive samplings showed only 10^2~10^4 CFU/g feces. Since values $< 10^4$ CFU/g feces were previously shown to have no antiviral effect [9], STEC treatment was discontinued in October.

BLV challenge

Sheep in groups 1, 2, and 3 were injected subcutaneously with single doses of 1.0×10^6 peripheral blood mononuclear cells (PBMC) from a BLV-positive cow. Group 3 was the STEC-untreated, BLV-infected control and Group 4 (no BLV) was the STEC-untreated, BLV-uninfected control.

Flow cytometry and histology

B cells in whole blood samples were identified by standard flow cytometry with murine monoclonal antibodies against B-cell markers B-B1 (BAS9A, IgM) and B-B2 (BAQ44A, IgM) (VMRD, USA) and secondary antibody conjugate (Caltag/Invitrogen, USA) [5]. Animals were killed by intravenous injection of potassium barbiturate at 12~14 months post BLV challenge, and autopsied. Gross pathology was noted, and tissue samples preserved in 4% buffered formaldehyde. Sections of lymph nodes (retropharyngeal, prescapular, submandibular, and mesentheric) were stained with hematoxylin-eosin and scored from 0~4 for neoplasia by a veterinary pathologist unfamiliar with the treatment assignments.

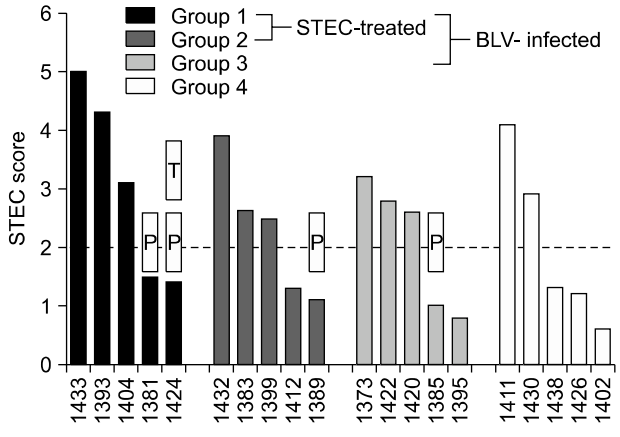

Fig. 1. Low Shiga toxin-producing *Escherichia coli* (STEC) score correlated with poor health at the advanced stage of bovine leukemia virus (BLV) infection. STEC scores were calculated form 6 samples (average logarithm of CFU/g feces, multiplied by proportion of STEC- positive samples). The horizontal broken line separates low (STEC score ≤ 1.5) from high (STEC score ≥ 2.3) rank. Animals presenting with symptoms of poor health are indicated by letter "P", and letter "T" indicates an animal with tumors.

Statistical analysis

Health status, pathology, and total B lymphocytes were analyzed independent of STEC treatment, among STEC treatment groups independent of STEC numbers, and between BLV-infected and BLV-free sheep carrying only naturally occurring STEC (i.e. not STEC treated). Statistical significance was assessed by non-parametric tests, and differences among experimental groups were assessed by analysis of variance (ANOVA). Analyses used Minitab 13 software (Minitab, USA).

Results

Low STEC scores correlated with poor condition of BLV-infected sheep. BLV-challenged sheep could be separated into two distinct subpopulations: those with STEC scores < 1.5 or > 2.3 (Fig. 1). All animals in poor health had low STEC scores (Chi-square test, DF = 1, $p = 0.004$) and failed to carry $\geq 10^4$ CFU/g more than once post BLV challenge. Also, these 4 animals never carried ≥ 4.5 log CFU STEC/g after BLV challenge, whereas two sheep (1412 and 1395) with low STEC scores < 1.5, that remained in good condition, had one fecal sample with ≥ 4.5 log CFU STEC/g after BLV challenge. Thus, carriage of ≥ 4.5 log CFU/g of intestinal STEC at least once during the early phase of infection appeared to protect sheep from BLV- induced disease for up to 12~14 months. Likewise, consistently low numbers of STEC ($< 10^4$ CFU/g) prior to and during the initial 2 months post BLV challenge were associated with deteriorating health. In the absence of BLV infection, low STEC scores were not associated with poor health.

STEC scores correlated with weight gain among the BLV-challenged sheep. At 6 months post BLV challenge (after 2 months of consistent weight gain by all BLV-negative control sheep), 9 animals with STEC score > 2.3 averaged 87.0 ± 2.6 kg, while 6 animals with STEC score < 1.5 averaged 75.0 ± 3.0 kg ($p = 0.001$, Mood median test). Among the STEC-treated groups 1 and 2, weight correlated weakly with STEC scores, but the correlation was strong in group 3 animals, carrying only naturally acquired STEC (Pearson coefficient 0.891, $p = 0.042$) (Fig. 2A). In the absence of BLV infection, STEC scores did not correlate with weight (Fig. 2B).

At autopsy, average lymph node neoplasia scores ranged from 1.8 to 2.2 for all sheep. Only one animal, 1424,

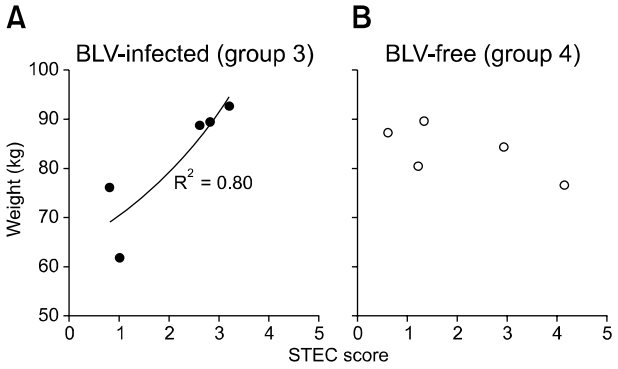

Fig. 2. Weight gain in sheep challenged with bovine leukemia virus (BLV) correlated with Shiga toxin-producing *Escherichia coli* (STEC) scores. Weight at 6 months post BLV challenge is plotted against STEC scores. (A) BLV-challenged sheep, (B) control sheep. Points in panel A were fitted with a second-power polynomial curve.

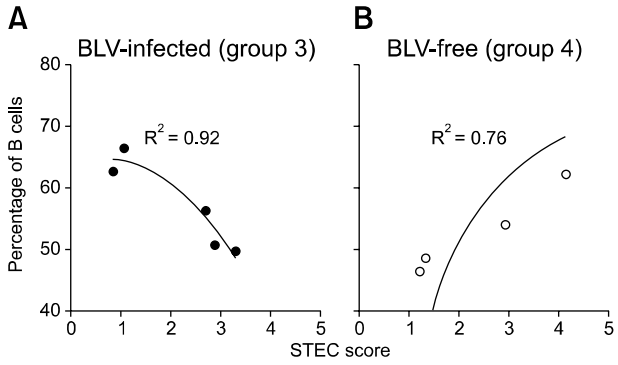

Fig. 4. Peak B-cell percentages differentially correlated with Shiga toxin-producing *Escherichia coli* (STEC) scores. (A) % B-cells in bovine leukemia virus (BLV)-challenged sheep were negatively correlated with STEC scores. (B) % B-cells from BLV-free control sheep were positively correlated with STEC scores.

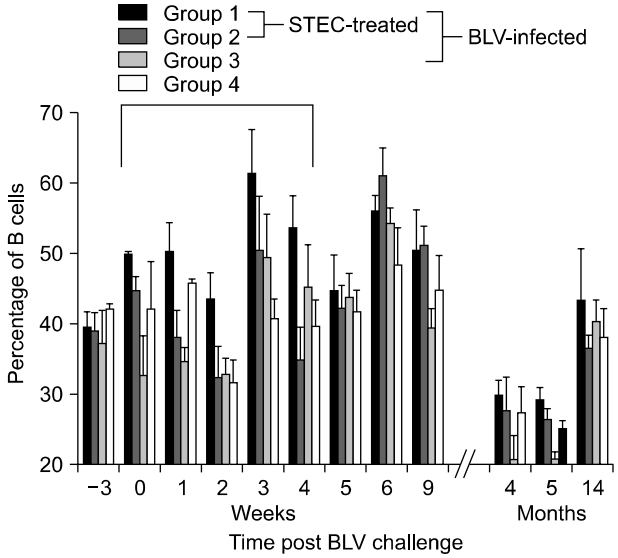

Fig. 3. Shiga toxin-producing *Escherichia coli* (STEC) treatment correlated with percentages of B cells in blood. Data are group averages + SEM of B cell percentages. A bracket indicates group 1 significantly different from control (ANOVA, $p < 0.05$).

presented an average lymph node score of 4.0, indicating the presence of a tumor in all lymph nodes examined, and with copious tumors located in the intestinal wall, and other tissues. This animal had the lowest fecal STEC counts post-BLV (0 to $< 10^3$ CFU/g feces).

Intestinal STEC differentially influenced the B-cell percentage in peripheral blood by BLV status. The percentages of B cells among PBMC from BLV-challenged sheep underwent major fluctuations indicative of viral expansion and immune suppression of viremia. The mean B-cell percentage post-BLV challenge was 39.6% ± 2.5% among all BLV-infected animals, higher than the control sheep mean (32.2% ± 4.5). In a majority of BLV infected sheep (11/15) values ranged from 52.4 to 70.5%, above the median value 50.9% for control animals. Among group 1 animals, the B-cell percentages were consistently higher than among the control animals (Fig. 3, bracketed time-points, $p = 0.031$, ANOVA). Peak B-cell percentage was noted at 5 weeks after commencement of STEC treatment in group 1 and at 6 weeks in group 2, suggesting that STEC treatment stimulated B-cell production in animals from both STEC-treated groups. In groups 3 and 4, that never received STEC treatment, correlations between STEC scores and maximal B-cell percentages were diametrically opposed: positive in BLV-free group 4 (Pearson coefficient = 0.986, $p = 0.014$) and negative in BLV-challenge group 3 (Pearson coefficient = -0.944, $p = 0.016$) (Fig. 4).

Discussion

Absence of disease in sheep exhibiting STEC scores > 2.3 agrees with our previous finding that carriage of $> 10^4$ CFU STEC/g feces for 2 months post challenge reduces early BLV viremia [9]. Suppression of early viremia may allow an effective immune response or STEC carriage at BLV challenge may influence interferon-γ and/or interleukin 12-dependent pathways, known to correlate with resistance to BLV [12]. STEC-associated weight gain in BLV-positive animals points to possible beneficial impact of STEC upon host physiology, beyond a strict antiviral effect.

STEC carriage was positively correlated with B-cell percentage in BLV-free animals, and negatively correlated in BLV-positive sheep, but only in a group that did not receive STEC. Thus, STEC may stimulate B-cell proliferation. In BLV-challenged animals this effect of STEC could be masked by STEC-mediated elimination of B cells harboring BLV. Although proviral BLV DNA was reported in T cells, monocytes, and other cell types, it appears that the virus is expressed only in B cells [15,18], and can stimulate these

cells to proliferate [6]. Thus, two opposing STEC-related factors, i.e. stimulation of B-cell expansion and elimination of BLV-positive B cells could confound the analysis of the impact of BLV infection and STEC carriage on B-cell percentages, especially in STEC-treated sheep. Moreover, B-cell expansion by STEC treatment increased the availability of BLV cellular targets, putting the sheep from groups 1 and 2 at a long-term disadvantage and making them more vulnerable to BLV, especially after cessation of STEC treatment at 4 months and removal of protective effects of Stxs, present in and/or produced by inocula. This conjecture is consistent with the lack of correlation between STEC scores and weight gain in groups 1 and 2, as opposed to group 3, and with the clustering of cases of poor health and tumor in group 1, that exhibited already elevated B-cell percentage upon BLV challenge.

Conclusions: 1) Elevated numbers of intestinal STEC carried at and after BLV challenge correlated with protection from BLV disease. High STEC scores were associated with good health and weight gain, and low STEC scores with poor health and low weight gain, among BLV-infected sheep. 2) Repeated oral treatments with STEC were associated with increased percentages of B cells in peripheral blood, although treatment did not consistently increase the numbers of fecal STEC. 3) STEC score provided a means of expressing time-averaged STEC colonization in sheep and was used effectively in statistical analysis. 4) The correlation between STEC score and B-cell percentage in blood was positive in BLV-free sheep, and negative in BLV-challenged sheep harboring only naturally acquired STEC. These results suggest that intestinal STEC can stimulate B-cell expansion. In BLV-positive animals, STEC presence may contribute to elimination of toxin-sensitive B cells harboring BLV, thereby reducing viral loads and disease progression.

Acknowledgments

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