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Evaluation of innate immune responses in bovine forestomachs

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ABSTRACT

Previous studies had indicated an active role of bovine forestomachs in the response to alimentary disorders as well as to inflammatory and infectious processes in both the gastro-intestinal (GI) tract and elsewhere. We investigated the potential of bovine forestomachs to receive, elaborate and produce signals and mediators of the innate immune response. Indeed, we detected the expression of Toll IL-1R8/single Ig IL-1-related receptor (TIR8/SIGIRR) and other receptors and cytokines, such as Toll-like receptor (TLR)4, interleukin (IL)-1β, IL-10 and Caspase-1 in the forestomach walls of healthy cows. Their presence suggests an active role of forestomachs in inflammatory disorders of the GI tract and other body compartments. Moreover, interferon (IFN)-γ was revealed in ruminal content. We confirmed and further characterized the presence of leukocytes in the rumen fluids. In particular, T-, B-lymphocytes and myeloid lineage cells were detected in the ruminal content of both rumen-fistulated heifers and diseased cows. An acidogenic diet based on daily supplements of maize was shown to inhibit leukocyte accumulation, as opposed to a control, hay-based diet, with or without a soy flour (protein) supplement. On the whole, results indicate that bovine forestomachs can receive and elaborate signals for the immune cells infiltrating the rumen content or other organs. Forestomachs can thus participate in a cross-talk with the lymphoid tissues in the oral cavity and promote regulatory actions at both regional and systemic levels; these might include the control of dry matter intake as a function of fundamental metabolic requirements of ruminants.

1. Introduction

The forestomachs of the ruminant species, such as the bovine, are expanded esophageal portions lined by stratified squamous epithelium. The esophagus enters the rumen, a large mixing organ that contains microorganisms for cellulose digestion and fermentation. Its epithelium allows the passage of water, electrolytes and other low molecular weight substances, mainly ammonia and short-chain fatty acids. The ruminal mucosa also contains several conical *papillae* to increase the surface area for absorption. Cellulose digestion, fermentation and absorption continue in the reticulum, whereas the omasum is intended for a large absorption of water through numerous mucosal fronds, which increase the surface area available for this purpose. The abomasum is the true stomach, intended for protein degradation, including that of ruminal microorganisms.

Scanty information is available about the organization of the mucosal immune system in bovine forestomachs as opposed to the impressive amount of data about the intestinal tract of both ruminant and non-ruminant species (Dommett et al., 2005). In general, the immune response in the mucosal areas is orchestrated by MALT (mucosal-associated lymphoid tissue), which is called GALT (gut-associated lymphoid tissue) in the gut. The activity of GALT relies on the capability of mucosal epithelial cells to discriminate between pathogenic and commensal bacteria. The recognition of commensal microorganisms is essential for the development and function of the immune system in the mucosal and peripheral districts (Macpherson and Harris, 2004). Signals provided by commensal bacteria determine a basal level of activation that promotes gene expression patterns required for normal development and function of immune cells (Rakoff-Nahoum et al., 2004). Microbial components are recognized by intestinal epithelial cells (IECs) by means of pattern-recognition receptors (PRRs) including Toll-like and NOD-like receptors (TLRs and NLRs). The activation of TLRs and NLRs results in the expression of pro-inflammatory cytokines, chemokines and antimicrobial peptides, initiating and regulating the immune response. Regulated expression (down-regulation under steady-state conditions) and

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localization (intracellular or basolateral localization) of TLRs and NLRs are proposed as possible mechanisms limiting the recognition of commensal bacteria (Abreu et al., 2001; Uehara et al., 2007). IECs also express TIR8/SIGIRR (Toll IL-1R8/single Ig IL-1-related receptor), a negative regulator of TLR and IL-1R signalling that influences the communication among IECs, immune system and commensal bacteria (Garlanda et al., 2004). On the other hand, commensal bacteria can inhibit the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Neish et al., 2000), thus exerting an anti-inflammatory control action. Commensal bacteria digest some complex polysaccharides, the metabolites of which may induce the expression of anti-inflammatory cytokines (Saemann et al., 2000). Also, IECs can regulate the activation of dendritic cells (DCs) present in the *lamina propria* by means of immunoregulatory molecules (Rimoldi et al., 2005). Whenever their default down-regulation is overwhelmed, intestinal epithelial cells are fully competent for the recognition of microbial components and secretion factors of diverse inflammatory and regulatory cytokines.

We suggest that the above concepts should be re-appraised in view of the similarities between rumen disturbances and those of monogastrics caused by microbial overgrowth in the intestine. Thus, in analogy to the colic problems of horses, high-yielding dairy cows could suffer from the same troubles (as previously hypothesized by Bertoni et al., 1989) when fed diets rich in starch. Also, as in the intestine, a major need for recognition and regulation activities of the immune system can be envisaged in forestomach, involved in controlled fermentation of digestible fiber by bacteria, fungi and ciliated protozoa (Baldwin and Allison, 1983). Since these functions are crucial for the global homeostatic balance in adult cattle, we reasoned that epithelial cells in forestomachs could display similar mechanisms for discriminating between commensal and pathogenic bacteria, as well as for recognizing dangers associated to abnormal fermentations of ruminal bacteria. This implies that epithelial cells of forestomachs can react to disturbances of the fermentation processes by the release of inflammatory and chemotactic molecules, intended for signalling at both local and systemic levels. Interestingly, it has been shown that inflammatory responses in forestomachs can be sustained by infiltrating leukocytes, that could secrete cytokines in the rumen liquor, affecting inflammatory and metabolic parameters in dairy cattle (Trevisi et al., 2009).

Owing to the above and our previous results on the expression of TIR8 in bovine forestomachs (Riva et al., 2010), the central objective of this study was to detect and characterize fundamental receptors and mediators of the innate immune system (i.e. cytokines) in the same organs. We also investigated innate immune responses in forestomachs in terms of leukocyte infiltration into the rumen liquor during both feeding disorders and disease conditions.

2. Materials and methods

2.1. Animals and samples

This study complied with Italian laws on animal experimentation and ethics. We collected samples from both cows at a slaughterhouse and three Frisian heifers with a surgical fistula of the rumen cavity housed at the Agricultural Experiment Station in Piacenza (Italy), under the supervision of the Italian National Veterinary Services.

2.1.1. Animals for tissue samples

At the beginning of our study, tissue samples for RNA and protein extraction were collected at a slaughterhouse (Calzi, Bertonico, Italy) from four healthy dairy cows. Samples (0.5 cm^3 pieces) from the three forestomachs and abomasum were collected from slaughtered cattle. They were immediately frozen (for protein extraction) or placed in sterile tubes containing 3 ml of RNAlater (Qiagen, Hilden, Germany) and stored at 4 °C, 24 h, for total RNA isolation before rapid freezing in dry ice.

2.1.2. Animals for rumen fluid samples

Ruminal fluids were collected from three fistulated Frisian heifers, fed a standard diet based on grass hay and 1 kg/d of a mixed concentrate fortified with minerals and vitamins. Samples of the ruminal fluids were mixed and used for subsequent *in vitro* fermentation (see Section 2.8) and *in vitro* assays (see Section 2.2). In this case, samples of ruminal fluids were deep frozen after addition of dimethyl sulfoxide (DMSO) and fetal calf serum (FCS) (10% and 40%, respectively), for immunological and molecular assays. Moreover, rumen fluid samples were taken in a subsequent period from one of the three heifers that showed joint swelling at the hocks. In this case, ruminal fluid samples were taken immediately before and two weeks after the pharmacological treatments (antinflammatory and antibiotic therapy) with the same aforementioned procedure. Finally, rumen fluid samples for immunological and molecular assays were also collected at a slaughterhouse from nine dairy cows affected by recent disease cases, as confirmed by post mortem examination.

2.2. Processing of ruminal fluids for immunological and molecular assays

The ruminal fluid samples were thawed at 38 °C and immediately placed on ice. Samples were diluted 1:3 with sterile PBS (without Ca^{++} and Mg^{++}). Then, non-bacterial mononuclear cells were isolated by centrifugation on Ficoll-Hypaque (density 1.083; Sigma–Aldrich Co, St. Louis, MO, USA) at 1687*g*, 20 min, 20 °C. The harvested cells were diluted 1:2 with sterile PBS and placed on ice. Cells were then pelleted (441 g, 10 min, 5 °C), resuspended in 2 ml of PBS and centrifuged through a 1-ml FCS layer at 300*g*, 10 min, 5 °C. The supernatant (containing bacteria) was discarded and the pellet was used for flow cytometry experiments or RNA extraction.

2.3. RNA extraction from tissue samples and ruminal fluid cells

Total RNA was isolated from tissue samples by the guanidine isothiocyanate method with minor modifications. Briefly, the samples were homogenized in a guanidine isothiocyanate buffer and the lysate was centrifuged through a caesium chloride layer. The RNA pellet was dissolved in sterile water and stored at −20 °C (Riva et al., 2010).

The cell pellet obtained from ruminal fluids was lysed in 0.5 ml of TRI Reagent (Sigma–Aldrich, St. Louis, MO, USA); total RNA was extracted according to the manufacturer's directions.

The concentration of RNA was determined using a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany) at 260 nm wavelength.

2.4. Reverse transcription and Real-time PCR

One μg of total RNA from tissues of forestomachs and ruminal fluid cells were reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA, USA), according to the manufacturer's instructions. The cDNA obtained from each sample was used as a template for Real-time PCR in an optimized 25-μl reaction volume as previously described (Riva et al., 2010). The primer pairs were designed using the Primer Express Software (Applied Biosystem, Foster City, CA, USA) and purchased from Invitrogen (Carlsbad, CA, USA). Their sequences are