Molecular mechanisms controlling bovine lymphocyte homing

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Molecular mechanisms controlling bovine lymphocyte homing

Bosworth, Brad Thomas, Ph.D.
Iowa State University, 1991
Molecular mechanisms controlling bovine lymphocyte homing

by

Brad Thomas Bosworth

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1991
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EXPLANATION OF DISSERTATION FORMAT

The alternative format was used in writing this dissertation. The first manuscript has been accepted for publication in Molecular Immunology. The second manuscript has been accepted for publication in Veterinary Immunology and Immunopathology. The third manuscript will likely be submitted to Immunology. The format used for this dissertation is that of Veterinary Immunology and Immunopathology. Literature cited in the General Introduction and General Summary and Discussion is listed at the end of the dissertation.

The candidate, Brad Bosworth, was the principal investigator for each of the studies. The coauthors of the first manuscript, Drs. Gallatin and St. John provided advice, reagents, and minor assistance in the writing of the manuscript. Dr. Harp, coauthor on all three manuscripts, provided advice and assistance in the studies and assisted in the writing of the manuscripts. Dr. Jutila kindly provided the antibody DREG-56 and Don Dowbenko provided one of the LECAM-1 cDNA clones used in the third manuscript.
GENERAL INTRODUCTION

Introduction

Infectious diseases in cattle are costly to the agricultural industry because of decreased productivity, cost of prevention and treatment of infectious diseases, and losses due to culling and death. The bovine immune system is important in mediating resistance to infectious diseases. The immune system involves a variety of cell types and soluble effector molecules which afford host protection against pathogenic organisms. The B and T lymphocytes are an integral part of the immune system because they are responsible for antigen recognition by the immune system and allow establishment of antigen-specific memory. Antigen-specific memory allows a more efficient immune response upon subsequent exposure to the same infectious agent (Tonegawa, 1985).

One interesting aspect of the immune system is a partial separation of the lymphocyte populations in mucosal organs (i.e.; intestine, respiratory tract, and reproductive organs) from that in peripheral organs. The basis for these somewhat separate lymphocyte populations is the preferential recirculation or homing of lymphocytes to either peripheral lymph nodes or mucosal lymphoid tissue (Gowans and Knight, 1964; Cahill et al., 1977; Scollay et al., 1976; Rose et al., 1976; Harp et al., 1988). Thus, the available repertoire of antigen-specific lymphocytes in mucosal lymphoid tissue is somewhat distinct from that in peripheral lymph nodes.
Mucosal immunity in non-ruminants

The major site of antigen uptake in the gut-associated lymphoid tissue is the Peyer's patch (Owen and Nemanic, 1978). The Peyer's patch consists of lymphoid aggregates in the intestinal wall covered by a layer of cuboidal epithelial cells on the lumenal surface. Specialized membrane epithelial cells (M cells) are present within the cuboidal epithelial cells that are involved in transporting foreign antigens into the Peyer's patches. Specialized epithelial cells similar in function to Peyer's patch M cells are also present in the bronchus-associated lymphoid tissue of the respiratory tract (Sheldrake and Husband, 1985).

After transport into the Peyer's patch, antigens are phagocytized by macrophages that travel to the lymphoid rich region of the Peyer's patch (Richman et al., 1981) and then initiate an immune response by presenting antigen to lymphocytes. After recognizing antigen and differentiating in the Peyer's patch, lymphocytes can enter the intestinal lymphatics, pass through mesenteric lymph nodes, enter the thoracic duct and then pass into the systemic circulation (Jalkanen, 1990). These lymphocytes recirculate through the blood and extravasate at mucosal sites. In the rodent, these mucosal sites include the Peyer's patches (McDermott and Bienenstock, 1979), lamina propria (Jeurissen et al., 1987), genital organs, respiratory tract (McDermott and Bienenstock, 1979), and mammary gland (Roux et al., 1977; Bienenstock et al., 1978).

The mucosal immune system of non-ruminants has unique characteristics that differentiate it from the peripheral immune system. Mucosal immunization results in local IgA production and parental immunization results in serum IgG (Tomasi et al., 1980). The predominant isotype present
in mucosal secretions of non-ruminants is IgA. Most of this IgA is produced locally by lymphocytes in the lamina propria of the intestine, the respiratory tract and other mucosal organs of non-ruminants (Tomasi et al., 1980).

In non-ruminants, not all of the IgA present in intestinal secretions is directly derived from local production by lymphocytes in the lamina propria. Some of the IgA produced by lymphocytes in the lamina propria escapes into the blood. This blood-borne IgA is transported into the bile. Bile is therefore rich in IgA and is another route by which IgA reaches the intestinal lumen in some non-ruminant species (Tizard, 1982).

Lymphocytes in Peyer’s patches, lamina propria, reproductive tract, respiratory tract, and mammary gland are derived from the same recirculating pool of lymphocytes in non-ruminants (Roux et al., 1977; Bienenstock et al., 1978; McDermott and Bienenstock, 1979; Jeurissen et al., 1987). Therefore, the antigen-specificity of lymphocytes and the antibody isotype present in these mucosal organs are somewhat similar and these organs are said to be part of a "common mucosal immune system" in non-ruminants.

Mucosal immunity in ruminants

The mucosal immune systems of cattle and sheep are markedly different from non-ruminants and care must be exercised when extrapolating information from non-ruminants to cattle and sheep. In cattle and sheep, intestinal fluids contain equal amounts of IgG1 and IgA (Lascelles et al., 1986). A majority of sheep intestinal IgG1 is derived from serum (Cripps et al., 1974), even though a large number of IgG1+ lymphocytes are present in the lamina propria (Lascelles et al., 1986). Data from experiments using
intravenous injections of radioisotope-labeled IgA suggest that sheep intestinal IgA is primarily produced locally (Cripps et al., 1974). In certain situations, some of sheep intestinal IgA may be derived from bile. Injection of antigen into the intestinal wall resulted in antigen-specific IgA in the bile that was likely derived by active transport from the blood (Hall, 1986).

In cattle and sheep, secretions in the upper respiratory tract are composed of equal amounts of IgG1 and IgA (Lascelles et al., 1986). The majority of IgG1 in sheep was derived from serum while the bulk of IgA present in upper respiratory secretions is produced locally (Scicchitano, 1984a). The similarities in the isotypes of antibodies in the intestine and upper respiratory tract suggest that these tissues may be part of the common mucosal immune system in ruminants. There is evidence to support this hypothesis, at least in sheep. Intestinal immunization resulted in the homing of antigen-specific lymphocytes to the upper respiratory tract of sheep (Scicchitano, 1984b). Therefore, the intestinal immune system contributes to upper respiratory immunity in sheep, via the homing of lymphocytes from the intestine to the upper respiratory system.

The predominant isotype of secretions in the lower respiratory tract (broncho-alveolar secretions) of sheep and cattle is IgG1 (Lascelles et al., 1986). It is not known whether this IgG1 is produced locally or derived from serum. The lower respiratory tract of sheep does not appear to be part of a common mucosal immune system as lymphocytes sensitized to antigen in the intestine do not home to the regional lymph nodes draining the lower respiratory tract (Scicchitano, 1984b).
Mammary immunity in ruminants

The mammary immune system of cattle and sheep is quite different from that of other species. IgG\textsubscript{1} is the most predominant isotype in milk and colostrum in cattle. The concentrations of IgG\textsubscript{1} and IgA in milk are 0.40 mg/ml and 0.11 mg/ml, respectively. The concentrations of IgG\textsubscript{1} and IgA in colostrum are 48.2 mg/ml and 4.70 mg/ml, respectively (Lascelles et al., 1986). Colostral IgG\textsubscript{1} is derived primarily from serum shortly before parturition (Brandon et al., 1971), but there is evidence that IgG\textsubscript{1} can be produced locally during intramammary infection (Chang et al., 1981). The small amount of IgA present in milk is produced locally during mammary infections (Lascelles et al., 1966).

The recirculation of lymphocytes to the mammary gland in cattle and sheep is quite different from rodents. The mammary gland of rodents is part of a common mucosal immune system as lymphocytes from the intestine will home to the mammary gland in rodents (Roux et al., 1977). Thus, intestinal lymphocytes sensitized to intestinal pathogens can home to the mammary gland in rodents, transferring protection against enteric disease to suckling offspring via the milk.

This does not appear to be the case in ruminants. Intestinal immunization does not result in any substantial homing of antigen-specific lymphocytes from the intestine to the mammary gland of sheep (Sheldrake et al., 1985; Sheldrake and Husband, 1985). Studies examining the in vivo homing of lymphocytes in cattle demonstrate that intestinal lymph node lymphocytes do not readily home to mammary lymph nodes (Harp et al., 1988). Lymphocytes from intestinal lymph nodes home primarily to intestinal lymph nodes, while tending to avoid homing to both mammary and prescapular lymph.
Bovine mammary lymph node lymphocytes tend to avoid homing to intestinal lymph nodes in vivo. These lymphocytes preferentially home to mammary and prescapular lymph nodes (Harp et al., 1988). The homing of mammary lymph node lymphocytes primarily to mammary lymph nodes and prescapular lymph nodes suggests that the mammary lymph node is a peripheral lymph node, not a mucosal lymph node. Therefore, the mammary immune system of cattle and sheep is probably not part of a common mucosal immune system.

Lymphocyte homing

The recirculation or homing of blood-borne lymphocytes is well documented and was first reported over 25 years ago (Cowans and Knight, 1964). Studies in rodents, sheep and cattle have demonstrated that this recirculation is not random as lymphocytes tend to recirculate or home to specific organs or regions of the body. Lymphocytes can recirculate or home to either peripheral lymph nodes or mucosal lymphoid tissue (Cahill et al., 1977; Harp et al., 1988; Rose et al., 1976). Lymphocyte homing allows antigen-specific lymphocytes to extravasate at distant body sites, and it facilitates interactions between T and B lymphocytes and other accessory cells of the immune system (Miyasaka and Trnka, 1986).

Recirculating lymphocytes leave the blood and enter the lymph node at specialized venules located in the paracortex (Cowans and Knight, 1964). In order to extravasate, lymphocytes must first bind to, migrate along and then extravasate through the endothelium lining these paracortical venules. The initial binding of lymphocytes to the endothelium of paracortical venules in the lymph node controls the tissue-specificity of lymphocyte
In rodents and humans, these paracortical venules are lined with high-walled endothelium and are therefore termed high-walled endothelial venules (HEV) (Gowans and Knight, 1964; Jalkanen and Butcher, 1985; Butcher, 1990). In cattle and sheep, the paracortical venules are lined with endothelium that is not as "high" as that in HEV of rodents and humans (Harp et al., 1990). While the paracortical venules in sheep are not lined with high-walled endothelium, lymphocytes extravasate through these paracortical venules in sheep (Harp et al., 1990).

**Homing of B and T cells**

It has been reported that in vivo B cell recirculation is slower than T cell recirculation in rodents (Howard, 1972; Sprent, 1976), but not in sheep. Sheep B and T lymphocytes recirculate with similar kinetics to intestinal and prescapular lymph in vivo (Reynolds et al., 1988). As sheep and rodent lymphocytes exit the blood and enter the lymph node primarily through the paracortical venules in vivo (Harp et al., 1990; Gowans and Knight, 1964), and murine B and T cells bind to paracortical venules in vitro (Stevens et al., 1982), it is possible that the molecular mechanisms controlling lymphocyte binding to and extravasation through paracortical venules are similar for both B and T cells.

After entry into the lymph node, B cells tend to migrate to follicles and germinal centers in the cortex and T cells tend to remain in the paracortex. The mechanisms controlling the migration of B and T lymphocytes within lymph nodes after extravasation are poorly defined (Jalkanen, 1990).
In vitro HEV assay

Stamper and Woodruff (1976) developed an in vitro HEV assay which allows study of the initial adherence event. In this assay, lymphocyte suspensions are placed on frozen sections of lymph nodes. The lymphocytes adhere specifically to HEV in lymph node sections. Using the in vitro HEV assay, it has been shown that the tissue-specificity of in vivo homing to either peripheral lymph nodes or to mucosal lymphoid tissue is due to receptor-mediated adherence of lymphocytes to HEV in either peripheral lymph nodes or mucosal lymphoid tissue. The molecules on lymphocytes that are involved in homing are called homing receptors and the HEV molecules are termed vascular addressins (Butcher, 1990; Stoolman, 1989). While these molecules are involved in the initial tissue-specific adherence, they do not mediate lymphocyte homing entirely by themselves. Other molecules (such as CD44 and LFA-1) play an accessory role in homing (Chin et al., 1991).

Using this in vitro HEV assay, some of the bovine, human, and murine homing receptors and vascular addressins have been identified and characterized (Gallatin et al., 1983; Streeter et al., 1988a, 1988b; Kishimoto et al., 1990; Bosworth and Harp, 1991; Bosworth and Harp, Part III of this dissertation). While differences exist between cattle and non-ruminants in paracortical venule morphology, antibody isotype in intestinal and mammary secretions, and lymphocyte homing to the mammary gland; some molecules controlling lymphocyte homing in cattle are very similar to those in mice and humans (Bosworth et al., 1991; Bosworth and Harp, 1991; Bosworth and Harp, Part III of this dissertation).

Further evidence for the conservation of molecules controlling lymphocyte homing in a variety of species comes from studies that
demonstrate that the receptor-mediated adherence of lymphocytes to HEV is highly conserved across species barriers. Bovine, murine, guinea pig, and human lymphocytes adhere in a tissue-specific manner to HEV in sections of murine lymphoid tissue in vitro (Wu et al., 1988; Bosworth and Harp, 1991; Bosworth and Harp, Part III of this dissertation). The tissue-specific pattern of human and guinea pig lymphocyte adherence to murine HEV mimics the pattern seen using human and guinea pig lymphocytes and homologous HEV (Wu et al., 1988). The pattern of bovine lymphocyte adherence to murine HEV in vitro reflects the in vivo homing patterns of bovine lymphocytes. Bovine mammary and prescapular lymph node lymphocytes bind in relatively equal numbers to murine peripheral lymph node HEV in vitro (Bosworth and Harp, Part III of this dissertation) and bovine mammary lymph node lymphocytes tend to home like peripheral lymph node lymphocytes in vivo (Harp et al., 1988).

Attempts to duplicate this in vitro HEV assay using bovine or sheep lymphocytes on homologous lymph node sections have been unsuccessful (Bosworth and Harp, personal observations; Miyasaka and Trnka, 1986). The reasons for this lack of success are not known. It may be due to low number of available binding sites for lymphocytes in paracortical venules in ruminants, since endothelial cells in these venules are not high in sheep and cattle (Miyasaka and Trnka, 1986; Harp et al., 1990).

In the following section of this review, the molecules involved in murine and human lymphocyte homing will be discussed. When possible, the relevance of similar molecules in cattle will be discussed.
Molecular Mechanisms Controlling Lymphocyte Homing

Mucosal lymphocyte homing receptors

Antibodies that recognize two distinct murine lymphocyte antigens, LPAM-1 and LPAM-2 (lymphocyte Peyer's patch HEV adhesion molecules, heterodimers with a common alpha subunit but different beta subunits), block the binding of murine lymphocytes to Peyer's patch HEV in vitro (Holzmann et al., 1989; Holzmann and Weissman, 1989). These antibodies have no effect on the binding of lymphocytes to peripheral lymph node HEV. This suggests that LPAM-1 and LPAM-2 may be mucosal homing receptors in the mouse. However, mere expression of LPAM-2 is not sufficient in itself to mediate lymphocyte binding to Peyer's patch HEV (Holzmann and Weissman, 1989). Possibly, adherence of lymphocytes to Peyer's patch HEV is due to the interactions of multiple adhesion receptors. There are no data on the role of bovine LPAM-1 or LPAM-2 in bovine lymphocyte homing.

Peripheral lymphocyte homing receptors

The human and murine peripheral lymphocyte homing receptors, recently termed LECAM-1 (lectin cell adhesion molecule-1), have been identified and characterized. The predicted amino acid sequences of human and murine LECAM-1 have a domain that is homologous to a number of calcium-dependent animal lectins (Bowen et al., 1989; Lasky et al., 1989; Siegelman et al., 1989; Siegelman and Weissman, 1989). As specific carbohydrates block the binding of human and murine lymphocytes to peripheral lymph node HEV in vitro (Stoolman and Rosen, 1983, Stoolman et al., 1984; Stoolman et al., 1987), the lectin domain of human and murine LECAM-1 is likely involved in binding of lymphocytes to peripheral lymph node HEV. Also, the monoclonal
antibody MEL-14, which blocks the binding of murine lymphocytes to peripheral lymph node HEV in vitro and homing of murine lymphocytes to peripheral lymph nodes in vivo, recognizes the lectin domain of LECAM-1 (Bowen et al., 1990).

Human and murine LECAM-1 also contain a domain with homology to proteins in the epidermal growth factor (EGF) family. This domain may have a role in lymphocyte homing. A monoclonal antibody that binds to the EGF-like domain of murine LECAM-1 reduced the binding of murine lymphocytes to peripheral lymph node HEV in vitro (Siegelman et al., 1990). The exact role the EGF-like domain plays in HEV binding has been speculated to be due a direct role in HEV binding. However, it is possible that the antibody that reacted with the EGF-like domain reduced HEV binding due to secondary changes induced on the lectin domain of LECAM-1 or due to steric effects of the antibody (Siegelman et al., 1990). Murine and human LECAM-1 contain two repeated domains that show homology to a number of proteins, most of which have a role in complement-binding (Bowen et al., 1989; Lasky et al., 1989; Siegelman et al., 1989; Siegelman and Weissman, 1989). The role of the LECAM-1 domains which are similar to complement-binding protein domains in lymphocyte homing has not been established.

The bovine peripheral lymphocyte homing receptor has lectin-like activity and is functionally similar to human and murine LECAM-1. Fructose-1-phosphate and fucoidin, which block the binding of human and murine lymphocytes to peripheral lymph node HEV, block the binding of bovine lymphocytes to murine peripheral lymph node HEV (Bosworth and Harp, 1991). We have recently isolated and sequenced cDNA clones that encode for bovine LECAM-1 (Bosworth and Harp, Part III of this dissertation). Bovine LECAM-1
has high amino acid sequence identity with human and murine LECAM-1. Bovine LECAM-1 contains domains that have high identity with the lectin domain, epidermal growth factor-like domain, and two repeated domains similar to complement-binding proteins present in human and murine LECAM-1. The antibody DREG-56, which recognizes human LECAM-1 and blocks the binding of human lymphocytes to peripheral lymph node HEV (Kishimoto et al., 1990), blocks the binding of bovine peripheral blood lymphocytes (PBL) to murine peripheral lymph node HEV (Bosworth and Harp, Part III of this dissertation). The effect of DREG-56 is tissue-specific in that it has no effect on the binding of human (Kishimoto et al., 1990) and bovine lymphocytes to Peyer's patch HEV (Bosworth and Harp, Part III of this dissertation). These data strongly suggest that bovine LECAM-1, like human and murine LECAM-1, is a peripheral lymphocyte homing receptor.

Differences are seen in the tissue distribution of bovine LECAM-1. Few lymphocytes from bovine Peyer's patch express LECAM-1 (Bosworth and Harp, Part III of this dissertation), whereas this molecule is present on most murine lymphocytes that home to either peripheral lymph nodes or Peyer's patches in vivo (Gallatin et al., 1983). Presumably, the LECAM-1+ lymphocytes that home to murine Peyer's patches in vivo express mucosal homing receptors; the LECAM-1 present on these Peyer's patch homing lymphocytes may be modified and unable to mediate homing to peripheral lymph nodes. Thus in the mouse, LECAM-1 is specific in function, but not distribution, to peripheral homing lymphocytes (Gallatin et al., 1983), whereas in the bovine both function and distribution are specific to peripheral homing cells (Bosworth and Harp, Part III of this dissertation).

The finding that bovine LECAM-1 is present in high amounts on
lymphocytes derived from peripheral and mammary lymph nodes, and low amounts on lymphocytes derived from Peyer's patch, provides an explanation at the molecular level for the previous observation that bovine mammary lymphocytes exhibit homing patterns similar to peripheral rather than mucosal lymphocytes (Harp et al., 1988).

In addition to its role in lymphocyte homing, LECAM-1 is involved in the binding of neutrophils to endothelium in areas of inflammation (Jutila et al., 1989; Hallmann et al., 1991). The antibody MEL-14 which recognizes murine LECAM-1, decreases the number of murine neutrophils that are recruited to sites of inflammation in vivo (Jutila et al., 1989). It is not known if bovine LECAM-1 plays a similar role in bovine neutrophil adherence to endothelium.

Vascular addressins

Streeter et al. (1988b) have described a monoclonal antibody that reacts with murine Peyer's patch HEV but not peripheral lymph node HEV. This monoclonal antibody, termed MECA-367, precipitates a 58-67 kD endothelial cell protein expressed at mucosal sites of lymphocyte extravasation. The monoclonal antibody MECA-367 probably recognizes the mucosal vascular addressin because it blocks the binding of murine lymphocytes to Peyer's patch HEV in vitro, and lymphocyte homing to Peyer's patches in vivo (Streeter et al., 1988b). There are no data on the bovine mucosal vascular addressins at this time. However, the bovine and murine mucosal vascular addressins may be similar as both bovine and murine lymphocytes bind to murine Peyer's patch HEV in vitro (Bosworth and Harp, 1991).
The monoclonal antibody MECA-79 recognizes human and murine peripheral lymph node vascular addressins (Butcher, 1990). The antigen detected by MECA-79 is expressed at high levels in murine peripheral lymph node HEV while its expression in Peyer's patch HEV is more restricted (Streeter et al., 1988a). The antibody MECA-79 blocks the binding of murine lymphocytes to peripheral lymph node HEV in vitro, and homing of murine lymphocytes to peripheral lymph node HEV in vivo, but has no effect on the interaction of lymphocytes with Peyer's patch HEV (Streeter et al., 1988a). A soluble recombinant form of LECAM-1 reacts selectively with murine peripheral lymph node HEV (Imai et al., 1991). The bovine peripheral lymph node vascular addressin is probably similar to that of human and mouse because the antibody MECA-79 and the soluble recombinant form of LECAM-1 cross react with bovine paracortical venules in peripheral lymph nodes (personal communication with Bruce Walcheck of Montana State University, Bozeman, MT; Mark Singer, University of California, San Francisco, CA).

Other molecules with an accessory role in lymphocyte homing

The β-2 integrin LFA-1, a heterodimer consisting of CD11a and the common β-2 subunit CD18, may have an accessory role in lymphocyte homing. Antibodies that recognize LFA-1 partially reduce murine and human lymphocyte binding to peripheral lymph node HEV and Peyer's patch HEV in vitro, and murine lymphocyte homing to peripheral lymph nodes and Peyer's patches in vivo (Hamann et al., 1988; Pals et al., 1988). A calf with a genetic defect in the expression of CD18, the β-2 subunit of LFA-1, appears to have some lymphocyte homing. Lymphocytes were present in the paracortical areas of lymph nodes in this calf (Kehrli et al., 1990). Possibly, the adhesive
functions of LFA-1 may be assumed by other lymphocyte adhesion molecules (Springer et al., 1987).

The glycoprotein CD44, has been reported to be involved in lymphocyte homing in humans. The monoclonal antibody Hermes-3 which recognizes CD44, blocks the binding of human lymphocytes to Peyer's patch HEV, but does not reduce the binding of lymphocytes to peripheral lymph node HEV. While the effect of Hermes-3 is limited to mucosal homing lymphocytes, polyclonal antibody to CD44 blocks the binding of human lymphocytes to both Peyer's patch HEV and peripheral lymph node HEV (Jalkanen et al., 1987).

Recent evidence has shown that distinct forms of CD44 exist. The shorter form of CD44 is present in most hematopoietic cells and has already been sequenced in many species, including the bovine (Bosworth et al., 1991). Longer forms of CD44 containing additional amino acids are present in keratinocytes, epithelial cells, a myelomonocytic cell line, and a pancreatic carcinoma cell line (Stamenkovic et al., 1991; Gunthert et al., 1991; Brown et al., 1991; Dougherty et al., 1991). These longer forms are likely derived by the addition of exons into the shorter form of CD44 (Brown et al., 1991). It has been speculated that glycosylation of the related core peptides could possibly be unique for each cell type and reflect functional differences of CD44 (Brown et al., 1991). Transfection of cells with the cDNA encoding for the shorter hematopoietic form of CD44 allowed a B cell line to bind to an endothelial cell line derived from HEV (Stamenkovic et al., 1991). This binding is blocked by polyclonal anti-CD44 antiserum, but not by Hermes-3. Transfection of the same B cell line with a longer form of CD44 did not cause an increase in binding to the cultured endothelial cells. Expression of a longer form of CD44, derived from a
pancreatic carcinoma cell line, conferred full metastatic behavior to a nonmetastasizing cell line (Gunthert et al., 1991). There are no reports on the existence of longer forms of bovine CD44. CD44 is clearly more than just a homing receptor as it is involved in a wide variety of cellular adhesion events and can be best described as a multipurpose adhesion protein (Stoolman, 1989).

Comparison of the predicted amino acid sequences of the shorter forms of bovine, human, and murine CD44 shows extensive overall similarities between species (Bosworth et al., 1991). However, there is an extracellular region of about 75 amino acids that has considerable variability between species. The location of this variable region is conserved between species. The antibody Hermes-3, which recognizes human CD44 and blocks the binding of human lymphocytes to Peyer's patch HEV (Jalkanen et al., 1987), recognizes an epitope of human CD44 that may lie within this variable region (Goldstein et al., 1989). While the antibody Hermes-3 cross reacts with bovine lymphocytes, it does not reduce the binding of bovine lymphocytes to peripheral lymph node HEV or Peyer's patch HEV (Bosworth, personal observations). The different effects of Hermes-3 on bovine and human lymphocyte binding to HEV may possibly be due to the amino acid differences in the variable region.

Conclusions

The recent advances in identifying some of the lymphocyte homing receptors and vascular addressins in the bovine have increased our understanding of the molecular basis of bovine lymphocyte homing.

The in vitro HEV assay has been used to characterize the molecules
controlling the binding of lymphocytes to HEV in lymph node sections in a variety of species (Bosworth and Harp, 1991; Bosworth and Harp, Part III of this dissertation, Wu et al., 1988; Gallatin et al., 1983). Experiments are currently being planned to verify that the binding of bovine lymphocytes to murine HEV in vitro represents the events controlling homing in vivo. Bovine lymphocytes will be treated with DREG-56 to determine if this antibody reduces the homing of bovine lymphocytes to bovine peripheral lymph nodes in vivo. Additionally, more research is needed to determine what role, if any, bovine CD44 plays in bovine lymphocyte homing.

The antibody DREG-56, which recognizes a peripheral lymphocyte homing receptor, bovine LECAM-1, should prove useful in studying the immune system in cattle. High levels of bovine LECAM-1 are present on lymphocytes from peripheral lymph nodes, little LECAM-1 is present on lymphocytes from Peyer's patches (Bosworth and Harp, Part III of this dissertation). This antibody could be used to determine if a lymphoid organ is part of the peripheral or mucosal immune system. Since lymphocytes from bovine Peyer's patches express little LECAM-1, it would be of interest to determine if lymphocytes in the upper respiratory tract and reproductive tract of cattle also express low levels of LECAM-1.

Future research should focus on the in vivo role of these molecules that control lymphocyte homing and tissue-specific immunity. These studies should further increase our understanding of lymphocyte homing and lay the groundwork for future methods that enhance tissue-specific immunity in the bovine and other animals.
PART I. SEQUENCE OF THE BOVINE CD44 cDNA:
COMPARISON WITH HUMAN AND MOUSE SEQUENCES
SEQUENCE OF THE BOVINE CD44 cDNA:
COMPARISON WITH HUMAN AND MOUSE SEQUENCES

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ABSTRACT

CD44 is a glycoprotein involved in lymphocyte homing to secondary lymphoid organs and may function in other intercellular adhesion events as well. We have isolated and sequenced a cDNA clone which encodes for bovine CD44. The predicted amino acid sequence of bovine CD44 had an overall high similarity with that of human and mouse CD44, 79.5% and 73.2%, respectively. In all three species, CD44 had a similar transmembrane region and cytoplasmic tail. In addition, all of the cysteine residues and a majority of the putative N-linked glycosylation sites in the extracellular domain were conserved between bovine, human and mouse. An area of low amino acid similarity between species was present within the extracellular domain. This area had a similarity of 34% between bovine and human, and 27% between bovine and mouse. The location of this area of low similarity was conserved between species.
INTRODUCTION

Antibodies recognizing the Pgp-1, (In[Lu]) related-p80, ECMRIII, and Hermes lymphocyte homing antigens have been shown to recognize the same molecule, designated CD44 at the Third International Workshop on Leukocyte Differentiation Antigens (Cobbold et al., 1987). Recently, the cDNAs encoding for human, baboon, and mouse CD44 have been sequenced (Stamenkovic et al., 1989; Goldstein et al., 1989; Idzerda et al., 1989; Nottenburg et al., 1989; Zhou et al., 1989). The CD44 membrane protein has been found on a wide variety of cells including lymphocytes, monocytes, granulocytes, fibroblasts, epithelial cells (Carter and Wayner, 1988; Flanagan et al., 1989), erythrocytes (Telen et al., 1983), and cells in the brain (Flanagan et al., 1989). CD44 has been reported to be involved in T-cell activation (Huet et al., 1989; Shimuzu et al., 1989; Haynes et al., 1988), to associate with cytoskeletal components and collagen (Carter and Wayner, 1988; Kalamoris and Bourguignon, 1988), recognize hyaluronate (Aruffo et al., 1990), and to regulate lymphocyte homing to secondary lymphoid organs in man (reviewed by Stoolman, 1989; Butcher, 1990).

Circulating lymphocytes exit the bloodstream and enter secondary lymphoid organs by attachment to and migration through post-capillary venules located in the paracortex (Gowans and Knight, 1964). The Hermes class of antibodies to CD44 may block homing by inhibiting the attachment of lymphocytes to the endothelium of these post-capillary venules in the lymph node paracortex (reviewed by Stoolman, 1989; Butcher, 1990). In humans and rodents these paracortical venules are lined with high-walled endothelium (Gowans and Knight, 1964; Jalkanen and Butcher, 1985; Smith and
Ford, 1983). While paracortical venules in ruminants do not have high-walled endothelium, it has been demonstrated that circulating lymphocytes attach to and migrate through paracortical venules lined with low-walled endothelium (Harp et al., 1990). Therefore, it is reasonable to assume that the molecular mechanisms regulating adhesion between circulating lymphocytes and endothelial cells are similar in ruminant and non-ruminant species.

In this paper, we report on the isolation and sequencing of a cDNA clone which encodes for bovine CD44. The predicted amino acid sequence of bovine CD44 demonstrated an overall high similarity with that of human and mouse CD44, 79.5% and 73.2%, respectively. While the overall similarity was high, an area of low similarity between bovine and other species (34% - 27%) was identified within the extracellular domain. Mouse and human also have a region of low similarity (35%) in the extracellular domain (Nottenburg et al., 1989). The location of this area of low similarity was conserved between species.
MATERIALS AND METHODS

Isolation of bovine CD44 cDNA

Approximately 30,000 recombinant clones from a bovine lymphocyte cDNA lambda gt11 library (ATCC, Rockville, MD) were screened by hybridization with a \(^{32}\)P-random primer labeled (Pharmacia LKB, Piscataway, NJ) baboon CD44 cDNA clone (Idzerda et al., 1989) as previously described (Sambrook et al., 1989). One clone that hybridized to the baboon CD44 cDNA was identified and the 1.4 kb insert cloned into the EcoR I site of the cloning vector plasmid pBluescript (Stratagene, La Jolla, CA).

Sequencing strategy

The DNA sequence of both strands of the bovine CD44 cDNA clone was determined as follows. The clone containing the 1.4 kb insert and subclones derived by digesting the 1.4 kb insert with restriction enzymes and subcloning into plasmid pBluescript (Stratagene) or pGem-5zf (Promega Biotec, Madison, WI) were used to generate single stranded filamentous bacteriophage using the helper bacteriophage M13K07 (Promega). Single-stranded phage and single-stranded DNA were recovered (Sambrook et al., 1989) and the sequence of the DNA was determined by the dideoxy chain termination method (Sanger et al., 1977) using T7 DNA polymerase (Pharmacia), primers specific for the cloning vectors and synthesized oligonucleotide primers specific for bovine CD44.

Northern blot analysis

Various bovine tissues were obtained at a local slaughter plant. Total
RNA was recovered by single extraction with an acid guanidinium thiocyanate-phenol-chloroform mixture (Chomczynski and Sacchi, 1987) and poly(A)^+RNA isolated using oligo dT chromatography (Aviv and Leder, 1972). Poly(A)^+RNA from these tissues was subjected to electrophoresis in a denaturing agarose gel and transferred to a nylon membrane (Nytran, Schleicher & Schuell, Keene, NH). Prehybridization was carried out in 50% formamide, 5X Denhardt’s, 5X SSPE, 0.5% SDS, and 0.1 mg/ml salmon sperm DNA for 4 hours at 42°C. Hybridization was carried out in the same buffer containing ^32P- random primer labeled bovine CD44 cDNA overnight at 42°C. The nylon membrane was washed twice in 6X SSPE/0.5% SDS at room temperature, twice in 1X SSPE/0.5% SDS at 37°C, and then exposed to X-ray film at -70°C.

Computer analysis

Sequence data were analyzed and amino acids deduced using the DNA sequence analysis programs written by Schwindinger and Warner (1984). Hydrophobicity of the deduced amino acid sequence was determined with the Hibio Prosis program (Pharmacia) using the method of Kyte and Doolittle (1982). Comparison of amino acid sequences was performed using the Align program (Scientific & Educational Software, Silver Spring, MD).
Characterization of the bovine cDNA encoding for CD44

The DNA sequence of the bovine CD44 cDNA clone is presented in Figure 1. The 1355 nucleotide cDNA contained one long open reading frame of 1089 bases starting at base number 101. This start location agreed well with the consensus translation initiation sequence described previously (Kozak, 1984). The 3' end of the cDNA terminated in a short poly(A) tail preceded by a possible polyadenylation sequence, CATAAA, 14 bases upstream (Birnstiel et al., 1985).

RNA expression analysis

The 1.4 kb bovine CD44 cDNA was hybridized in Northern blot analyses to 1.5 μg of poly(A)^+RNA from three different bovine tissues (Figure 2). Three RNA bands of approximately 4.5, 2.3, and 1.7 kb were detected in RNA derived from mesenteric lymph node and liver. The two smaller bands were of similar intensity and were more intense than the faint 4.5 kb band. Similar sized CD44 mRNAs were detected in spleen, spinal cord, mammary lymph node, and lung (data not shown). The number and size of bovine CD44 mRNAs were similar to those found in human hematopoietic cells (Stamenkovic et al., 1989; Goldstein et al., 1989). Murine CD44 mRNAs have only two sizes and do not purify with polyadenylated RNAs (Nottenburg et al., 1989). No CD44 mRNA was detected in poly(A)^+RNA derived from bovine heart (Figure 2).

Predicted amino acid sequence of bovine CD44

The first 20 amino acids of the open reading frame (Figure 1) resembled
a possible leader peptide (von Heijne, 1986). Cleavage of the leader peptide would result in a mature protein with an apparent predicted molecular mass of 37.7 kD. The 253 amino acid long extracellular protein domain (amino acids 21-273) was rich in serine (12.6%) and threonine (9.8%) residues and contained seven potential N-linked glycosylation sites. The putative hydrophobic transmembrane region (amino acids 274-294) consisting of 21 neutral amino acids was followed by a 72 amino acid cytoplasmic domain (amino acids 295-366). The putative transmembrane region contained a single cysteine which may be a potential fatty acylation site (Kaufman, 1984).

**Comparison of the predicted amino acid sequence of bovine, human and mouse CD44**

A comparison of the predicted amino acid sequence of bovine, human and mouse CD44 is shown in Figure 3. Bovine CD44 had 79.5% and 73.2% amino acid sequence similarity with that of human and mouse CD44, respectively. The areas of highest similarity extended from bovine amino acids 1-187 and 261-366. All three contained a putative leader peptide; the mouse leader peptide was 4 amino acids longer than that of bovine and human. Of the seven potential N-linked glycosylation sites in the bovine extracellular domain, six were present in human and five in mouse. The N-linked glycosylation sites that were present in bovine CD44, but not in mouse or human CD44, were within the area of low inter-species similarity. All cysteine residues in the extracellular and transmembrane regions, the amino acid sequence and location of the transmembrane region, and the length of the cytoplasmic tail were conserved between species.

An area of low similarity between bovine, human and mouse was present
in the extracellular domain and extended from bovine amino acids 188-260. This region of low amino acid similarity had 34% and 27% identity with that of human and mouse, respectively. This same area is also the region of lowest similarity (35%) between human and mouse (Nottenburg et al., 1989). Minor differences included three-base insertions in bovine and murine CD44 cDNA that did not alter the reading frame.

The region of low similarity does not appear to be due to use of different exons in various species. In the mouse, one exon has been found to encompass amino acids 152-227. The area of low inter-species similarity starts within this exon at mouse amino acid 187 and extends well beyond it to amino acid 259 (Nottenburg et al., 1989). This region of low inter-species similarity is contained in the region possibly involved in the binding of human lymphocytes to high-walled endothelial venules (Goldstein et al., 1989). If some of the adhesive functions of CD44 involve specific polypeptides in the region of low similarity (amino acids 188-259), this region may mediate certain species-specific types of cellular adhesion. Another possibility is that the area of lowest amino acid similarity has a limited role in adhesion, allowing variation between species without affecting certain adhesive functions of CD44.

In conclusion, we have isolated and sequenced a bovine CD44 cDNA clone and subsequently deduced the amino acid sequence. The overall similarity between the predicted amino acid sequence of bovine, human and mouse CD44 was extensive. Conservation of N-linked glycosylation and cysteine residues suggests that the proteins may assume similar three dimensional structures. There was a region of low amino acid similarity between species which may alter protein folding; however, the location of this region was conserved
between species.
Figure 1. cDNA sequence of bovine CD44. Potential N-linked glycosylation sites are double underlined; putative leader peptide and transmembrane region are underlined.
<table>
<thead>
<tr>
<th>Position</th>
<th>Codon</th>
<th>Amino Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>928</td>
<td>ATG TTG GCA TCT CTC CTG GCC TTG GCT TTG ATT CTC GCG GTT TGC ATT GCT GTC</td>
<td>Ile Leu Ala Ser Leu Ala Leu Ala Val Cys Ile Ala Val</td>
</tr>
<tr>
<td>982</td>
<td>AAC AGT AGG AGG TGT GGG CAG AAG AAA AAA CTG GTG ATC AAC AAT GGG AAT</td>
<td>Asn Ser Arg Arg Cys Gly Gin Lys Lys Leu Val Ile Asn Asn Gly Asn</td>
</tr>
<tr>
<td>1036</td>
<td>GGA ACC ATG GAG GAG AAG CCC AGC GGA CTC AAC GGA GAA GCT AAG TCT</td>
<td>Gly Thr Met Glu Glu Arg Lys Pro Ser Gly Leu Gly Glu Ala Ser Lys Ser</td>
</tr>
<tr>
<td>1144</td>
<td>CAG GAG ATG GTG CAT TTG GTG AAC AAG GGG TCG TCA GAG ACC CCA GAC CAG TTC</td>
<td>Gln Glu Met Val His Leu Val Asn Lys Gly Ser Ser Glu Thr Pro Asp Glu Phe</td>
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</tr>
<tr>
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<tr>
<td>1338</td>
<td>TTCAACATCGAATGCGTACTGATTGTTCATTGGGATCTTTTTTTTTTTTACGATAAAAATTTCTATTC</td>
<td></td>
</tr>
<tr>
<td>1355</td>
<td>CTTAAAAAAAAAAAAA</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2. RNA analysis of the CD44 gene. 1.5 μg of poly(A)^+RNA from bovine heart (A), liver (B), and mesenteric lymph node (C) was electrophoresed, transferred to a nylon membrane, and hybridized with ^32P-labeled bovine CD44 cDNA. The numbers on the left correspond to the sizes of a RNA ladder.
Figure 3. Comparison of the amino acid sequences of CD44. The amino acid sequence (one letter designation) determined from the bovine CD44 cDNA is shown on the top line. Amino acid differences in human (Stamenkovic et al., 1989) and the C3H strain of mouse (Nottenburg et al., 1989) are shown. Amino acid similarity is designated with a dot (.) and deletions with a dash (-).
REFERENCES


PART II. EVIDENCE FOR THE CONSERVATION OF PERIPHERAL LYMPHOCYTE HOMING RECEPTORS IN THE BOVINE, HUMAN AND MURINE SPECIES
EVIDENCE FOR THE CONSERVATION OF PERIPHERAL LYMPHOCYTE HOMING RECEPTORS IN THE BOVINE, HUMAN AND MURINE SPECIES

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In rodents and humans, lymphocytes circulate throughout the body and return preferentially to their tissues of origin via a process termed homing. The specificity of homing is controlled by the binding of tissue-specific receptors on lymphocytes to ligands on specialized high-walled endothelial venules (HEV) found in lymphoid tissue. The murine and human peripheral lymphocyte homing receptors (PLHR) have been characterized and shown to be similar to each other. We present evidence for a similar receptor in the bovine. Bovine peripheral blood lymphocytes (PBL) bound to the HEV of murine peripheral lymph node tissue in vitro. The same carbohydrates that have been shown to decrease the binding of murine or human lymphocytes to murine HEV also decreased the binding of bovine PBL to murine HEV. Neuraminidase treatment affected lymphocyte binding in a similar manner in the bovine, murine, and human species. Phorbol myristate acetate (PMA) stimulation, which has been shown to reduce the expression of murine and human PLHR, also reduced the binding of bovine PBL to murine HEV. These data suggest conservation of PLHR between these species.
INTRODUCTION

Lymphocyte homing is the process by which lymphocytes recirculate throughout the body and return to lymphoid tissues (Gowans and Knight, 1964; Butcher, 1990; Stoolman, 1989). In rodents and humans, the specificity of homing is controlled by adhesive interactions of homing receptors on lymphocytes with vascular addressins on high-walled endothelial venules (HEV) (Gallatin et al., 1983; Kishimoto et al., 1990; Streeter et al., 1988a, 1988b). Murine and human lymphocyte homing receptors and vascular addressins have been studied and characterized using an in vitro HEV binding assay. This HEV assay detects tissue-specific adhesive interactions of lymphocytes to HEV in frozen sections of either peripheral lymph nodes or Peyer's patches and predicts migratory activity in vivo (for review, see Butcher, 1990; Stoolman, 1989).

The murine and human peripheral lymphocyte homing receptors (PLHR) have been characterized and shown to be very similar to each other (Gallatin et al., 1983; Bowen et al., 1989; Kishimoto et al., 1990; Lasky et al., 1989; Siegelman et al., 1989; Siegelman and Weissman, 1989). Sequence analysis of the cDNA encoding for murine and human PLHR reveals an extracellular lectin-like domain (Bowen et al., 1989; Lasky et al., 1989; Siegelman et al., 1989; Siegelman and Weissman, 1989). Evidence that this lectin domain is involved in homing of peripheral lymphocytes includes the ability of carbohydrates, such as mannose-6-phosphate (M6P), fructose-1-phosphate (F1P), and fucoidin, to inhibit the binding of lymphocytes to peripheral lymph node HEV (Stoolman and Rosen, 1983; Stoolman et al., 1984; Stoolman et al., 1987). The aforementioned carbohydrates compete with each other for
binding to purified PLHR (Imai et al., 1990). Also, the antibody MEL-14, which blocks homing of murine peripheral lymphocytes, binds to the lectin domain of PLHR (Bowen et al., 1990).

In this report, we present evidence for a receptor in the bovine that is similar to murine and human PLHR. Bovine peripheral blood lymphocytes (PBL) bound preferentially to HEV of murine peripheral lymph node tissue in vitro. Carbohydrates that have been shown to decrease binding of murine or human lymphocytes to murine HEV also decreased the binding of bovine PBL to murine HEV. Neuraminidase treatment affected lymphocyte binding in a similar manner in the bovine, murine, and human species. Phorbol myristate acetate (PMA) stimulation, which has been shown to reduce the expression of murine and human PLHR (Huang et al., 1989; Jung and Dailey, 1990; Kishimoto et al., 1990), reduced the binding of bovine PBL to murine HEV. These data suggest that structure and function of PLHR are conserved between these species.
MATERIALS AND METHODS

Cell preparation

Bovine PBL were collected by centrifugation at 450 g for 40 min over Percoll (sp gr = 1.084) as previously described (Whitmire and Harp, 1991). Platelets were removed from the PBL by centrifugation through Percoll (sp gr = 1.029). The PBL were then washed in assay buffer (RPMI 1640; Gibco Laboratories, Grand Island, NY; supplemented with 5% fetal bovine serum). Bovine PBL in assay buffer were then labeled with Hoechst 33342, a fluorescent dye that binds DNA (Sigma Chemical Co, St. Louis, MO; 0.6 μg/ml, 30 min at 39°C). Murine splenocytes and Peyer's patch lymphocytes from adult BALB/c mice were labeled with fluorescein isothiocyanate (FITC; Sigma; 50 μg/ml, 30 min at 37°C). After labeling, lymphocytes were pelleted by centrifugation through fetal bovine serum, to remove unbound label and dead cells, and then washed in assay buffer.

HEV assay

Axillary, mesenteric and popliteal lymph nodes, and Peyer's patches were removed from adult BALB/c mice for use in the HEV binding assay described previously (Butcher et al., 1979; Stamper and Woodruff, 1976; Stoolman et al., 1987). Briefly, lymphoid tissue imbedded in O.C.T. (Miles Inc, Elkhart, IN) was frozen on dry ice and sectioned with a cryostat. Tissue sections were placed on glass slides. The O.C.T. was removed with forceps and sections were overlaid with 100 μl of lymphocyte suspension in assay buffer. Sections were rotated at 60 RPM on a horizontal rotary shaker for 30 min at 5°C. The sections were then fixed in 3% glutaraldehyde in
phosphate-buffered saline (PBS) for 45 min, rinsed in PBS, and examined microscopically with darkfield/fluorescent illumination.

**Tissue specificity of bovine PBL binding**

Hoechst-labeled bovine PBL (2 X 10^7/ml) were mixed at a 10 to 1 ratio with FITC-labeled murine splenocytes or Peyer's patch lymphocytes (2 X 10^6/ml). Samples containing bovine PBL and murine splenocytes were used to overlay sections of murine peripheral lymph nodes (axillary and popliteal). Samples containing bovine PBL and murine Peyer's patch lymphocytes were used to overlay sections of murine Peyer's patches. The numbers of bovine and murine lymphocytes bound to HEV were determined by visually identifying and then counting lymphocytes bound to HEV. The specific adherence ratio (SAR) was calculated as previously described by dividing the ratio of bovine to murine lymphocytes bound to HEV by the ratio of bovine to murine lymphocytes used to overlay the lymph node sections (Butcher et al., 1979).

**Neuraminidase, PMA, and carbohydrate treatment**

Bovine PBL in assay buffer (1 X 10^7/ml) were incubated for 30 min at 39°C with 0.5 U/ml neuraminidase, 1 μg/ml phorbol PMA, or medium alone. The bovine PBL were then washed in assay buffer and resuspended along with FITC-labeled murine splenocytes at a 10 to 1 ratio (2 X 10^7/ml bovine, 2 X 10^6/ml murine). The bovine and murine lymphocytes were overlaid on murine mesenteric lymph node sections, and the SAR was determined as described above.

Murine lymph node sections were pre-incubated for 30 min at 37°C with either PBS alone or PBS containing 0.5 U/ml of neuraminidase as described (Rosen et al., 1985), washed in PBS, and then overlaid with bovine PBL (2
X \(10^7/ml\) in assay buffer. Murine lymphocytes were not used as an internal control in these experiments, since the treatment of lymph node sections would have affected the binding of both murine and bovine lymphocytes.

In experiments testing the effects of carbohydrates, bovine PBL in assay buffer (2 \(X\) \(10^7/ml\)) were incubated for 15 min on ice with 5 mM fructose-1-phosphate (F1P), 5 mM fructose-6-phosphate (F6P), 1 \(\mu\)g/ml fucoidin, or medium alone before being overlaid on mesenteric lymph node sections. The aforementioned carbohydrates were included with the bovine PBL during the incubation on murine lymph node sections. Murine lymphocytes were not added as an internal control since the carbohydrates would also have interfered with their binding to HEV. The neuraminidase, PMA, and carbohydrates used in these experiments were obtained from Sigma.

**Experimental Design and Data Analysis**

Experiments were performed on separate days for each treatment regimen or tissue-specific HEV assay. Each experiment consisted of an average of eight replicate sections. The binding of bovine PBL relative to the internal control (murine lymphocytes) was determined by calculating the SAR as described above.

When murine lymphocytes could not be used as an internal control, consecutively cut tissue sections containing HEV of similar size and number were used to assess lymphocyte binding. The total number of lymphocytes bound to HEV in all treated or control samples for each experiment was counted and compared to determine the percent binding in treated, relative to control, samples.

The standard error of the mean (SEM) represents the variation between
separate experiments done on different days. Treatments that the lymphocytes or lymph node sections had received were not known to the person determining the number of lymphocytes bound to HEV. Over 90% of the treated lymphocytes were viable, based on their ability to exclude trypan blue dye.
RESULTS

Bovine PBL bound preferentially to murine peripheral node HEV compared with murine Peyer's patch HEV. The average SAR for binding to peripheral nodes and Peyer's patches was 0.83 and 0.13, respectively (Table 1). Bovine PBL bound primarily to HEV of lymph nodes with a limited amount of binding to medullary sinuses (Figure 1a).

Hoechst 33342 did not affect binding of murine or bovine lymphocytes to HEV. In one experiment, the labels were reversed; i.e., bovine PBL were FITC-labeled and murine splenocytes were Hoechst-labeled (Figure 1b and Table 1, Experiment 3). There were no differences in the SAR from this experiment compared with the SAR in experiments with Hoechst-labeled bovine PBL and FITC-labeled murine splenocytes (Figure 1a and Table 1, Experiments 1 and 2). Hoechst 33342 appeared to be a better label than FITC, as the intensity was greater, the cells retained fluorescence for an extended period of time, and a 100-fold increase in the concentration of Hoechst 33342 did not affect bovine PBL binding in the HEV assay (data not shown).

The presence of FIP and fucoidin in the HEV assay reduced the binding of bovine PBL to murine HEV to about one-third of control levels (Table 2). Conversely, F6P did not affect the binding of bovine PBL as compared with controls (Table 2). Treatment of bovine PBL with 0.5 U/ml neuraminidase caused a 150% increase in HEV binding relative to controls.

Stimulation of bovine PBL with PMA decreased their binding to HEV (Table 3). The SAR of bovine PBL treated with PMA was reduced to 26% and 24% of the control SAR in experiments 1 and 2, respectively.
Figure 1a. Binding of bovine and murine lymphocytes to murine HEV. Hoechst-labeled bovine PBL (arrows) and FITC-labeled murine splenocytes (arrowheads) are binding to murine HEV. The murine HEV is darker than the surrounding lymphoid tissue.
Figure 1b. Binding of bovine and murine lymphocytes to murine HEV. FITC-labeled bovine PBL (arrows) and Hoechst-labeled murine splenocytes (arrowheads) are binding to murine HEV. Reversal of Hoechst and FITC labels had no effect on the specificity of lymphocyte binding in the HEV assay.
TABLE 1. Binding of bovine PBL to murine HEV

<table>
<thead>
<tr>
<th>Exp. #</th>
<th>Peripheral Lymph Node HEV&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Peyer's Patch HEV&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>121/16 (0.76)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>91/82 (0.11)</td>
</tr>
<tr>
<td>2</td>
<td>559/61 (0.92)</td>
<td>123/80 (0.15)</td>
</tr>
<tr>
<td>3</td>
<td>474/59 (0.80)</td>
<td>ND&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Average</td>
<td>0.83 ± 0.05&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.13 ± 0.02</td>
</tr>
</tbody>
</table>

<sup>a</sup>Bovine PBL and murine splenocytes (10 to 1 ratio) were used to overlay murine peripheral lymph node sections.

<sup>b</sup>Bovine PBL and murine Peyer's patch lymphocytes (10 to 1 ratio) were used to overlay murine Peyer's patch sections.

<sup>c</sup>Numbers of bovine PBL/murine lymphocytes bound to HEV.

<sup>d</sup>SAR (in parentheses), determined as previously described (Butcher et al., 1979).

<sup>e</sup>ND, not done.

<sup>f</sup>Average SAR + SEM.
TABLE 2. Effects of carbohydrates on bovine PBL binding to murine HEV

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Exp. #</th>
<th>F1P</th>
<th>F6P</th>
<th>Fucoidin</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>89/248% (36%)</td>
<td>320/317 (101%)</td>
<td>267/732 (36%)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>196/535 (37%)</td>
<td>522/535 (98%)</td>
<td>108/404 (32%)</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td>36.5% + 1%</td>
<td>99% + 2%</td>
<td>34% + 2%</td>
</tr>
</tbody>
</table>

*Bovine PBL were incubated with 5 mM fructose-1-phosphate (F1P), 5 mM fructose-6-phosphate (F6P), 1 ug/ml fucoidin, or medium alone, and then overlaid on murine mesenteric lymph node sections.

Numbers of treated bovine PBL/control bovine PBL bound to HEV.

The percent binding of treated PBL, relative to PBL treated with medium alone (in parentheses).

Average percent binding of treated PBL, relative to PBL treated with medium alone, + SEM.
TABLE 3. Effect of PMA treatment on bovine PBL binding to murine HEV

<table>
<thead>
<tr>
<th>Exp. #</th>
<th>Control</th>
<th>PMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>437/103(^b) (0.42)(^c)</td>
<td>86/79 (0.11)</td>
</tr>
<tr>
<td>2</td>
<td>400/81 (0.49)</td>
<td>84/67 (0.12)</td>
</tr>
<tr>
<td>Average</td>
<td>0.44 + 0.04(^d)</td>
<td>0.12 + 0.01</td>
</tr>
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</table>

\(^a\)Bovine PBL were incubated with PMA or medium alone (control), mixed 10 to 1 with murine splenocytes, and overlaid on murine mesenteric lymph node sections.

\(^b\)Numbers of bovine PBL/murine splenocytes bound to HEV.

\(^c\)SAR (in parentheses), determined as previously described (Butcher et al., 1979).

\(^d\)Average SAR + SEM.
DISCUSSION

Murine and human PLHR are similar; both contain a lectin domain and the binding of lymphocytes to HEV in both species is inhibited by specific carbohydrates (Bowen et al., 1989; Lasky et al., 1989; Siegelman et al., 1989; Siegelman and Weissman, 1989; Stoolman et al., 1987). In the present study, FIP and fucoidin inhibited bovine PBL binding to murine HEV. These same carbohydrates inhibit binding of murine peripheral lymphocytes to murine HEV (Stoolman et al., 1987). The carbohydrate F6P, which does not affect binding of murine lymphocytes to HEV (Stoolman et al., 1987), had no effect on bovine PBL binding in the present study. The similar effects of carbohydrates on lymphocyte binding suggest conservation of a lectin domain in PLHR of all three species.

Removal of sialic acid residues from bovine lymphocytes with neuraminidase increased binding to murine HEV. Previous studies examining the binding of human PBL to murine HEV also reported an increase in binding after neuraminidase treatment (Stoolman et al., 1987). This increased binding after sialic acid removal may be due to the decrease in negative charge on the lymphocyte which could enhance binding to HEV (Stoolman et al., 1987). Conversely, neuraminidase treatment of murine lymph node sections has been reported to decrease binding of murine lymphocytes to peripheral HEV (Rosen et al., 1985). We saw a similar reduction in binding of bovine PBL following neuraminidase treatment of lymph node sections (data not presented). It has been suggested that sialic acid residues on murine peripheral HEV interact with PLHR, and that neuraminidase treatment decreases lymphocyte binding by removing the sialic acid residues from the
HEV vascular addressins (Rosen et al., 1985; True et al., 1990). The reduction in the binding of both bovine PBL and murine lymphocytes to murine HEV after neuraminidase treatment of the HEV suggests that PLHR of both species recognize the same vascular addressins.

Murine and human PLHR are rapidly down-regulated by a variety of stimulatory agents, including PMA (Huang et al., 1989; Jung and Dailey, 1990; Kishimoto et al., 1990). In the present study, binding of bovine PBL to murine HEV was decreased by pretreatment of the PBL with PMA. This suggests that the bovine receptor may possibly be down-regulated by PMA.

In summary, the ability of bovine PBL to bind to murine HEV and the similarities between the effects of carbohydrates, neuraminidase, and PMA on binding of bovine, murine, and human lymphocytes in the HEV assay suggest that PLHR are conserved in all three species. Definitive identification of the bovine receptor will be facilitated by isolation and sequencing of a cDNA encoding the bovine equivalent of the murine and human PLHR.
REFERENCES


PART III. CHARACTERIZATION OF A PERIPHERAL LYMPHOCYTE HOMING RECEPTOR: BOVINE LECAM-1
CHARACTERIZATION OF A PERIPHERAL LYMPHOCYTE 
  HOMING RECEPTOR: BOVINE LECAM-1 

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The bovine cDNA clones encoding for bovine LECAM-1 (lectin cell adhesion molecule-1) were isolated and sequenced. The predicted amino acid sequence of bovine LECAM-1 showed high identity with that of human and murine LECAM-1. The antibody DREG-56, which recognizes human LECAM-1, blocked the binding of bovine peripheral blood lymphocytes to high-walled endothelial venules in murine peripheral lymph nodes. Surface expression of bovine LECAM-1 was high in lymphocytes isolated from peripheral lymph nodes and low in lymphocytes isolated from Peyer's patches. This evidence strongly suggests that bovine LECAM-1 is a peripheral lymphocyte homing receptor.
INTRODUCTION

Lymphocyte homing is the process by which lymphocytes recirculate throughout the body and return to lymphoid tissues (Cowans and Knight, 1964; Gallatin et al., 1986). In rodents and humans, the specificity of homing is controlled by adhesive interactions of homing receptors on lymphocytes with vascular addressins on high-walled endothelial venules (HEV) (Gallatin et al., 1983; Kishimoto et al., 1990; Streeter et al., 1988a, 1988b). Lymphocytes home to secondary lymphoid organs such as peripheral lymph nodes, or mucosal lymphoid tissues like the Peyer's patch (for review, see Stoolman, 1989; Butcher, 1990).

The murine and human peripheral lymphocyte homing receptors, recently termed LECAM-1 (lectin cell adhesion molecule-1), have been characterized and the cDNAs encoding for them sequenced (Siegelman et al., 1989; Bowen et al., 1989; Lasky et al., 1989; Siegelman and Weissman, 1989). The predicted amino acid sequences of human and murine LECAM-1 have a domain that is homologous to a number of calcium-dependent animal lectins (Bowen et al., 1989; Lasky et al., 1989; Siegelman et al., 1989; Siegelman and Weissman, 1989). As specific carbohydrates block the binding of human and murine lymphocytes to peripheral lymph node HEV (Stoolman and Rosen, 1983, Stoolman et al., 1984; Stoolman et al., 1987), the lectin domain of human and murine LECAM-1 is likely involved in the binding of lymphocytes to peripheral lymph node HEV. Also, the monoclonal antibody MEL-14, which blocks the binding of murine lymphocytes to peripheral lymph node HEV in vitro and lymphocyte homing to peripheral lymph nodes in vivo, recognizes the lectin domain of LECAM-1 (Bowen et al., 1990).
Human and murine LECAM-1 also contain a domain with homology to proteins in the epidermal growth factor (EGF) family. This domain may have a role in lymphocyte homing. A monoclonal antibody that reacts with the EGF-like domain of murine LECAM-1 reduced the binding of murine lymphocytes to peripheral lymph node HEV in vitro (Siegelman et al., 1990). The exact role the EGF-like domain plays in HEV binding has been speculated to be due a direct role in HEV binding. However, it is possible that the antibody that reacted with the EGF-like domain reduced HEV binding due to secondary changes or steric influences induced on the lectin domain of LECAM-1 (Siegelman et al., 1990). Murine and human LECAM-1 contain two repeated domains that show homology to a number of proteins, most of which have a role in complement-binding (Bowen et al., 1989; Lasky et al., 1989; Siegelman et al., 1989; Siegelman and Weissman, 1989). The role of the domains similar to complement-binding proteins in lymphocyte homing has not been established.

Previously, we have shown that the bovine peripheral lymphocyte homing receptor is an evolutionarily conserved molecule with lectin activity. Carbohydrates that block the binding of murine and human lymphocytes to peripheral lymph node HEV also block the binding of bovine lymphocytes to peripheral lymph node HEV and both neuraminidase and phorbol myristate acetate have similar effects in all three species (Bosworth and Harp, 1991). In the present study, we have isolated and sequenced bovine LECAM-1 cDNA and blocked the binding of bovine lymphocytes to peripheral lymph node HEV with an antibody to human LECAM-1. Taken together, these data strongly suggest that bovine LECAM-1 is a peripheral lymphocyte homing receptor.
MATERIALS AND METHODS

Cell preparation

Lymphocytes from bovine mammary and prescapular lymph nodes and Peyer's patches were isolated as previously described (Harp et al., 1988). Briefly, bovine lymphoid tissues were removed from an adult female bovine and placed immediately on ice. The tissue was gently minced over a wire screen and cells that passed through were collected in medium (RPMI with 5% fetal bovine serum; Gibco Laboratories, Grand Island, NY). Bovine peripheral blood lymphocytes (PBL) were isolated by centrifugation at 450 g for 40 min over Percoll (sp gr = 1.084) as previously described (Whitmire and Harp, 1991). Platelets were removed from the PBL by centrifugation at 250 g for 10 minutes through Percoll (sp gr = 1.029).

Antibodies and fluorescence-activated cell sorter (FACS) analysis

Bovine lymphocytes (1 x 10^6) in 100 μl medium were incubated with 50 μg/ml of DREG-56 (kindly provided by Dr. Mark Jutila, Montana State University, Bozeman, MT), an IgG1 monoclonal antibody that recognizes human LECAM-1 (Kishimoto et al., 1990; Jutila et al., 1990), or an isotype-matched control antibody. After a 15 min incubation at room temperature, lymphocytes were washed twice in phosphate-buffered saline (PBS). Next, lymphocytes were incubated with goat anti-mouse IgG-fluorescein isothiocyanate (FITC) conjugate (Sigma Chemical Co., St. Louis, MO) for 15 min at room temperature. After washing twice in PBS, cells were fixed with 1% paraformaldehyde. Fluorescence analysis was performed on 10,000 cells at Iowa State University, Ames, IA using a EPICS 752 flow cytometer (Coulter...
Electronics, Inc., Hialeah, FL). Lymphocytes were identified by their characteristic pattern of forward and right angle light scatter. Fluorescence data are presented as the percentage staining above control antibody background and as histograms.

**HEV assay**

Murine popliteal lymph nodes or Peyer's patches were used in an HEV binding assay similar to that described previously (Butcher et al., 1979; Stamper and Woodruff, 1976; Stoolman et al., 1987; Bosworth and Harp, 1991). Briefly, murine lymphoid tissue imbedded in O.C.T. (Miles Inc., Elkhart, IN) was frozen on dry ice and sectioned with a cryostat. Tissue sections were placed on glass slides. The O.C.T. was removed with forceps and sections were overlaid with $1 \times 10^6$ bovine lymphocytes suspended in 100 µl medium. Sections were rotated at 60 RPM for 30 min at 5°C. The sections were then fixed in 3% glutaraldehyde in PBS for 45 min, rinsed in PBS, and examined microscopically with darkfield/fluorescent illumination.

To determine the relative affinity of lymphocytes from prescapular and mammary lymph nodes for peripheral node HEV, FITC-labeled lymphocytes from prescapular lymph nodes and mammary lymph node lymphocytes labeled with Hoechst 33342, a fluorescent dye that binds DNA, were mixed at a 1:1 ratio in medium and then overlaid on peripheral lymph node sections (Bosworth and Harp, 1991). To determine the effect of DREG-56 on lymphocyte binding in the HEV assay, lymphocytes were incubated for 30 min on ice with 50 µg/ml of DREG-56 or an isotype-matched control antibody. These lymphocytes were then overlaid on either popliteal lymph node sections or Peyer's patch sections.
Isolation of bovine LECAM-1 cDNA

A bovine LECAM-1 cDNA clone isolated from a bovine lymph node cDNA lambda gt10 library was kindly provided by Don Dowbenko, Genentech Inc., San Francisco, CA. The 2.0 kb insert from this clone was subcloned into the cloning vector plasmid pBluescript (Stratagene, La Jolla, CA). Approximately 300,000 recombinant clones from a bovine lymphocyte cDNA phagemid library were screened with $^{32}$P-labeled cDNA that encodes for the lectin domain of bovine LECAM-1 (kindly provided by Bruce Walcheck, Montana State University, Bozeman, MT) as previously described (Sambrook et al., 1989). One positive clone was isolated which contained a 1.1 kb fragment.

As cDNA sequencing showed that neither of these clones contained the entire 5' region of bovine LECAM-1, we produced cDNA encoding for the 5' region using the rapid amplification of cDNA ends (RACE) procedure (Frohman et al., 1988). Briefly, 1 μg of poly(A)+RNA was reverse transcribed using a primer complementary to LECAM-1 mRNA (5'-CCACACATTGTCCATGGCCGCTG-3'). The excess primer was removed with a Centricon 100 spin filter (W. R. Grace and Co., Danvers, MA) and then the cDNA was poly(A) tailed using terminal deoxynucleotidyl-transferase (Bethesda Research Laboratories, Gaithersburg, MD). Five μl of the tailed cDNA, 25 pmol each of adaptor primers (5'-GACTCGAGTCGACAAGCTTTTTTTTTTTTTTTTTT-3' and 5'-CGGAATTCTGTGTCAGGCATCATCATTCC-3'), 1.5 mM deoxynucleotides, 10% dimethyl sulfoxide, 1 unit of the polymerase enhancer Perfect Match (Stratagene), and 1X Taq polymerase buffer were denatured at 95°C for 5 min and then cooled to 72°C. Then 2.5 units of Taq DNA polymerase (Perkin-Elmer-Cetus, Norwalk, CT) were added, and the temperature was reduced to 57°C for 1 min. The cDNA was extended for 40 min at 72°C and then the mixture was amplified in a DNA thermal cycler (Perkin-
Elmer-Cetus) using a step program (35 cycles; 94°C, 40 sec; 59°C, 2 min; 72°C, 3 min). The amplified cDNA was digested with Xho I and EcoR I and then subcloned into plasmid pBluescript. This RACE procedure was done on two separate occasions and 3 clones from one experiment and 2 clones from the other experiment were sequenced.

Double-stranded plasmid and single-stranded phage DNA were isolated as previously described (Sambrook et al., 1989) from the LECAM-1 cDNA clones. The sequence of both strands of bovine LECAM-1 cDNA was determined by the dideoxy chain termination method (Sanger et al., 1977) using T7 DNA polymerase (United States Biochemical, Cleveland, OH), primers specific for the cloning vectors, and synthesized oligonucleotide primers specific for bovine LECAM-1 cDNA.

**Northern Blot Analysis**

Mammary and prescapular lymph nodes and Peyer's patches were removed from an adult cow and immediately placed on dry ice. Total RNA was recovered by single extraction with an acid guanidinium thiocyanate-phenol-chloroform mixture (Chomczynski and Sacchi, 1987) and poly(A)^+RNA isolated using oligo dT chromatography (Aviv and Leder, 1972). Ten µg of poly(A)^+RNA from these tissues were subjected to electrophoresis in a denaturing agarose gel and transferred to a nylon membrane (Nytran, Schleicher and Schuell, Keene, NH). Prehybridization was carried out in 50% formamide, 5X Denhardt's, 5X SSPE, 0.5% SDS and 0.1 mg/ml salmon sperm DNA for 4 hr at 42°C. Hybridization was carried out in the same buffer using ^32P-random primer labeled (Pharmacia LKB, Piscataway, NJ) bovine LECAM-1 cDNA overnight at 42°C. The nylon membrane was washed and exposed to X-ray film at -70°C.
The membrane was stripped of LECAM-1 cDNA probe, then reprobed with $^{32}$P-ran-om primer labeled bovine actin cDNA (kindly provided by Dr. David Morris, University of Washington, Seattle, WA) and exposed to X-ray film in order to visually determine if comparable amounts of RNA were present in each lane.
RESULTS

DNA and predicted amino acid sequence of bovine LECAM-1

The DNA sequence and predicted amino acid sequence of bovine LECAM-1 are presented in Figure 1. The 2650 nucleotide cDNA contained one long open reading frame of 1110 bases starting at base 129. The 3' end of the cDNA terminated in a short poly(A) tail. The cDNA clone from the bovine lymphocyte cDNA phagemid library extended from base 272 to base 1327. The clone from the bovine lymph node lambda gt10 library extended from base 551 to base 2650. The sequence for bases 1 to 272 was determined from the five RACE produced clones. The multiple rounds of amplification in the RACE procedure resulted in an error rate of less than 0.1%.

The first 38 amino acids of the open reading frame (Figure 1) resembled a leader peptide (von Heijne, 1986). This was unusually long for a eukaryotic protein but the same length as the leader peptides of human and mouse LECAM-1 (Lasky et al., 1989; Siegelman and Weissman, 1989; Siegelman et al., 1989). Cleavage of the leader peptide would result in a mature protein with a predicted molecular mass of 37.4 kD. Comparison of the predicted amino acid sequence of bovine LECAM-1 to human and murine LECAM-1, revealed that bovine LECAM-1, like human and murine LECAM-1, had a lectin domain, an epidermal growth factor-like domain, and two repeated domains similar to complement-binding proteins (Figure 2). Bovine LECAM-1 had an overall amino acid identity of 76% and 69% with human and mouse, respectively.

Bovine LECAM-1 had nine putative N-linked glycosylation sites in the extracellular domain (amino acids 1-355, Figure 1), eight of which were
present in human and/or murine LECAM-1. The transmembrane region (amino acids 334-355) had a high level of inter-species identity while the cytoplasmic tail of bovine LECAM-1 (amino acids 356-370) was truncated and dissimilar when compared to human and murine LECAM-1 (Figure 2).

**Tissue distribution of bovine LECAM-1**

Lymphocytes from mammary lymph nodes, prescapular lymph nodes and Peyer's patches were analyzed for surface expression of LECAM-1 with flow cytometry (Figure 3). The percentages of DREG-56 positive cells in mammary lymph nodes, prescapular lymph nodes and Peyer's patches were 35%, 45%, and 5%, respectively. Little LECAM-1 mRNA was present in Peyer's patches and high levels were seen in mammary and prescapular lymph nodes (Figure 4). The size of bovine LECAM-1 mRNA was approximately 3 kb.

**In vitro binding to HEV**

Lymphocytes from mammary and prescapular lymph nodes bound to HEV in murine peripheral lymph node sections. Lymphocytes from the mammary lymph node had slightly reduced binding to peripheral lymph node HEV compared with lymphocytes from the prescapular lymph node. In two separate experiments, the numbers of lymphocytes from mammary lymph nodes that bound to HEV was 81% and 84% of the numbers of lymphocytes from prescapular lymph nodes that bound to HEV. This slightly reduced lymphocyte binding was likely due to the lower percentage of LECAM-1⁺ lymphocytes in mammary lymph node relative to prescapular lymph node (Figure 3).

The antibody DREG-56 reduced the binding of PBL to peripheral node HEV to about 25% of the binding seen with PBL that were treated with control
antibody (Figure 5). The antibody DREG-56 affected only peripheral homing lymphocytes and did not affect the binding of bovine Peyer's patch lymphocytes to murine Peyer's patch HEV (Figure 5).
Figure 1. Sequence of bovine LECAM-1 cDNA. Nucleotides and predicted amino acids are shown. Potential N-linked glycosylation sites are in bold and capitalized; the putative leader peptide and transmembrane region are underlined.
Figure 2. Comparison of predicted amino acid sequences of bovine, human and murine LECAM-1. The amino acid sequence (one letter designation) for bovine LECAM-1 is shown on the top line. The lectin domain is underlined, the epidermal growth factor-like domain is double underlined and the two domains similar to complement-binding proteins are in bold. Amino acid differences found in human (Seigelman and Weissman, 1989) and murine LECAM-1 (Seigelman et al., 1989) are shown. Identical amino acids are designated with a dash (-).
Figure 3. Expression of LECAM-1 on bovine lymphocytes. Fluorescence of bovine lymphocytes incubated with control antibody (left side) or DREG-56 (right side) is shown. The lymphocyte populations examined are shown under the histograms. The cell count is on the vertical axis and the log fluorescence intensity (LFL1) is on the horizontal axis.
Figure 4. Northern blot of LECAM-1 RNA in bovine lymphoid organs. Ten μg of poly(A)^+RNA from mammary lymph node (A), prescapular lymph node (B), and Peyer's patch (C) were electrophoresed, transferred to a nylon membrane, and hybridized with ^32P-labeled bovine LECAM-1 cDNA. Actin mRNA levels were determined to ensure comparable amounts of RNA were present in each lane. The numbers on the left correspond to the position of the ribosomal subunit bands.
Figure 5. The effect of DREG-56 on bovine lymphocyte binding to HEV. The percent binding of DREG-56 treated PBL to peripheral node HEV (hatched bar) and the percent binding of DREG-56 treated Peyer's patch lymphocytes to Peyer's patch HEV (open bar), relative to lymphocytes treated with control antibody are shown.
DISCUSSION

We have been studying lymphocyte homing and lymphocyte homing receptors in the bovine (Harp et al., 1988; Bosworth et al., 1991, Bosworth and Harp, 1991). While the molecular mechanisms controlling lymphocyte homing are similar in many species (Wu et al., 1988), cattle differ in some aspects of lymphocyte homing. In rodents, and probably other species, the mammary lymph node is part of a common mucosal immune system, which includes the intestine and respiratory tract (Roux et al., 1977; McDermott and Bienenstock, 1979). Rodent lymphocytes sensitized to intestinal antigens in the Peyer's patches are able to traffic or home to the mammary gland. This allows the mother's immune responses to pathogens in her intestine to be transferred, via the homing of lymphocytes sensitized to antigens in the intestine to the mammary gland, to colostrum and milk. Thus, the milk and colostrum are able to protect the newborn offspring from enteric disease.

In ruminant species, including cattle, this process is less efficient. Bovine mammary lymphocytes tend to home like peripheral lymph node lymphocytes (Harp et al., 1988). This suggests that the mammary immune system is not part of the common mucosal immune system in ruminants (Sheldrake and Husband, 1985; Harp et al., 1988; Harp and Moon, 1987). Also, the lymph node venules that are the site of lymphocyte extravasation in rodents and humans are lined with cuboidal high-walled endothelium (Gowans and Knight, 1964; Jalkanen and Butcher, 1985; Butcher, 1990) but in cattle these vessels are lined with relatively flat, low-walled endothelium (Harp et al., 1990). In rodents and humans, these endothelial cells are esterase positive (Freemont and Jones, 1983; Anderson et al., 1976), in
cattle they are esterase negative (Bosworth, personal observations).

Despite these differences, the control of lymphocyte homing in ruminants may involve conserved molecules (Bosworth et al., 1991; Bosworth and Harp, 1991). We have previously shown that the bovine peripheral lymphocyte homing receptor has lectin-like activity and is functionally similar to human and mouse (Bosworth and Harp, 1991). In the present study, we identified the bovine equivalent of LECAM-1. The predicted amino acid sequence of the molecule was 76% and 69% identical with human and murine LECAM-1, respectively. The predicted amino acid structure of bovine LECAM-1, like human and murine LECAM-1, included a lectin domain, an epidermal growth factor-like domain, and two repeated domains similar to complement-binding proteins. The cytoplasmic tail of bovine LECAM-1 showed little similarity with that of human and murine LECAM-1, suggesting that the cytoplasmic tail has little involvement in evolutionarily conserved functions. These differences in the cytoplasmic tails of LECAM-1 may have arisen from different exon usage as the beginning of this area correlates with the beginning of the region encoded by exon IX of the human LECAM-1 gene (Ord et al., 1990).

Messenger RNA which hybridized with the bovine LECAM-1 cDNA was present in higher amounts in peripheral lymph nodes than in Peyer's patches (Figure 4). The monoclonal antibody DREG-56, which recognizes human LECAM-1 (Kishimoto et al., 1990), reacted with a molecule present on bovine lymphocytes in peripheral, but not mucosal lymphoid tissue. The differences in tissue distribution on bovine LECAM-1 surface protein levels were likely due to transcriptional regulation. This antibody also blocked the in vitro binding of bovine lymphocytes to murine peripheral lymph node HEV. Taken
together, the evidence from this and our previous study indicates that bovine LECAM-1 is a peripheral lymphocyte homing receptor and is structurally and functionally similar to human and murine LECAM-1.

Differences were seen in the tissue distribution of bovine LECAM-1. Few lymphocytes from bovine Peyer's patches expressed LECAM-1, whereas this molecule is present on most murine lymphocytes that home to either peripheral lymph nodes or Peyer's patches in vivo (Gallatin et al., 1983). Possibly, the LECAM-1⁺ lymphocytes that home to murine Peyer's patches in vivo also express the mucosal homing receptors necessary for homing to murine Peyer's patches. An additional possibility is that the LECAM-1 present on these murine Peyer's patch homing lymphocytes is modified and therefore unable to mediate homing to peripheral lymph nodes. Thus in the mouse, LECAM-1 is specific in function, but not distribution, to peripheral homing cells (Gallatin et al., 1983), whereas in the bovine both function and distribution are specific to peripheral homing cells. In this respect, the finding of bovine LECAM-1 on mammary lymph node lymphocytes was not surprising. In fact, it provides an explanation at the molecular level for our previous observations that bovine mammary lymphocytes exhibit homing patterns similar to peripheral rather than mucosal lymphocytes (Harp et al., 1988).

In summary, although some of the patterns of lymphocyte homing in the bovine differ from those seen in human and mouse, at least one of the molecules controlling peripheral homing of lymphocytes appears to be conserved in both structure and function between the three species.
REFERENCES


GENERAL SUMMARY AND DISCUSSION

In this dissertation, data are presented on lymphocyte molecules that control the preferential recirculation or homing of bovine lymphocytes to either peripheral lymph nodes or mucosal lymphoid tissue. The cDNA sequences and predicted amino acid sequences of bovine CD44 and LECAM-1 (lectin cell adhesion molecule-1) are similar to those of human and murine CD44 and LECAM-1. Bovine LECAM-1, like human and murine LECAM-1, controls the homing of lymphocytes to peripheral lymph nodes, but not to mucosal lymphoid tissue. Bovine LECAM-1 is expressed at high levels on lymphocytes from peripheral lymph nodes (mammary and prescapular lymph nodes), but it is expressed at low levels on lymphocytes from mucosal lymphoid tissue (Peyer's patch). The expression of high levels of bovine LECAM-1 on lymphocytes from both mammary and prescapular lymph nodes provides a molecular basis for previous observations that bovine mammary lymph node lymphocytes are peripheral homing, not mucosal homing, lymphocytes. The differential expression of bovine LECAM-1 on peripheral homing lymphocytes (those from peripheral lymph nodes) and mucosal homing lymphocytes (those from mucosal lymphoid tissue) may be useful in determining the best method of inducing an immune response in a particular tissue. The prevalence of LECAM-1^+ lymphocytes in a particular tissue would determine if mucosal or peripheral immunization would stimulate lymphocytes that would eventually home back to that tissue. It has not been determined if bovine CD44 has a role in lymphocyte homing (as does human CD44). The identification of some of the molecules involved in bovine lymphocyte homing has increased understanding of lymphocyte homing and provides the background for methods
that could control the immune response in various tissues by selectively regulating peripheral homing or mucosal homing lymphocytes.

The in vitro HEV assay has been used to characterize the molecules controlling lymphocyte homing in a variety of species, including cattle (Gallatin et al., 1983; Kishimoto et al., 1990; Streeter et al., 1988a, 1988b; Bosworth and Harp, 1991; Bosworth and Harp, Part III of this dissertation). This in vitro HEV assay reflects the in vivo homing patterns of bovine lymphocytes. Bovine mammary and prescapular lymph node lymphocytes bind in relatively equal numbers to murine peripheral lymph node HEV (Bosworth and Harp, Part III of this dissertation) and bovine mammary lymph node lymphocytes tend to home like peripheral lymph node lymphocytes in vivo (Harp et al., 1988). Experiments are currently being planned to determine if the antibody DREG-56, which recognizes the peripheral lymphocyte homing receptor and reduces the binding of bovine lymphocytes to murine peripheral lymph node HEV in vitro (Bosworth and Harp, Part III of this dissertation), reduces the in vivo homing of bovine lymphocytes to peripheral lymph nodes in cattle. The in vitro HEV assay (Bosworth and Harp, 1991; Bosworth and Harp, Part III of this dissertation) should prove a valuable tool in studying molecules involved in bovine lymphocyte homing and should be useful in predicting the in vivo homing patterns of bovine lymphocytes from various lymphoid organs.

While some aspects of bovine lymphocyte homing differ from human and mouse, it appears that the peripheral lymphocyte homing receptor, LECAM-1, is similar in structure and function in all three species (Bosworth and Harp, 1991; Bosworth and Harp, Part III of this dissertation). Carbohydrates that block the binding of human and murine lymphocytes to
peripheral lymph node HEV also block the binding of bovine lymphocytes to peripheral lymph node HEV in the in vitro HEV assay (Bosworth and Harp, 1991). This suggests that similar molecules with lectin activity control the homing of peripheral lymphocytes in the bovine, human and mouse. The presence of a lectin domain has been verified in all three species as the predicted amino acid sequences of bovine, human, and murine LECAM-1 contain a domain with high homology to number of lectin proteins (Bowen et al., 1989; Lasky et al., 1989; Siegelman et al., 1989; Siegelman and Weissman, 1989; Bosworth and Harp, Part III of this dissertation).

Lymphocytes from bovine peripheral lymph nodes (mammary and prescapular lymph nodes) express higher levels of LECAM-1 than do lymphocytes from bovine Peyer's patches (Bosworth and Harp, Part III of this dissertation). The presence of similar levels of LECAM-1 on bovine lymphocytes from both mammary and prescapular lymph nodes provides a molecular basis for previous observations that bovine mammary lymphocytes exhibit homing patterns similar to peripheral rather than mucosal lymphocytes (Harp et al., 1988). The level of LECAM-1 on lymphocytes from the upper respiratory tract, lower respiratory tract, reproductive tract and other organs may be useful in determining the homing pattern of bovine lymphocytes. This would be extremely useful in determining if a population of lymphocytes are part of the common mucosal immune system or part of the peripheral immune system. This information would be useful in determining what route of immunization (mucosal or peripheral) would likely stimulate an effective immune response in these various organs in cattle. Additionally, presence or absence of LECAM-1 could be compared to surface antibody isotype present on B cells to determine what role lymphocyte homing has in the isotype of antibodies.
present in various organs in cattle.

The predicted amino acid sequence of bovine CD44 has high identity with that of human and mouse CD44 (Bosworth et al., 1991). Human CD44 has been reported to have a role in lymphocyte homing as Hermes-3, a monoclonal antibody that reacts with CD44, blocks the binding of human lymphocytes to Peyer's patch HEV and polyclonal antiserum to CD44 blocks the binding of human lymphocytes to both peripheral lymph node and Peyer's patch HEV (for review, see Butcher, 1990). Hermes-3 has no effect on the binding of bovine Peyer's patch lymphocytes to murine Peyer's patch HEV (Bosworth, personal observations). The role of CD44 in bovine and murine lymphocyte homing is not known at this time. Studies using polyclonal antiserum to bovine and murine CD44 may be helpful in determining what role, if any, CD44 plays in bovine and murine lymphocyte homing. As human CD44 has been reported to be involved in a wide variety of cellular adhesion events (Haynes et al., 1989), the role of bovine CD44 in other adhesion events should also be investigated.

The bovine mucosal lymphocyte homing receptor has not yet been identified. Possibly, bovine homologues to the murine mucosal homing receptors, LPAM-1 and LPAM-2, control the homing of bovine lymphocytes to mucosal lymphoid organs. The in vitro HEV assay will be quite helpful in the screening of antibodies to bovine LPAM-1, LPAM-2 or other lymphocyte molecules to determine if these molecules are mucosal lymphocyte homing receptors in the bovine.

The recent advances in identifying and characterizing some of the molecules controlling lymphocyte homing in the bovine (Bosworth et al., 1991; Bosworth and Harp, 1991; Bosworth and Harp, Part III of this
dissertation) have significantly increased understanding of bovine lymphocyte homing. Previously, the majority of data available on bovine lymphocyte homing concerned the in vivo homing patterns of lymphocytes derived from various locations in the bovine. The identification, characterization and sequencing of a bovine peripheral lymphocyte homing receptor, bovine LECAM-1 (Bosworth and Harp, 1991; Bosworth and Harp, Part III of this dissertation), are significant as these accomplishments provide the first insights into molecular control of bovine lymphocyte homing and form a solid basis for future studies that will attempt to further understand bovine lymphocyte homing. Future research should focus on further characterization of the homing receptors and vascular addressins, and the in vivo role of these molecules in controlling lymphocyte homing and tissue-specific immunity. These studies should further increase understanding of lymphocyte homing and lay the groundwork for methods that enhance tissue-specific immunity in the bovine and other animals.
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