Biochemical and functional analysis of a novel lineage-specific gd T cell surface antigen (GD3.5Ag)
by Ward McAlister Jones

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Molecular Biology MONTANA STATE UNIVERSITY Bozeman, Montana
Montana State University
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Abstract:
Gamma-delta (γδ) T cell lymphocytes were discovered over a decade ago. Since that time, a great deal of research has been done to gain an understanding of them. Here, I define a novel γδ T cell lineage-specific antigen (GD3.5Ag) at the biochemical, regulatory, and functional level. GD3.5Ag is expressed on 90% of the bovine peripheral blood γδ T cell population. GD3.5Ag is approximately 230kD and considerable evidence suggests that it does not represent one of the known WC1 isotypes. GD3.5Ag and WC1 exhibit disparate sensitivity to V-8 protease digestion as well as to PNGase-F, O-sialoglycoprotein endopeptidase, and neuraminidase treatment. GD3.5Ag expression on the cell surface is extremely sensitive to low-dose chymotrypsin treatment, while WC1 is not. I show that large numbers of γδ T cells that express lower levels of GD3.5Ag are found in the spleen. Treatment of γδ T cells with PHA or ConA results in the downregulation of GD3.5Ag expression, mimicking the findings in the spleen. Functional analyses revealed that chymotrypsin removal of GD3.5Ag from the cell surface in a dose-dependent manner correlates well with the loss of ability to roll on 24hr activated endothelium. Also, polyclonal antibodies against GD3.5Ag partially inhibit γδ T cell interactions with 24hr activated endothelium. Although GD3.5Ag is not an E-selectin ligand itself, I confirmed that γδ T cells express a 250kD E-selectin ligand and possibly 200kD and 300kD E-selectin ligands. Additionally, I define three potentially relevant E-selectin ligands of 120kD, ~220kD, and 260kD on human lymphocytes.

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APPROVAL

of a thesis submitted by

Ward McAlister Jones

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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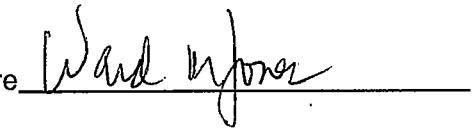
Signature 

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PREPARATION OF THE DISSERTATION

To assist the reader, I decided to include this section to describe how the dissertation was compiled and formatted. In the Introduction (Chapter 1), a general overview is given to provide sufficient background information for the understanding of subsequent chapters. The chapters that follow the Introduction also include an introductory section. The purpose for using this format is to allow those chapters that have been published or submitted for publication to be integrated into the dissertation in a logical manner. Chapters 2 and 4 represent data that has been published or submitted for publication. The manuscripts included in Chapters 2 and 4 , respectively, are as follows:

Jones, W. M., B. Walcheck, and M. A. Jutila. 1996. Generation of a new γδ T cell-specific monoclonal antibody (GD3.5): biochemical comparisons of GD3.5 antigen with the previously described workshop cluster 1 (WC1) family. *J. Immunol. 156:3772.*

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During the process of obtaining my degree, several collaborative projects within the lab were established. We performed experiments involving several individuals, including the study of E-selectin ligand and GD3.5Ag expression on bovine lymphocytes. Figures 9 and 20 are the result of these collaborative studies. Therefore, I would like to specifically acknowledge Gayle Watts and Karen Sipes for their assistance in generating Figures 9 and 20, respectively. In addition, I would like to acknowledge Martyn Robinson and Dietmar Vestweber for providing critical reagents (P11.4 chimera and ESL-1 polyclonal antibody). Finally, I would like to acknowledge Bruce Walcheck who generated the GD3.5 mAb.

I dedicate this thesis to my wife, Betsy Jones, and children, Krystal Fix and Erica Fix, for their unending patience in my endeavors to obtain this degree.

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ABSTRACT

Gamma-delta (γδ) T cell lymphocytes were discovered over a decade ago. Since that time, a great deal of research has been done to gain an understanding of them. Here, I define a novel γδ T cell lineage-specific antigen (GD3.5Ag) at the biochemical, regulatory, and functional level. GD3.5Ag is expressed on 90% of the bovine peripheral blood γδ T cell population. GD3.5Ag is approximately 230kD and considerable evidence suggests that it does not represent one of the known WC1 isotypes. GD3.5Ag and WC1 exhibit disparate sensitivity to V-8 protease digestion as well as to PNGase-F, O-sialoglycoprotein endopeptidase, and neuraminidase treatment. GD3.5Ag expression on the cell surface is extremely sensitive to low-dose chymotrypsin treatment, while WC1 is not. I show that large numbers of γδ T cells that express lower levels of GD3.5Ag are found in the spleen. Treatment of γδ T cells with PHA or ConA results in the downregulation of GD3.5Ag expression, mimicking the findings in the spleen. Functional analyses revealed that chymotrypsin removal of GD3.5Ag from the cell surface in a dose-dependent manner correlates well with the loss of ability to roll on 24hr activated endothelium. Also, polyclonal antibodies against GD3.5Ag partially inhibit γδ T cell interactions with 24hr activated endothelium. Although GD3.5Ag is not an E-selectin ligand itself, I confirmed that γδ T cells express a 250kD E-selectin ligand and possibly 200kD and 300kD E-selectin ligands. Additionally, I define three potentially relevant E-selectin ligands of 120kD, ~220kD, and 260kD on human lymphocytes.

CHAPTER 1

INTRODUCTION

Historical Background

A large portion of T cells found in circulation express the αβ T cell receptor (TCR), which is associated with the "invariant components" of the CD3 signalling complex (1). Importantly, the TCR recognizes, in conjunction with coreceptors CD8 or CD4, processed antigen that is presented in the context of MHC Class-I or MHC Class-II molecules, respectively (2). In 1984, cDNAs encoding the αβ TCR were identified. Initially, subtractive hybridization and differential screening revealed a cDNA clone that encoded the β chain molecule (3,4). Soon after this discovery, a second cDNA clone was identified and thought to encode the α chain (5). Paradoxically, this particular clone did not encode any potential N-linked glycosylation sites which were known to exist in the α chain (1). Subsequent studies uncovered the "true" α chain cDNA; the protein encoded by the original "α" chain cDNA was termed the "γ" chain (6-8).

Although the α and β chain cDNAs had been identified, the function of the third "γ" chain remained unknown. Several investigators suggested that the γ chain was expressed in less mature thymocytes and represented an αβ T cell progenitor (9,10). Others suggested that cloned αβ T cells possessed "nonproductive γ rearrangements" and, therefore, γ genes played no role in αβ T cell function (8). In 1986, several groups described TCR heterodimers associated with the CD3 complex that contained the γ chain (11-13), and some suggested that the γ chain was associated with an

unidentified fourth chain (11,13). Finally, in 1987, the fourth unidentified gene, termed the "δ" chain, was discovered in studies looking at C, D, and J region gene rearrangements in $CD4^-$ and $CD8^-$ thymocytes (14,15). In addition, the human δ chain cDNA was discovered using subtractive hybridization (16). This new heterodimeric TCR, comprised of the γ and δ chain molecules, was found on a subset of $CD4^-$ and $CD8^-$ T cells that were collectively termed γδ T cells.

Determining the biological function of this novel T cell subset continues to generate a great deal of attention. Although the function of γδ T cells has not been completely defined, several investigators suggest that these cells are involved in the initial host response to infectious agents, contributing to what is referred to as "the first line of defense," which will be discussed later.

T cell receptor γ and δ chain: Lineage-specific γδ T cell marker

As described earlier, γδ T cells received their namesake from the γ and δ TCR gene products on their cell surface. Therefore, γδ T cells can be identified by mAbs directed against the TCR. As found for αβ T cells, the TCR on γδ T cells is associated with an array of proteins referred to as the CD3 complex. The complex is similar to CD3 on αβ T cells and consists of γ, δ, ε, and ζ CD3 proteins on most cells (17) though some reports indicate that epithelial γδ T cells do not express ζ chain proteins (18-21). αβ T cell TCR function involves the recognition of peptide in the context of Class I or Class II MHC. The $CD4^+$ αβ T helper cells recognize antigen expressed with MHC Class II, while $CD8^+$ T cytolytic cells recognize antigen in conjunction with Class I MHC (22). Both CD4 and CD8 are referred to as coreceptors assisting in the TCR recognition of MHC complexes (22). γδ T cells appear to have a less restricted antigen recognition event. Interestingly, most γδ T cells lack CD4 or CD8 and apparently are not restricted by MHC Class-I or Class-II recognition. Although some γδ T cells have been isolated that react

with classical MHC I and II, many appear to react with other nonclassical MHC gene products (23-26).

Recently, some reports indicate that γδ T cell antigen recognition may be independent of antigen processing, suggesting a direct recognition event (27,28). It has been suggested that γδ TCRs are more closely related to immunoglobulins than to αβ TCRs based upon CDR3 length analysis (27,29). γδ T cells demonstrate greater diversity in CDR3 length than do αβ T cells. It is thought that αβ T cells demonstrate limited variability in CDR3 length because of MHC-restricted interactions with antigen presenting cells (APCs). Therefore, CDR3 length variability may explain the apparent lack of MHC restriction demonstrated by some γδ T cells. The authors suggest that this may mediate γδ T cell cytotoxicity in the absence of APCs with a direct response to antigen (18,27).

Several reports suggest that γδ T cells recognize unconventional antigens, such as heat shock proteins (HSP) and nonpeptide antigens (18,23,28,30-36). Very small (<1kD) protease-resistant mycobacterial antigens are capable of activating γδ T cell subsets (32-34). In addition, human γδ T cell lines have been shown to respond to PPD and recombinant HSP (36). More recently, γδ T cell response to heat-killed mycobacteria has been attributed to small, nonpeptide, phosphate-containing antigens (28,35). Specifically, a 1-3kD phosphate-containing carbohydrate has been identified as the stimulatory component in mycobacterium (28,37). Therefore, γδ T cells have the capacity to respond to rather unusual or unconventional antigens.

Workshop Cluster 1 (WC1): Lineage-specific γδ T cell marker

In 1986, MacKay et al. described a unique, lineage-specific, γδ T cell marker in sheep (38). This cell surface marker (T19) is found on as many as 60% of ovine peripheral blood lymphocytes (PBLs). In comparison, only 1-3% of lymph node (LN)

lymphocytes and thymocytes express T19 (38). Later reports show that ovine $T19^+$ cells comprising 30-60% of PBLs are positive for the γδ TCR and negative for CD4 and CD8 (39).

T19 can be distinguished from TCRs by SDS-PAGE analysis. The T19 antigen migrates under reducing and nonreducing conditions at ~215kD and ~300kD, while γδ TCRs demonstrate a migratory pattern of approximately 40kD under reducing conditions and 70-75kD under nonreducing conditions (39). The γδ TCR and T19 appear at different times during fetal and neonate development. Cells expressing γδ TCR are detected at 40 days gestation while $T19^+$ cells are not detected until 15 days later (39). The percentage of γδ TCR^+ cells in all tissues is higher than the percentage of $T19^+$ cells, indicating that some γδ TCR-bearing T cells are $T19^-$ (39).

Soon after the discovery of T19 in sheep, several groups identified a similar antigen on bovine γδ T cells (40-43). Many mAbs were generated against this antigen and are now clustered into a group collectively called Workshop Cluster 1 (WC1) (44). As in the sheep, bovine WC1 is characterized by ~210kD and ~300kD bands (43). Additionally, as described for T19 in sheep, the percentage of $WC1^+$ cells in tissues is lower than the percentage of γδ TCR^+ cells (43,45).

By 1992, it became apparent that the multitude of mAbs that recognized WC1 were widely variable relative to the percentage of the population stained in a single individual. This prompted studies looking for the DNA that codes for WC1. A full length cDNA clone of WC1 was reported in bovine by Wijngaard et al. (46). Sequence analysis revealed a predicted protein containing a N-terminal leader peptide with a transmembrane region located in proximity to the C-terminus (46). In addition, 17 potential N-linked glycosylation sites are located in the extracellular domain. The extracellular domain contains 11 homologous regions that demonstrate strong identity to the scavenger receptor cysteine-rich (SRCR) family (46). In this same report,

Southern blots indicate that bovine genomic DNA contains several similar sequences, suggesting the potential for a WC1 gene family. Goats, pigs, sheep, and horse genomes appear to present similar complexity, while man and rodents appear to have fewer related sequences (46).

A plethora of mAbs to WC1 are clustered into two groups according to their reactivity to two different WC1 cDNA clones transfected into mouse L-cells (47). Several mAbs react with both WC1.1 and WC1.2 L-cell transfectants, while other antibodies react with either transfectant, but not both. Simultaneous reports indicated that WC1.1 and WC1.2 can be differentiated into two groups by apparent molecular weights of 205 and 215kD, respectively (48). Subsequently, a third cDNA was isolated (WC1.3) which shares significant nucleic acid identity with the first two cDNAs (49). Upon comparison, all three cDNAs share approximately 90% nucleic acid identity. Interestingly, a portion of the first SRCR domain, the most N-terminal, demonstrates only 50% identity among the three cDNA clones (49). Thus, this portion of the molecule could provide a unique N-terminal domain for each isotype, thereby directing its function and suggesting one reason that multiple WC1 species exist. Most importantly, mAbs IL-A29 and CC15 stain all three cDNA transfectants, providing pan-markers for the three known WC1 isotypes (49).

Although molecular biological techniques and mAbs have somewhat elucidated WC1 structure and tissue distribution, little is known about its function. MacKay et al. suggest that WC1 may play a role in tissue-specific homing, since WC1 is lost once some $\gamma\delta$ T cell populations enter certain tissues (39). Taking it one step further, Wijngaard et al. suggest that expressing different WC1 genes may provide a mechanism for tissue-specific homing (49). However, direct evidence supporting a role for WC1 in homing has not been shown.

Some authors speculate that WC1 may play a role similar to CD4 and CD8 on αβ T cells (49,50), whereas others suggest that WC1 does not "substitute" for these antigens (51). Binding of WC1 with mAbs augments proliferation of γδ T cells under certain circumstances (51). More specifically, cell-bound IL-A29 specifically enhances γδ T cell proliferation in autologous mixed leukocyte reactions. Additionally, immobilized IL-A29 enhances γδ T cell proliferation when cells are treated with anti-CD3 mAb, suggesting that WC1 engagement may augment TCR signalling events (51). Another report indicates that WC1 signals cell-cycle arrest, but not apoptosis (52). In this report, anti-WC1 antibodies induce cell-cycle arrest in G0/G1 in a dose-dependent manner that is reversible upon removal of the antibody (52). The antibody-induced cell arrest could be overcome by treating with anti-CD3 mAb. Therefore, engagement of the TCR and WC1 may provide an enhanced stimulatory event while immobilized WC1 alone signals cell-cycle arrest.

Other lineage-specific antigens

In our lab, we (my colleagues and I) have endeavored to generate mAbs against bovine γδ T cell surface antigens by injecting mice with purified γδ T cells. During this process, as expected, many mAbs against γδ TCR and WC1 were generated. However, one of the mAbs generated in a fusion piqued our interest. Preliminary results suggested that this mAb (GD3.5) stained a unique population of γδ T cells and recognized a unique antigen by Western Blotting. This antigen (GD3.5Ag) is described in Chapters 2 and 3.

γδ T cell distribution and function

Even though many laboratories have performed elegant studies of the function of γδ T cells, a clear consensus of their importance has not been established. We and others

hypothesize that their tissue distribution may lend some clues to their function. In ruminants, γδ T cells comprise 60-80% of the peripheral blood lymphocyte (PBL) population (45,50), but do not appreciably accumulate in the organized lymphatic tissues, such as the lymph node (39,43,53). Studies have demonstrated considerable homing of bovine γδ T cells into epithelial tissues, such as the skin and gut (50,53,54). In addition to epithelial tissues, bovine γδ T cells home to sites of inflammation. For example, intradermal injection of PPD results in accumulation of γδ T cells at the inflammatory site (55).

Many studies have been conducted in mice describing γδ T cells in association with extralymphoid sites, including the mucosal epithelial of the gut, tongue, vaginal areas and the skin (56-58). Interestingly, tissue localization of these cells seems to correlate with TCR gene usage in some cases (31,58,59). For example, murine γδ T cells expressing the Vγ5 Vδ1 TCR migrate to the skin (30,58,60). Therefore, localization may be either linked to or correlate with TCR gene usage (58,59). Several authors have hypothesized that specific homing receptors are expressed by the different T cell subsets directing their tissue-specific homing (30,58).

Human γδ T cell tissue localization is different in comparison to the bovine and mouse. Human γδ T cells are found in lymphoid as well as nonlymphoid tissues and are not as tightly associated with epithelial tissues as seen in the mouse and bovine models (35,53). Human TCR gene usage has been associated with circulating γδ T cell populations (35,61,62). Vγ9 Vδ2 γδ T cells represent a large subset of human peripheral blood γδ T cells (35,62). Importantly, this γδ T cell subset binds small molecular mass, non-protein antigens (32,35). Hence, it has been suggested that these cells respond quickly to antigen, performing as sentinels (35).

Since γδ T cells locate in areas where invading pathogens would first be encountered (portals of entry into the body) in what appears to be "directed" localization of γδ T

cells to epithelial tissues, many hypothesize that γδ T cells play a major role in "the first line of defense" and, in fact, recent reports support this hypothesis. In studies using γδ and αβ TCR-knockout mice, the role of the γδ T cells in malarial infection appears to be during initial protection (63). γδ TCR-deficient mice exhibit early, acute malaria, but eventually clear the infection. In contrast, αβ-deficient mice exhibit chronic, almost subclinical malaria (63). Other reports indicate that γδ T cells may play a role in eradication of intracellular bacterial pathogens, such as *Listeria* and *Mycobacteria* species (28,64,65). γδ T cells are crucial in reducing bacterial growth during early infection (28). Additionally, αβ T cells appear to play a role in bacterial eradication in the later stages of infection (28,65). Therefore, γδ T cell function during these bacterial infections appears to be similar to their response during malarial infections. Lastly, γδ T cells are involved in protection against viral infections (66,67). In one report, cytotoxic γδ T cells were shown to recognize and lyse HSV-infected target cells (66).

In general, γδ T cells appear to provide protection against intracellular viral, bacterial, and parasitic pathogens, thus supporting the "first line of defense" hypothesis. In addition, their tissue distribution is consistent with "the first line of defense" theory. Finally, unlike αβ T cells, their ability to recognize unprocessed antigens may suggest that these cells provide a rapid response against invading pathogens (18,27).

Lymphocyte trafficking

To get to specific tissues, lymphocytes follow very distinct steps in a process called trafficking. The study of lymphocyte trafficking greatly enhances our understanding of lymphocyte function. It is known that expression of specific receptors and their ligands regulates lymphocyte trafficking of discrete T cell subsets. αβ T cells originate from

the hematopoietic stem cells of the bone marrow and develop to maturity in the thymus. Although mature, these cells are naive in relation to exposure to antigen and expression of cell-surface antigens. For instance, these cells express peripheral lymph node homing receptor (L-selectin) and the naive cell-surface marker CD45RA. Naive αβ T cells recirculate through secondary lymphoid tissues, such as PLN, MLN, and Peyer's patches, using very specific molecular interactions.

Naive αβ T cells can bind to specific sites in the PLN using the lymphocyte homing receptor L-selectin. In the PLN, specialized high-endothelial venules (HEV) express peripheral node addressin (PNAd) which is recognized by surface-expressed L-selectin (68). This interaction can be inhibited by functionally blocking L-selectin or PNAd. Specifically, anti-L-selectin mAbs inhibit binding of lymphocytes to PNAd (69). Additionally, mAb MECA-79, which recognizes PNAd, blocks binding of lymphocytes to PLN HEV (70). Therefore, L-selectin plays a key role in naive αβ T cell trafficking into the PLN via binding PNAd expressed on HEVs.

Recirculation of naive αβ T cells through the gut-associated secondary lymphoid tissues, such as the MLN and Peyer's patches, involves a more complex homing mechanism. As described above, PNAd is expressed on PLNs, which plays a major role in lymphocyte homing into PLN. In experiments using L-selectin-deficient mice, lymphocyte homing to PLN was significantly inhibited while inhibition of homing to MLN and Peyer's patch was less severe (71). Thus, a significant portion of homing to gut-associated secondary lymphoid tissues occurs in the absence of L-selectin. Importantly, low levels of L-selectin ligand (MECA-79-reactive molecules) are expressed in MLNs and Peyer's patches, indicating that L-selectin binding can occur (72,73). However, L-selectin alone is not the only naive lymphocyte ligand that can mediate rolling on MLNs and Peyer's patches (73,74). The integrin α4β7 can initiate rolling on MLNs or Peyer's patches via binding mucosal addressin cell-adhesion

molecule (MAdCAM-1) (72,73). Thus, homing of naive αβ T cells to gut-associated lymphoid tissues can be initiated by L-selectin and α4β7 interactions with ligands expressed on these tissues (72-74). In fact, it has been proposed that rolling on L-selectin ligands, in Peyer's patch, is too fast to allow cell arrest and α4β7 serves as a "bridge" between rolling and cell arrest by significantly reducing rolling velocity (73,74). Furthermore, Peyer's patch-HEVs express a unique form of MAdCAM-1 which possesses ligands for both L-selectin and α4β1 (74,75).

Once the cells have homed to a particular tissue, they must either perform their function or continue to recirculate. Naive cells that enter the secondary lymphoid tissue and encounter antigen are retained in the tissue where they undergo conversion to memory/effector T cells. If, however, the naive cell does not encounter antigen, it must recirculate throughout the body to continue its search for foreign antigen. The naive cells exit the lymphoid tissue via the efferent lymph. Here, through the lymphatic system, the naive cells are returned to the blood by way of the thoracic duct where they can recirculate through the body and once again home to the secondary lymphoid tissues (76,77).

As opposed to naive T cells, memory T cells express CD45RO and varying levels of L-selectin (72,78). In addition, several adhesion molecules, such as cutaneous leukocyte-associated antigen (CLA), α4β1, and α4β7 are expressed on memory cells (72,79,80). Tissue-specific homing receptor expression on these cells appears to be dependent upon the site where naive to memory conversion occurs (72,81-83). For example, memory T cell production in PLN correlates with CLA upregulation or L-selectin on some memory T cells (83). Importantly, CLA^+ lymphocytes represent the skin-homing T cell population (79,84). Therefore, conversion to memory in PLN correlates with skin or PLN homing T cells.

Memory T cells can migrate through the secondary lymphoid tissues (73,76), but

unlike naive T cells, they also have the capacity to migrate into extralymphoid tissues. In sites of inflammation, where upregulation of various adhesion molecules occurs, memory T cells have the capacity to bind the endothelium and enter. For example, memory cells expressing $\alpha 4\beta 1$ (VLA-4) can bind vascular cell adhesion molecule-1 (VCAM-1), which is expressed by endothelium at sites of inflammation (68,73,85). In addition, memory cells expressing CLA have the capacity to bind E-selectin, allowing their migration into cutaneous sites of inflammation (79,84,86). Recently, several reports state that recruitment of specific memory T cell subsets is possible. E- and P-selectin are expressed by inflamed endothelium and are capable of initiating leukocyte/endothelial cell rolling interactions. Importantly, Th1 cells bind to these vascular selectins, while Th2 cells do not (87,88). Thus, Th1 cells can be specifically recruited into inflammatory sites where E- and P-selectin are expressed.

In other extralymphoid sites, such as the lamina propria, memory cells have the capacity to bind MAdCAM-1 via $\alpha 4\beta 7$. Interestingly, naive cells cannot bind this form of MAdCAM-1 due to its lack of L-selectin ligand expression and lower level of naive T lymphocyte $\alpha 4\beta 7$ expression (73,89). Therefore, memory cells can home to lamina propria, while naive T cells cannot.

Similarities in the trafficking of memory $\alpha\beta$ T cells and $\gamma\delta$ T cells

Upon initial observation, it becomes apparent that memory $\alpha\beta$ T cells and $\gamma\delta$ T cells possess the ability to home to nonlymphatic epithelial tissues. In addition, $\gamma\delta$ T cells and memory $\alpha\beta$ T cells express CD45RO, possessing a memory-like phenotype (90). Importantly, memory $\alpha\beta$ T cells and $\gamma\delta$ T cells are "primed" for antigen recognition and respond more rapidly then naive $\alpha\beta$ T cells; however, $\alpha\beta$ T cells recognize antigen in the context of MHC while some $\gamma\delta$ T cells recognize antigen in the absence of antigen processing and MHC restriction (27,28). In addition, both subsets appear to be

surveying the portals of entry into the body thereby providing protection from invading pathogens.

In cattle, young animals possess large numbers of circulating γδ T cells (43). As the animals mature, these numbers drop dramatically (43). Young animals do not possess a large repertoire of memory αβ T cells, therefore, these animals would need an initial defense mechanism to compensate for the slow response to infection due to the lack of memory αβ T cells. It is tempting to propose that perhaps the higher number of γδ T cells in young animals are needed until a sufficient repertoire of memory αβ T cells can be generated. Thus, later in life, the more responsive memory T cells could respond faster and to a wider variety of antigens thereby requiring fewer γδ T cells. In fact, it has been suggested that γδ T cells and αβ T cells complement and in some cases slightly compensate for each other (28). Therefore, we suggest that αβ T cells become more like γδ T cells upon conversion to the memory phenotype. Both subsets migrate to epithelial tissues and possess a heightened response to antigen. Although these subsets do possess some unique features, perhaps their complementary and potentially compensatory functions and rapid response would allow more efficient immune responses in older animals which possess fewer γδ T cells.

As stated above, memory αβ T cells and γδ T cells are capable of entering extralymphoid tissues. In some cases, expression of specific molecules, such as E-selectin, can initiate recruitment of γδ T cells (98) and specific memory αβ T cell subsets (87,88). For example, as discussed above, memory Th1 αβ T cells preferentially bind E-selectin (87,88). Thus, the ligands expressed on lymphocytes that bind this vascular selectin are important.

Leukocyte ligands for vascular E-selectin

Leukocyte adhesion to endothelium is sometimes initiated by an inflammatory response. The expression of multiple adhesion molecules on endothelial cell surfaces and their interactions with leukocyte ligands results in the recruitment of leukocytes into an inflamed site. Leukocyte adhesion and transmigration through the endothelium into inflammatory sites has been described as a multistep process (72,91,92). These events are very complex and include initial tethering and rolling interactions between the leukocyte and endothelium followed by tight adhesion to the endothelium and finally transmigration into the underlying tissue (91,92). Many of the initial adhesion events between leukocytes and endothelial cells require a unique group of molecules called selectins (68,72,93,94). To date, three proteins have been identified and classified as selectins: CD62E or E-selectin, CD62P or P-selectin, and CD62L or L-selectin. The selectins are defined by an NH_2-terminal Ca^{2+}-dependent (C-type) lectin domain, an EGF-like region, several consensus repeats and a transmembrane region with a cytoplasmic tail (68,94,95).

E-selectin is expressed on endothelial cells while P-selectin is expressed on endothelium and platelets. L-selectin is expressed on leukocytes and is characterized by a different molecular mass depending upon the expressing cell type. The two vascular selectins support interactions of myeloid cells and unique subsets of T cells, including memory cells and $\gamma\delta$ T cells (79,96-100). P-selectin is stored preformed in Weibel-Palade bodies and α-granules of endothelial cells and platelets, respectively (101-104). Upon stimulation with a variety of activators; P-selectin is rapidly expressed within minutes on the cell surface (103,104). In contrast, E-selectin upregulation upon stimulation with cytokines or LPS requires transcriptional activation exhibiting peak expression 4-6 hours after stimulation (105-108). Therefore, P-selectin expression is capable of mediating rolling interactions minutes after stimulation while

E-selectin interactions occur hours after stimulation. Additionally, E-selectin expression is variable depending upon species and anatomic location (109).

Recent in vitro results indicate that leukocyte rolling on chronically inflamed endothelium appears to be independent of E- or P-selectin, suggesting novel adhesion systems that mediate leukocyte/endothelial cell interactions (85,109,110). Because of their critical role in the initial leukocyte tethering to endothelium, many investigators have devoted a great deal of time to defining the ligands for each selectin on various target cells.

E-, L-, and P-selectin ligands have been described in several systems. All three selectins bind to both sialyl Lewis a (SLe^a) and sialyl Lewis x (SLe^x) tetrasaccharide structures which are expressed by many different cell types (111-117). Specific high molecular mass glycoproteins present these carbohydrates in appropriate fashion to support cell/cell adhesion (113,118).

E-selectin ligands on myeloid cells have been well defined. Early reports have demonstrated that L-selectin can bind to E-selectin. In vitro reports have shown that neutrophil adhesion to inflamed endothelium, which expresses E-selectin, is blocked by anti-L-selectin mAb (119-121). In addition, adhesion of neutrophils on E-selectin-transfected L-cells was inhibited by both anti-E-selectin and anti-L-selectin mAb, suggesting that E-selectin and L-selectin may represent a receptor/counterreceptor pair (119). Of interest, neutrophil but not lymphocyte L-selectin is sLe^x positive and only neutrophil L-selectin binds E-selectin-transfected L1.2 cells (122). Extensive analyses, including reprecipitation experiments, have revealed that neutrophil L-selectin binds to E-selectin (123). More specifically, an affinity-purified E-selectin ligand of 80-90kD was reprecipitated with anti-L-selectin mAb, revealing that soluble L-selectin binds to E-selectin.

Another neutrophil E-selectin ligand, called ESL-1, has recently been described.

ESL-1, a 150kD ligand under reducing conditions and 130kD under nonreducing conditions, was affinity-isolated from mouse myeloid cells (124,125). In these reports, immobilized E-selectin/Ig chimera immunoprecipitated a 150kD ligand from metabolically labeled PMN lysates. In addition, a weak 250kD ligand was also purified from mature neutrophils. Later reports described two additional but weaker E-selectin ligands of 130kD and 230kD on mouse neutrophils, which were also reactive with a P-selectin chimera (125). Furthermore, these authors indicated that human neutrophils possess similar ligands (125).

Since E- and P-selectin bind the same tetrasaccharides, it is possible that E- and P-selectin would bind the same glycoprotein expressing these carbohydrates. Supporting this possibility are recent reports indicating that PSGL-1 (P-selectin glycoprotein ligand-1), a known P-selectin ligand (126-130), can also serve as an E-selectin ligand (124,129-134). Specifically, transfection of CHO cells with PSGL-1 cDNA and a combination of various glycosyltransferase cDNAs conferred binding to E- and P-selectin, indicating that PSGL-1/E-selectin interactions occur (134). In addition, tyrosine sulfation of PSGL-1 seems to be required for binding to P-selectin, but not E-selectin (134). These results suggest that the PSGL-1 protein backbone provides the support upon which post-translational modifications confer selectin binding specificity.

In contrast to neutrophils, far less is known about the glycoprotein ligands for the vascular selectins expressed by lymphocytes. As stated before, E- and P-selectin support interactions of unique subsets of T cells, including memory cells and $\gamma\delta$ T cells (79,96-100). An appropriate post-translationally modified form of PSGL-1 is likely to be the lymphocyte ligand for P-selectin (99,135); however, the lymphocyte ligands for E-selectin are unknown. Lymphocyte PSGL-1 does not appear to interact appreciably with E-selectin (97,136,137), as has been shown with neutrophil PSGL-1 (124,129-134). Indeed, O-sialoglycoprotease treatment of lymphocytes, which

hydrolyzes PSGL-1, blocks their binding to P-selectin, but not E-selectin (97,136,137). Of importance, mAb HECA-452 recognizes the E-selectin-binding human lymphocytes, but HECA-452 staining does not correlate with the P-selectin-binding subset, further distinguishing the two (84,86,136). The HECA-452 epitope is a carbohydrate structure called cutaneous lymphocyte-associated antigen (CLA) that decorates many different cell surface ligands (79) and correlates with E-selectin binding (84,86). This antigen is associated with cutaneous T lymphocytes and HECA-452 specifically blocks the interaction of CLA+ T cells with E-selectin-transfected COS cells (79,84,86). In addition, purified CLA coated onto glass supports specific binding of E-selectin-transfected L1.2 cells to CLA (86). Thus, in humans, CLA is capable of binding E-selectin. In initial reports describing HECA-452-reactive antigens, Picker et al. showed that it reacts with 125kD and 200kD glycoproteins on T lymphocytes (79). Which of the different T lymphocyte CLA glycoproteins serve as the predominant E-selectin binding molecule is unknown at this time.

Recently, we showed that virtually all bovine γδ T cells in newborn animals have the capacity to bind E-selectin (96). In contrast, αβ T cells acquire this binding capacity only after conversion to a memory phenotype (79,84,100). We isolated a 250kD E-selectin-binding molecule from γδ T cells by affinity isolation (96) using purified, recombinant E-selectin immobilized on Sepharose beads by a nonblocking anti-E-selectin mAb. Silver staining of the column fractions revealed a 250kD ligand expressed by these cells. In addition, using an in vitro shear-dependent recirculating loop assay developed while studying neutrophil interactions (138,139), we have shown that bovine γδ T cells bind E- and P-selectin avidly and in newborn calves γδ T cells are the only lymphocyte population that bind to E-selectin (98).

In pursuit of the bovine E-selectin ligands, we have proposed that lineage-specific cell-surface antigens may represent selectin ligands. More specifically, GD3.5Ag and

WC1, which share biochemical characteristics with other adhesion ligands, may bind E-selectin.

Summary

Historically, lineage-specific cell-surface markers have been shown to be functionally important. Since the discovery of the γδ T cell, definition of γδ T cell lineage-specific markers continues to be a topic of interest. Unfortunately, in the past ten years, only two lineage-specific markers have been described on γδ T cells (WC1 and TCR). Here, I will describe a new lineage-specific γδ T cell marker (GD3.5Ag) and determine if this marker is an E-selectin ligand.

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CHAPTER 2

GENERATION OF A NEW γδ T CELL-SPECIFIC MONOCLONAL ANTIBODY (GD3.5): BIOCHEMICAL COMPARISONS OF GD3.5 ANTIGEN WITH THE PREVIOUSLY DESCRIBED WC1 FAMILY

Introduction

Although gamma/delta (γδ) T cells were discovered a decade ago, their specific biological function has yet to be fully defined. Some suggest that these cells contribute to the initial host response to various infectious agents, which include parasitic, bacterial, and viral pathogens, forming what is referred to as "the first line of defense" (1-3). This hypothesis is consistent with their anatomical location at the portals of entry in nonlymphatic tissues, such as the skin and gut (4-6). In studies using γδ or αβ T-cell-deficient mice, the role of γδ T cells in malarial infection appears to occur during initial host responses against the liver-stage of parasite development (1). In another report, $\beta_2$microglobulin-deficient mice infected with live avirulent mycobacteria appear to respond with increased numbers of γδ T cells when compared to normal mice indicating that these cells may possess an early compensatory function in the absence of MHC I molecular expression (2). Further reports indicate that γδ T cells respond to and destroy virally infected target cells (3).

Although γδ T cells that react with classical MHC I and II have been isolated, they appear to react with other nonclassical MHC gene products, possibly providing a faster and more encompassing immunological response (7-10). Several reports suggest that γδ T cells may recognize "unconventional" antigens, such as nonpeptide (carbohydrate) antigens and heat-shock proteins (11-16). Recent reports indicate that γδ T cell antigen recognition may be independent of antigen processing, suggesting a direct recognition event (17).

Historically, lineage-specific cell surface markers have been indispensable in describing various cell functions. In the case of γδ T cells, only one non-TCR lineage-specific marker (SBU-T19), originally described in sheep, has been defined (4,18). This marker, later demonstrated in cattle, goats, pigs, horses, humans and rodents, is collectively called WC1 (6,19-26). Bovine genomic DNA contains several similar sequences to WC1, suggesting the potential for a WC1 gene family (23). Three WC1 isotypes have been identified to date (23,26) and the IL-A29 monoclonal antibody recognizes all three native species, including gene products of the 3 WC1 cDNAs. At the protein level, bovine WC1 exhibits bands at approximately 215kD and 300kD under reducing conditions (recognized by IL-A29); while under nonreducing conditions, the smaller band migrates somewhat faster (200kD) (4,6,22,23,25,27). WC1 is clearly distinguishable from γδ TCR which migrates at approximately 45kD under reducing and 70-75kD under nonreducing conditions. Unfortunately, little is known about the function of WC1. Some speculate that WC1 may play a role similar to CD4 and CD8, while others suggest the expression of different WC1 isotypes may provide a mechanism for tissue-specific homing (4,23,26,28).

We have sought the identification of additional lineage-specific markers to better study γδ T cell function. We chose the bovine model because γδ T cells represent a major portion of the circulating T cell pool in neonates; thus, their biological function may be

more easily studied. We have raised many specific monoclonal antibodies against purified γδ T cells with most being shown to recognize either WC1 or TCR. In this report, we provide the characterization of one antibody (GD3.5) that recognizes a γδ T cell-restricted surface antigen that is distinct from IL-A29-reactive WC1 and TCR. GD3.5 stains approximately 90% of the circulating γδ T cell population. SDS-PAGE analysis indicates that GD3.5 antigen migrates slightly slower than WC1 under nonreducing conditions and does not exhibit a 300kD band. Crossprecipitation experiments indicate that mAb GD3.5 and mAb IL-A29 immunoprecipitate distinct molecules that exhibit distinct proteolytic digestion profiles. Glycosidase and endopeptidase treatment further characterize and distinguish GD3.5 antigen and WC1 demonstrating unique profiles. Interestingly, GD3.5 antigen and WC1 are sensitive to O-sialoglycoprotease treatment indicating that these molecules possess sialomucin-like characteristics. In addition, GD3.5 is exquisitely sensitive to low-dose α-chymotrypsin, while WC1 is resistant to equivalent protease concentrations.

Materials and Methods

γδ T cell purification and ^{125}I labeling

Blood was obtained by jugular venipuncture from Holstein calves housed at the Montana State University Large Animal Facility. Lymphocytes were purified by Histopaque gradient centrifugation (Sigma Chemical Co., St. Louis, MO). Lymphocytes were resuspended in HBSS and platelets were removed by Percoll gradient centrifugation (Sigma Chemical Co., St. Louis, MO) with subsequent incubation of lymphocytes in T175 flasks for 30min at 37^{o}C to allow monocytes to adhere and be removed from suspension. γδ T cell lymphocytes were purified from whole lymphocytes by incubation for 30min at RT in T175 flasks coated with 7 x 10^{6} adherent E-selectin L-cell transfectants.

Remaining unbound cells were discarded and bound γδ T cells lifted with 2mM EDTA (29). This purification protocol provided >90% pure γδ T cells (29). 1 x 10^8 purified γδ T cells were $Na^{125}I$ (ICN Biomedicals, Inc., Irvine, CA)/lactoperoxidase-labeled according to published methods (30) and lysed for 30min on ice with NP40 lysis buffer (2% NP-40, 100mM NaCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 5mM NaN_3, 10mM Hepes, and protease inhibitors: pepstatin A, 1,10-phenanthroline, phenylmethylsulfonyl fluoride, benzamidine, antipain, leupeptin, and chymostatin (Sigma Chemical Co., St. Louis, MO).

mAbs and FACS analysis

Monoclonal antibodies used included IL-A29 (anti-WC1) and two mouse mAbs CC21 (145kD B-cell marker) and CC42 (αβ T cell-CD2) that recognize bovine and caprine lymphocytes (kindly provided by Chris Howard, Institute for Animal Health, Compton, UK). In house mAbs, GD3.5, GD3.8, and GD197 were produced by immunization (i.p.) of Balb/C mice with 6 x 10^7 purified γδ T cells in Gerbu Adjuvant (Biotech, Poway, CA). Mouse serum was screened by FACS analysis to determine reactivity with γδ T cells after two weeks. Two subsequent immunizations were performed as described at two week intervals. After the third immunization, mouse spleens were aseptically removed 72hrs later and fused as described (31). Fusion wells containing hybrids that positively stained γδ T cells by FACS analysis were subsequently subcloned and screened by FACS analysis. mAb production resulted in various γδ T cell-positive mAbs including GD3.5, GD197 (γδ TCR), and GD3.8 (pan-γδ TCR). (B. Walcheck and M.A. Jutila, unpublished observations; and Wilson, E. et al., manuscript in preparation).

FACS analysis was performed on ice as follows: cells were blocked with 2% horse serum/PBS for 10min, washed, and incubated with appropriate primary Ab for 30min. Cells were washed and incubated with appropriate FITC-conjugated or PE-conjugated

goat α-mouse secondary Ab (Jackson ImmunoResearch, West Grove, PA) for 30min, washed again, and analyzed. Two-color analysis was performed using PE-conjugated secondary Ab followed by a 10min block with 10% mouse serum and subsequent 30min incubation with FITC-conjugated second-stage Ab. All analyses were performed using a FACScan flow cytometer (Becton Dickinson).

Western Blot

Purified γδ T cells were lysed with NP-40 lysis buffer (see above), mixed with nonreducing loading buffer and electrophoresed through 8% polyacrylamide gels. Gels were transferred to nitrocellulose (NC) overnight. Resulting NC was washed, blocked for 30min with horse serum, washed again, and placed in a Miniblotter 25 (Immunetics, Cambridge, MA). Lanes were loaded with GD3.5 or IL-A29 supernatants and incubated for 30min. Primary antibody was washed away and α-mouse alkaline phosphatase-conjugated second stage (Sigma Immuno Chemicals, St. Louis MO) applied for 30min. The NC was washed and removed from Miniblotter 25 and developed to desired intensity using Nitro Blue Tetrazolium and 5-Bromo-4-Chloro-3-Indolyl Phosphate (Sigma Chemical Co., St. Louis, MO) in AP buffer (100mM Tris pH 9.5, 100mM NaCl, and 5mM $MgCl_2$).

Immunoprecipitation/Crossprecipitation

To eliminate nonspecific protein interactions, ^{125}I-labeled lysates were incubated for 1hr at RT with 5% rabbit serum and then incubated at 4°C with protein G beads (Boehringer Mannheim Biochemicals, Indianapolis, IN). After overnight incubation, protein G beads were removed and lysates were used in subsequent procedures. Precleared lysates were incubated with 75ug/ml GD3.5 or IL-A29 for 1hr at RT. Ab/Ag complexes were precipitated with protein G at 4°C overnight. Several subsequent

clearing steps with protein G were performed on the lysates to insure removal of all Ab/Ag complexes. IL-A29 and GD3.5 mAbs were added to lysates which had been cleared of GD3.5 and WC1 Ab/Ag complexes, respectively. Ab/Ag complexes were precipitated as described earlier and mixed with standard 2x reducing or nonreducing loading buffer, boiled for two minutes, and loaded onto 8% SDS-polyacrylamide gels. Gels were electrophoresed and dried at 80°C. Gels were exposed 1-2 weeks in a phosphorimaging storage cassette, and images were developed using a phosphorimager (both from Molecular Dynamics).

V-8 protease peptide mapping

^{125}I-labeled γδ T cell lysates, described above, were prepared and immunoprecipitated with IL-A29 and GD3.5. IL-A29 or GD3.5 immunoprecipitates were mixed with reducing buffer, boiled for two minutes, cooled to RT and centrifuged to remove beads. Supernatants were aliquoted (25ul) and incubated with 10, 50, and 100ng of V-8 protease for 40min at RT, loaded, and electrophoresed. Gels were dried at 80°C and exposed/developed using the MD phosphorimager, as described above.

Enzymatic deglycosylation of GD3.5 antigen and WC1

^{125}I-labeled γδ T cell lysates, described above, were prepared and immunoprecipitated with 30ug of IL-A29 or GD3.5. Immunoprecipitates were boiled for 3min after adding 80ul 50mM pH 6.0 phosphate buffer, 20ul 1% SDS, and 20ul .5M β-mercaptoethanol (Sigma Chemical Co., St. Louis, MO) and cooled to RT. After cooling, 35ul 200mM pH 6.0 phosphate buffer, 5ul 100mM $CaCl_2$, and 40ul 10% NP40 (Sigma Chemical Co., St. Louis, MO) were added to each reaction which were then centrifuged and the supernatants aliquoted (35ul) for treatment with 0.2U PNGase F (Boehringer Mannheim Biochemicals, Indianapolis, IN), 9.6ug O-sialoglycoprotein endopeptidase/O-

sialoglycoprotease (Accurate Chemical & Scientific Corp., Westbury, NY), or 2mU neuraminidase (Boehringer Mannheim Biochemicals, Indianapolis, IN). Aliquots of GD3.5 antigen and WC1 were adjusted to pH 6.0 (control and neuraminidase) or pH 7.4 (PNGase F and O-sialoglycoprotease) with 15ul of 500mM phosphate buffer, mixed with appropriate amount of enzyme, and incubated overnight at 37°C (13-15hrs). Reactions were stopped with 6x reducing loading buffer, boiled for 3min and electrophoresed. Gels were dried at 80°C and exposed/developed using the MD phosphorimager, as described above.

Chymotrypsin Sensitivity Assay

Lymphocytes were purified as described above. Cells ($1x10^7$) were resuspended in HBSS with Ca^{2+} and Mg^{2+} and 0, 62.5, 125, or 250 mU α-chymotrypsin (Sigma Chemical Co., St. Louis, MO)/ml and incubated for 1hr at 37°C (32). After completion of incubation, cells were washed with 2% horse serum/PBS and analyzed by single-color indirect FACS analysis for GD3.5 antigen and WC1 using PE-conjugated second stage, as described earlier.

Results

Surface expression of the GD3.5 antigen on bovine lymphocytes

GD3.5 was originally identified by flow cytometric analysis as a γδ T cell-specific staining monoclonal antibody (See Materials and Methods mAb production and see below). Using two-color FACS analysis, we determined the staining characteristics of GD3.5 on bovine peripheral blood lymphocytes. Cells were stained with GD3.5, IL-A29 (WC1), CC21 (B-Cell) or CC42 (αβ T cell), followed by a PE secondary Ab, and then stained with FITC-labeled GD3.5 or GD3.8 mAb. As shown in Figure 1A, GD3.5 stained 91.8% of

the total GD3.8 (pan-TCR) γδ T cell population. In comparison, panel 1B shows that IL-A29 stained 78% of the total γδ T cell population. Figure 1C indicates that a substantial percentage of the GD3.5-positive population was WC1 negative. Additionally, single-color FACS analysis further supported the two-color data indicating that GD3.5 stains approximately 90% of the total γδ T cell population while IL-A29 stained 70-80% of the total γδ T cell population (data not shown). To further demonstrate that GD3.5 specifically stained γδ T cells, cells were stained with B-cell (CC21) or αβ T cell (CC42) mAbs. Figure 1D and 1E illustrate that GD3.5 stained a population of lymphocytes that were distinct from both B cells (CC21) and αβ T cells (CC42). Furthermore, using the distinguishing light-scatter profiles of neutrophils and monocytes, gated FACS analysis of these cell populations in whole bovine leukocyte preparations revealed that mAbs GD3.5 and GD3.8 did not stain either cell population (data not shown).

Using single-color FACS analysis of peripheral blood, crossreactivity was determined using dog, horse, human, sheep, pig, and rabbit. GD3.5 crossreacted only with the sheep lymphocytes (data not shown).

Western Blot and immunoprecipitation analysis of the GD3.5 antigen

Western blot analysis was done to determine a Mr for the GD3.5 antigen. As shown in lanes 3 and 4 of Figure 2, GD3.5 recognized a single band migrating at 220-240kD under nonreducing conditions. Interestingly, using this technique sometimes resulted in a single GD3.5-reactive band at approximately 80kD which is likely due to the exquisite sensitivity of GD3.5 antigen to proteolysis (data not shown and see Figure 7). In comparison, lanes 5 and 6 show bands recognized by IL-A29 (anti-WC1) at 200-220kD and 300kD under nonreducing conditions. Therefore, under these conditions, GD3.5 antigen was electrophoretically distinct from IL-A29 antigen (WC1).

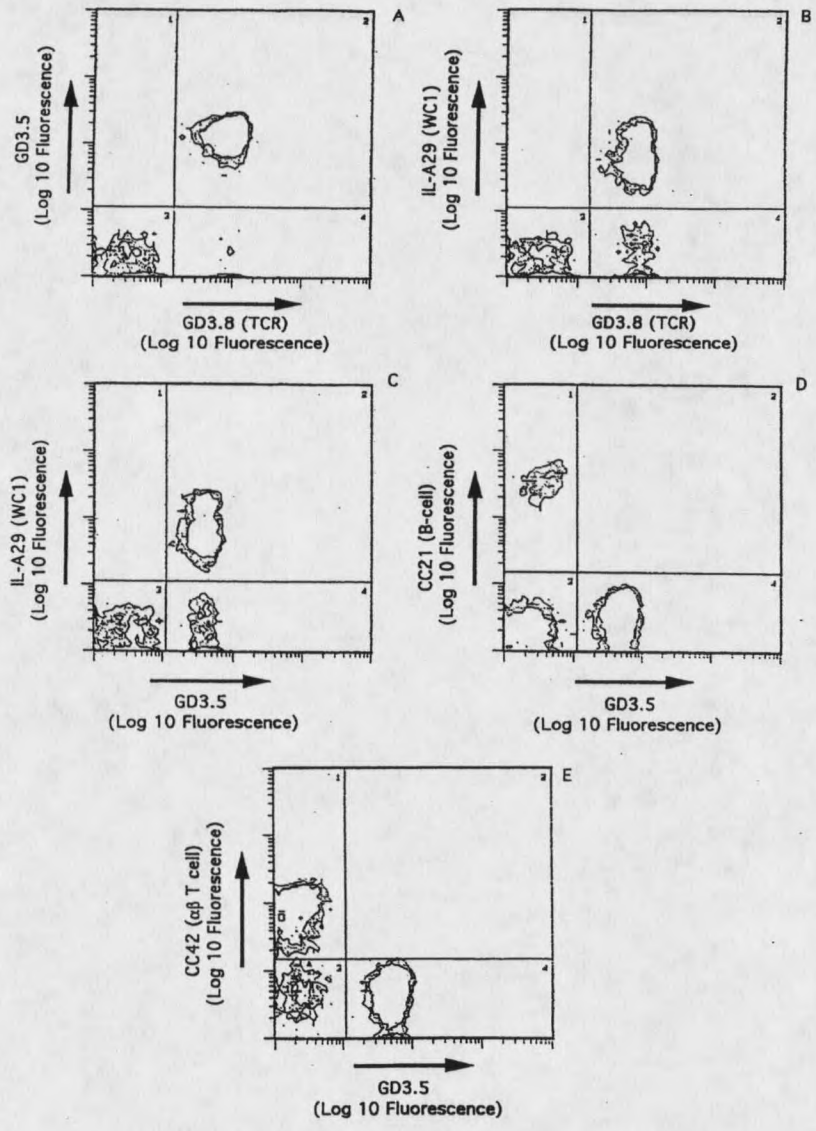

Figure 1. Two-color FACS analysis illustrating GD3.5 staining patterns of peripheral blood lymphocytes from a single individual. Two-color analysis was performed according to Materials and Methods. Panel 1A shows cells stained with GD3.5 and GD3.8 (pan-γδTCR). Panel 1B shows cells stained with IL-A29 (WC1) and GD3.8. Panel 1C shows cells stained with IL-A29 and GD3.5. Panel 1D shows cells stained with CC21 (B-cell) and GD3.5. Panel 1E shows cells stained with CC42 (αβ T cell) and GD3.5. Figure 1 is representative of at least three separate experiments.

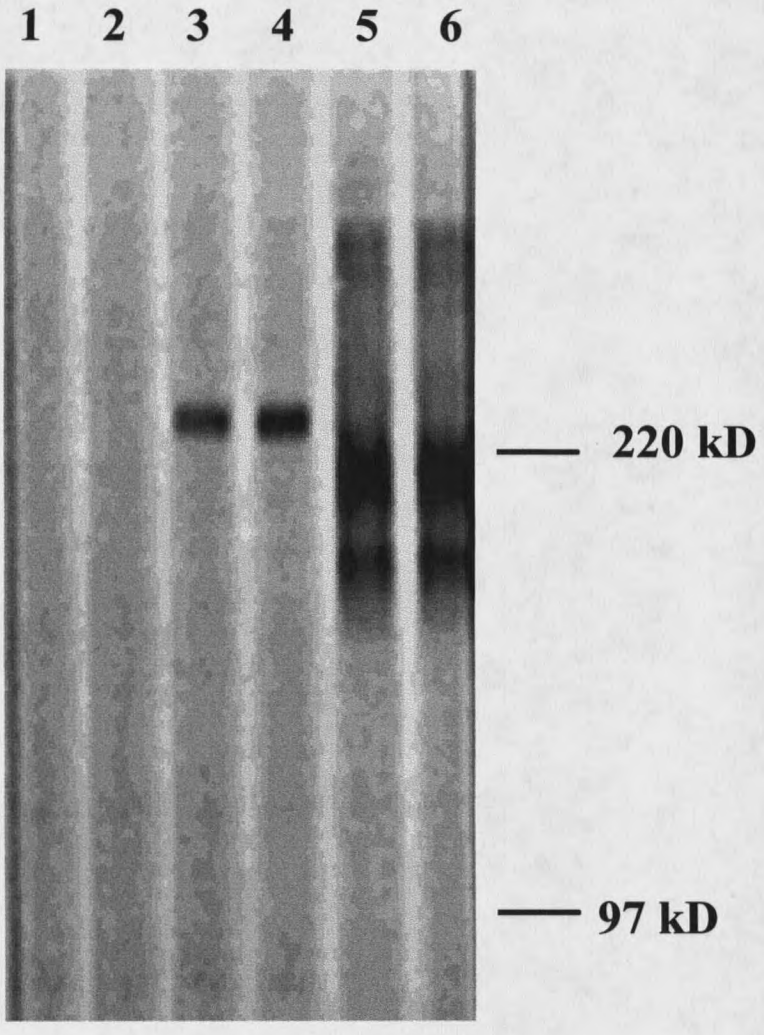

Figure 2. Nonreducing Western Blot of γδ T cell lysate, illustrating electrophoretic mobility of GD3.5 antigen and WC1. Western blotting was performed as described in the Materials and Methods. Lanes 1 and 2 represent media control. Lanes 3 and 4 were blotted with GD3.5, and lanes 5 and 6 were blotted with IL-A29. Molecular standards are as marked to the right. Figure 2 is representative of at least three separate experiments.

Interestingly, another IL-A29-reactive band appeared at approximately 180kD. This same band was not present in IL-A29 immunoprecipitates in Figure 3 (see below), indicating that it was not surface labeled by ^{125}I. This suggested that it was exposed after cell lysis, possibly representing an immature molecule that was not yet expressed on the cell surface but was IL-A29-reactive. Neither IL-A29 or GD3.5 worked by Western blot under reducing conditions.

Immunoprecipitation analysis was also done to confirm the Mr of the GD3.5 antigen, particularly under reducing conditions. Purified γδ T cells were ^{125}I-labeled, lysed, immunoprecipitated with IL-A29 (anti-WC1), GD197 (anti-γδ TCR), or GD3.5, and separated by SDS-PAGE, as described in Materials and Methods. As shown in lane 2 of Figure 3, IL-A29 immunoprecipitate (WC1) was characterized by two distinct bands at 200-220kD and 300kD under nonreducing conditions. Lane 3 shows that GD3.5 immunoprecipitated a protein that migrated at approximately 220-240kD, slightly slower than the 200-220kD WC1 band. These results were the same as those obtained by Western blotting. As expected, TCR migrated at approximately 75kD under nonreducing conditions (lane 4). Under reducing conditions, the GD3.5 antigen was indistinguishable from the lower (220-240kD) WC1 species (compare lanes 6 and 7) and TCR migrated at 40-50kD (lane 8). Importantly, earlier reports have shown that immunoprecipitation of labeled γδ T cell lysates with framework anti-CD45 mAb results in a single band that is substantially smaller than the 220kD IL-A29-reactive band (4) showing that GD3.5 does not recognize CD45. In a separate study, we are characterizing the expression of CD45RO on bovine γδ T cells and have confirmed that GD3.5 is molecularly distinct from CD45 (M. A. Jutila et al., unpublished observations).

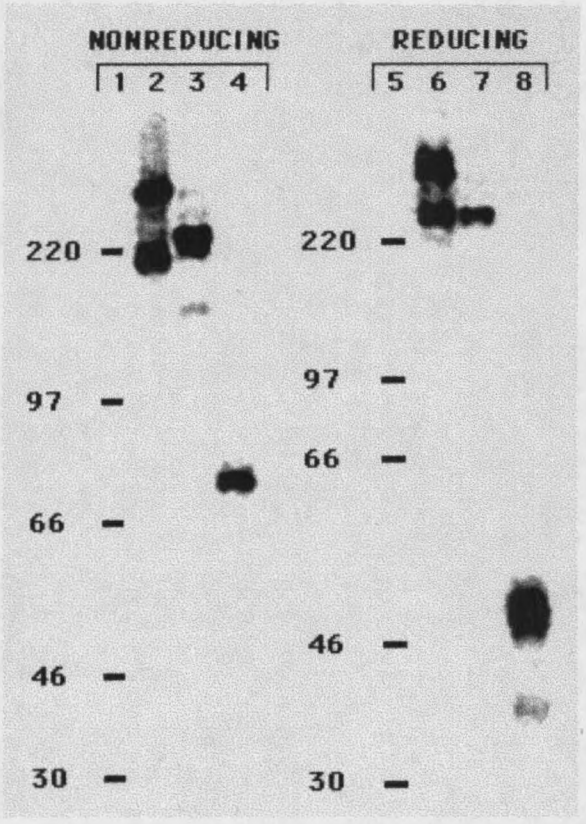

Figure 3. Comparisons of ^{125}I-labeled lysate immunoprecipitations of WC1, GD3.5 antigen, and TCR under nonreducing and reducing conditions. Lysates were immunoprecipitated with IL-A29, GD3.5, and GD197, electrophoresed and analyzed, as described in Materials and Methods. Lanes 1 and 5 show the molecular standards as marked. Lanes 2, 3, and 4 show lysates immunoprecipitated with IL-A29, GD3.5 and GD197, respectively, and electrophoresed under nonreducing conditions. Lanes 6, 7, and 8 show lysates immunoprecipitated with IL-A29, GD3.5 and GD197, respectively, and electrophoresed under reducing conditions. Figure 3 is representative of three separate experiments.

Crossprecipitation analysis, V-8 protease peptide mapping, enzymatic deglycosylation, and WC1 and GD3.5 antigen cell surface expression

Even though the molecular mobility of the GD3.5 antigen was distinct from WC1 under nonreducing conditions, their similarity under reducing conditions prompted us to further characterize the difference in the two antigens. Crossimmunoprecipitation experiments were used to confirm if IL-A29 and GD3.5 immunoprecipitated different

molecules, and the results are shown in Figure 4. Excess IL-A29, GD3.5, and GD197 was added to ^{125}I-labeled γδ T cell lysates. Lanes 2, 3, and 4 show the first round of immunoprecipitation with IL-A29, GD3.5, and GD197, respectively. Lanes 5, 6, and 7 were loaded with immunoprecipitates from the same lysates above that had been cleared 4 additional times of Ag/Ab complex, demonstrating that lysates were cleared of IL-A29/WC1, GD3.5Ab/GD3.5Ag, and GD197/TCR complexes, respectively. Lysates precleared of IL-A29/WC1 were then crossimmunoprecipitated with GD3.5. Lane 8 shows that GD3.5 immunoprecipitated the characteristic 220-240kD protein from a lysate precleared of IL-A29/WC1. In addition, IL-A29 was used to crossimmunoprecipitate the lysate precleared of GD3.5Ab/GD3.5Ag. Lane 9 shows that IL-A29 immunoprecipitated the characteristic 200-220kD and 300kD proteins. Lanes 12-19 represent the same experiments done under reducing conditions and the results were identical.

As another means of comparing WC1 and the GD3.5 antigen, V-8 protease digestion profiles were generated and compared. Immunoprecipitated WC1 was digested with 0ng, 10ng, 50ng, and 100ng V-8 protease/sample and separated by SDS-PAGE. In Figure 5, lanes 1, 3, 5, and 7 show the effects of increasing concentrations of protease treatment on WC1, and lanes 2, 4, 6, and 8 represent immunoprecipitated GD3.5 treated with the same protease regimen. Lanes 5 and 7 indicate protease digestion products unique to WC1, while the arrows indicate products unique to GD3.5 antigen digestion (lanes 6 and 8).

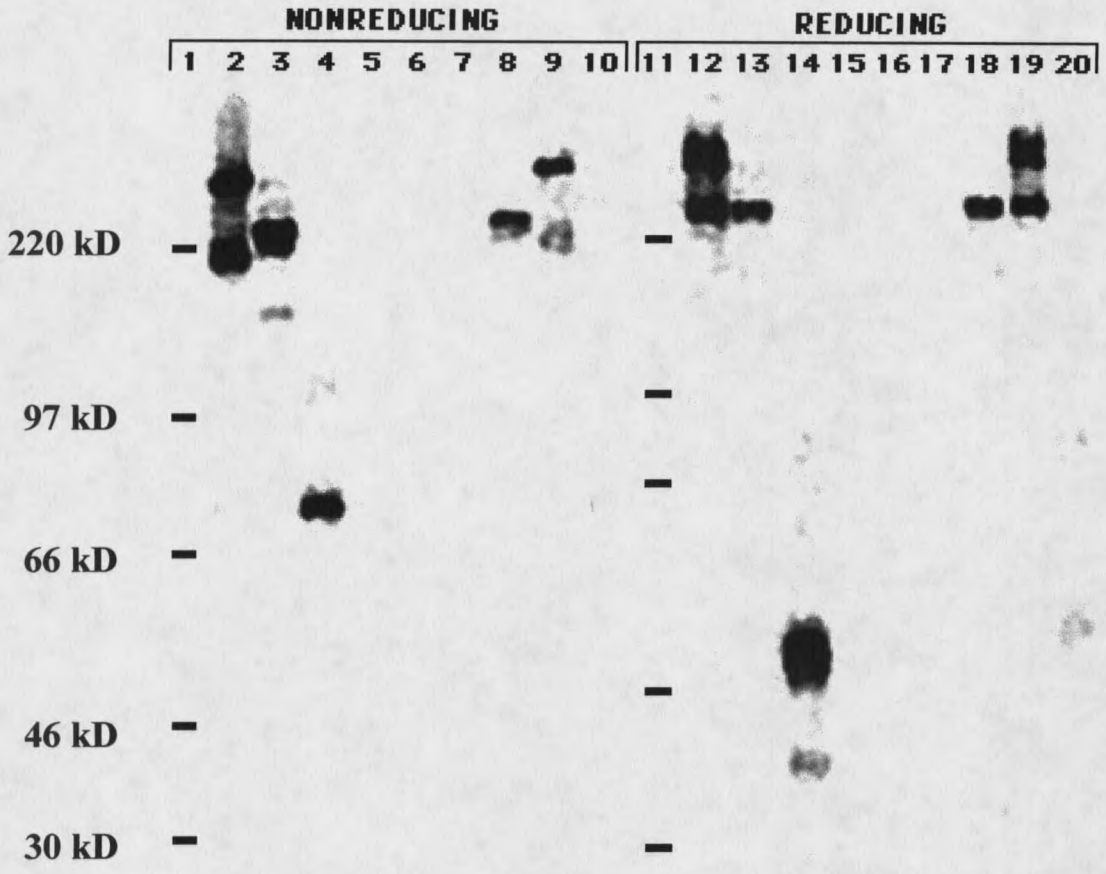

Figure 4. ^{125}I-labeled lysate immunoprecipitation/crossprecipitation experiment. Lanes 1-10 represent antigens electrophoresed under nonreducing conditions, while lanes 11-20, represent antigens electrophoresed under reducing conditions. Lanes 1 and 11 show molecular standards as marked. Lanes 2, 3, and 4 represent three separate lysates immunoprecipitated with IL-A29, GD3.5, and GD197, respectively. Lanes 12, 13, and 14 represent the same treatment electrophoresed under reducing conditions. Lanes 5, 6, and 7 represent the lysates/immunoprecipitations from lanes 2, 3, and 4 that have been cleared 5x, using protein G beads, of IL-A29, GD3.5, and GD197 antigen/antibody complexes, respectively. Lanes 15, 16, and 17 represent the same treatment under reducing conditions. Lanes 8 and 9 represent the crossimmunoprecipitation treatment. Lane 8 shows lysate cleared 5x of IL-A29/WC1 crossimmunoprecipitated with GD3.5. Lane 9 shows lysate cleared 5x of GD3.5Ab/GD3.5Ag and crossimmunoprecipitated with IL-A29. Lanes 18 and 19 represent the same treatment under reducing conditions. Lanes 10 and 20 represent control lanes showing a second attempt to immunoprecipitate TCR with additional GD197 mAb added to lysates cleared 5x of GD197/TCR complexes. Figure 4 is representative of three separate experiments.

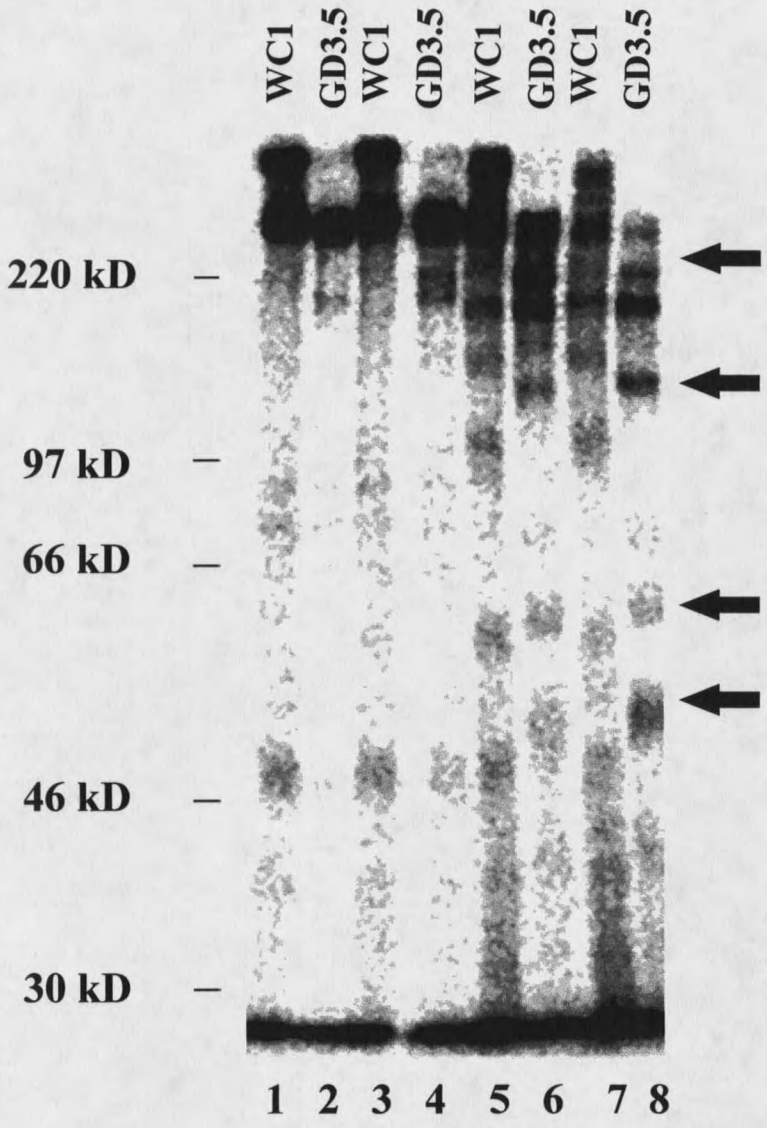

Figure 5. GD3.5 antigen and WC1 immunoprecipitates treated with varying concentrations of V-8 protease and electrophoresed under reducing conditions to illustrate/compare digestion profiles. Lanes 1 and 2 represent WC1 and GD3.5 antigen controls, respectively (no protease). Lanes 3 and 4 represent WC1 and GD3.5 antigen, respectively, treated with 10ng of V-8 protease/lane. Lanes 5 and 6 represent WC1 and GD3.5 antigen, respectively, treated with 50ng of V-8 protease/lane. Lanes 7 and 8 represent WC1 and GD3.5 antigen, respectively, treated with 100ng/lane. Arrows indicate unique GD3.5 digestion bands in lanes 6 and 8. Molecular standards are as marked to the left. Figure 5 is representative of three separate experiments.

To provide additional characterization of the GD3.5 antigen, we used PNGase F, O-sialoglycoprotease, and neuraminidase to determine glycosylation characteristics of the molecule. In addition, comparisons of GD3.5 and WC1 were also performed. CD45 was used as a control protein to ensure specific activity of the various enzymes (data not shown). Immunoprecipitated antigen was treated with PNGase F (0.2U/rxn), O-sialoglycoprotease (9.8ug/rxn), or neuraminidase (2mU/rxn). In Figure 6, lanes 1 and 5 represent control WC1 and GD3.5 antigen under reducing conditions. Lanes 2 and 6 represent WC1 and GD3.5 digested with O-sialoglycoprotease, respectively. Interestingly, O-sialoglycoprotease treatment resulted in the loss of both the 220-240kD and 300kD WC1 bands and the appearance of two bands at ~200-220kD and ~100kD (indicated by arrows, lane 2). In contrast, GD3.5 antigen was digested into two bands of ~220kD and ~160kD (indicated by arrows, lane 6). Of note, repeated gels indicated that the upper GD3.5 antigen digestion band may be comprised of 2 separate molecular species. The activity of O-sialoglycoprotein endopeptidase on both WC1 and GD3.5 antigen indicated that both of these molecules are decorated with O-linked sugars containing terminal sialic acid residues characteristic of mucin-like glycoproteins. In addition, the O-sialoglycoprotease data further distinguished GD3.5 antigen from WC1. Lanes 3 and 7 represent digestion patterns of WC1 and GD3.5 antigen with PNGase F, respectively. Both the 220-240kD and 300kD WC1 bands demonstrated sensitivity to PNGase F indicating that WC1 is also N-glycosylated. Both the 300kD and 220-240kD bands were digested into apparently one broad band (lane 3). GD3.5 antigen was deglycosylated with PNGase F resulting in a single band at 160-170kD, representing a greater shift than the lower Mr species of WC1. The results with PNGase F indicated that both WC1 and GD3.5 antigen possess distinguishing N-linked sugars that comprise a significant portion of their total molecular mass. Lanes 4 and 8 illustrate digestion of WC1 and GD3.5 antigen with neuraminidase, respectively. Interestingly, both WC1

bands shift slightly (downward) indicating decoration with sialic acid. The 220-240kD WC1 band has a more pronounced shift than the 300kD band. GD3.5 antigen shifts downward (~10kD) after treatment with neuraminidase, indicating that this molecule is also decorated with sialic acids. These data are not surprising since O-sialoglycoprotease requires sialic acid residues for activity (33). Also, the 220-240kD WC1 band demonstrated a greater shift than did the GD3.5 antigen, consistent with the greater sensitivity of WC1 to O-sialoglycoprotease.

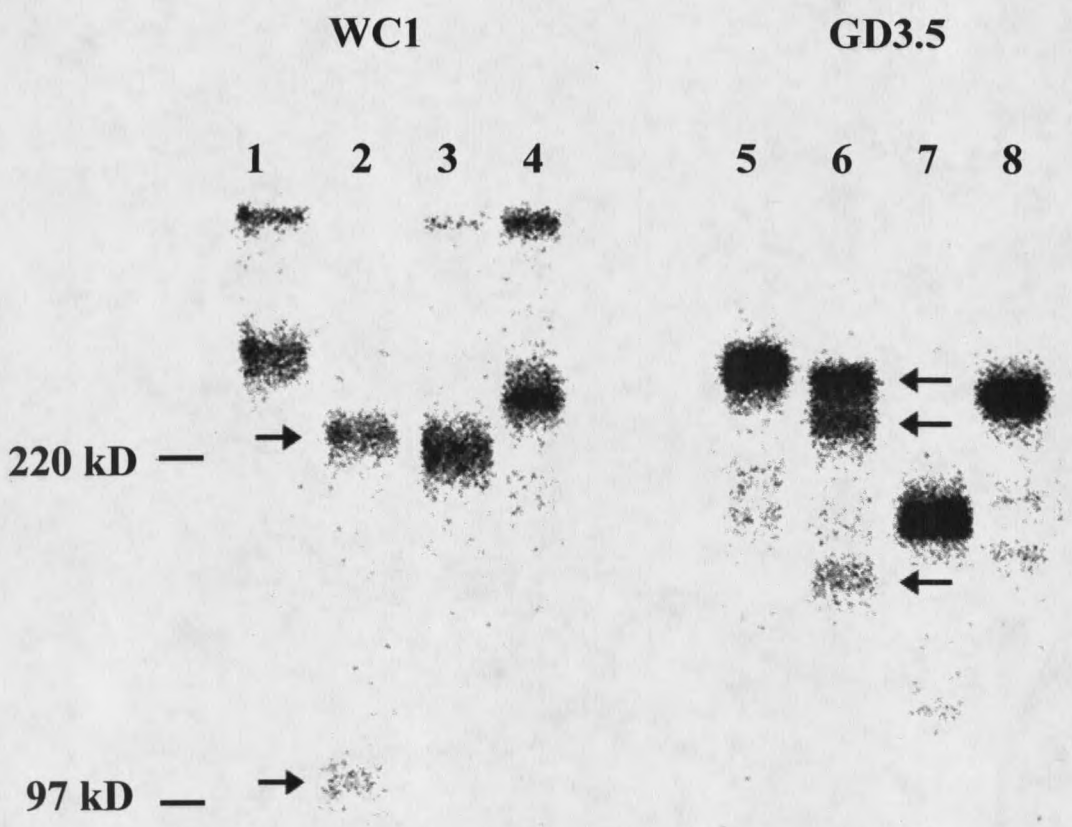

Figure 6. Comparison of ^{125}I-labeled lysates of WC1 (lanes 1-4) and GD3.5 antigen (lanes 5-8) treated with O-sialoglycoprotease, PNGase F, or neuraminidase. Lanes 1 and 5 are untreated controls. Lanes 2 and 6 show lysates treated with O-sialoglycoprotease, lanes 3 and 7 indicate lysates treated with PNGase F, and lanes 4 and 8 show lysates treated with neuraminidase. Lysates were incubated overnight at 37^{o}C, electrophoresed, and analyzed as described in Materials and Methods. Arrows indicate O-sialoglycoprotease degradation products of WC1 and GD3.5 antigen as discussed in the Results section. Molecular standards are as marked to the left. Figure 6 is representative of three separate experiments.

Effects of low-dose chymotrypsin treatment on cell surface expression of GD3.5 antigen and WC1 was used to further distinguish these antigens. Cells were incubated with α-chymotrypsin for 1hr at 37°C and then FACS analysis was done. Figure 7 demonstrates that GD3.5 antigen was very sensitive to low-dose chymotrypsin. GD3.5 antigen mean fluorescence, indicated by the cross-hatched bars, consistently declined as the concentration of chymotrypsin was increased from 62.5 to 250 mU/ml, illustrating dose-response characteristics. In contrast, WC1 (solid bars) mean fluorescence remained similar to control levels (no chymotrypsin) regardless of chymotrypsin concentration.

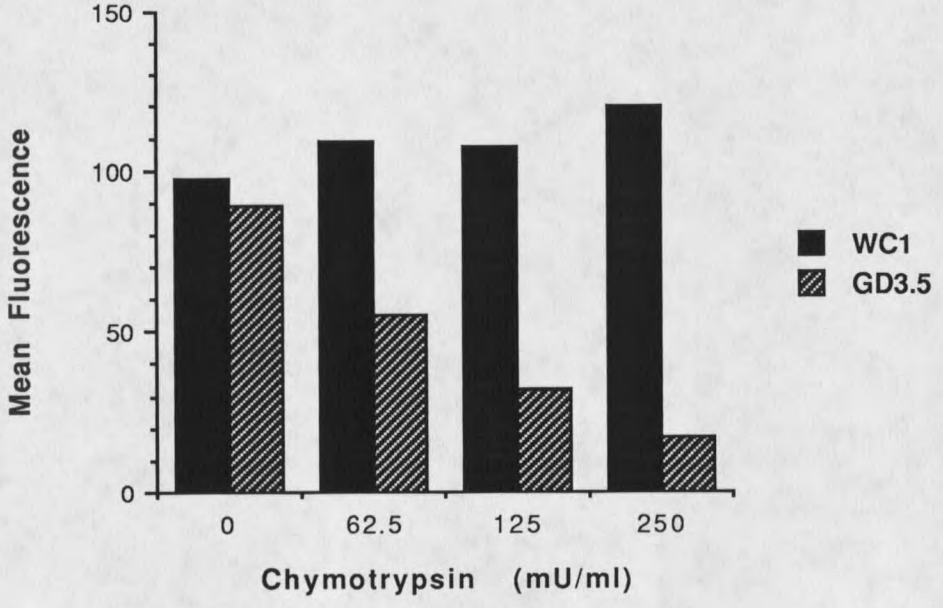

Figure 7. Effects of low-dose chymotrypsin on cell surface expression of WC1 and GD3.5 antigen. Purified lymphocytes were treated with 0-250mU α-chymotrypsin/$1x10^7$ cells for 1hr at 37°C and analyzed by FACS for GD3.5 antigen and WC1 expression. GD3.5 antigen is indicated by cross-hatched bars and WC1 by solid bars. Figure 7 is representative of three separate experiments indicating average mean fluorescence of duplicate samples.

Therefore, crossprecipitation analysis, V-8 protease digestion, enzymatic deglycosylation, and cell surface chymotrypsin sensitivity conclusively show that the GD3.5-reactive antigen is clearly distinct from the molecules recognized by IL-A29, even though under reducing conditions the smaller Mr WC1 species is similar to GD3.5 antigen by size. Since IL-A29 is thought to recognize all WC1 species, our results suggest that GD3.5 represents a completely distinct lineage-specific antigen or a unique WC1 family member not recognized by IL-A29.

Discussion

The identification and characterization of γδ T cell-specific surface molecules will be useful in understanding the biology and function of this cell type. In this report, we have demonstrated a novel γδ T cell-specific marker (GD3.5 antigen), which is distinct from both TCR and other known WC1 species. GD3.5-reactive lymphocytes represent a distinct γδ T cell-specific population in comparison to IL-A29-reactive WC1. In addition, GD3.5 stains a major subset of γδ TCR-expressing cells.

Although GD3.5 antigen and IL-A29-reactive WC1 share similar migratory patterns under reducing conditions, they represent distinct molecules. Indeed, because of the similarity in Mr of the GD3.5 antigen and WC1 under reducing conditions, we did an exhaustive analysis of the biochemical nature of the antigens. The most direct evidence supporting our conclusion are the crossprecipitation studies in which we show that GD3.5 antigen can be precipitated from a lysate cleared of IL-A29-reactive WC1 and vice versa. Analysis of the protease and glycosidase sensitivities of GD3.5 antigen further confirmed the molecular differences between the GD3.5 antigen and IL-A29-reactive WC1 species. From our studies, it is clear that GD3.5 antigen does not represent one of the IL-A29-reactive WC1 species. In addition, preclearing lysates

with three different WC1 mAbs and then crossprecipitating with GD3.5mAb did not diminish the immunoprecipitation of GD3.5Ag further suggesting that GD3.5Ag is not a known WC1 isotype (data not shown and see chapter five).

Of particular interest is the analysis of the glycosylation patterns of the GD3.5 antigen and WC1. As described in the results section, GD3.5 antigen can be distinguished from WC1 when comparing treatment of these molecules with O-sialoglycoprotease, PNGase F, and neuraminidase (Figure 6). Both molecules are sensitive to O-sialoglycoprotease, suggesting that these molecules possess O-linked carbohydrates with sialic acid residues which is characteristic of mucin-like molecules (33). Our study represents the first demonstration that WC1 may represent a family of mucin-like molecules on γδ T cells. This is important information, which may give clues to the potential function of both molecules. For example, it has been previously reported that a T-lymphocyte ligand and myeloid PSGL-1, which mediate adhesion to P-selectin, possess O-linked carbohydrates that are sensitive to O-sialoglycoprotease treatment, which eliminates binding to P-selectin (34,35). We have previously shown that γδ T cells bind P-selectin via an unknown receptor that is sensitive to O-sialoglycoprotease (36). Additionally, we found that O-sialoglycoprotease treatment of γδ T cells inhibits their binding to another selectin (L-selectin) which is important in regulating leukocyte/leukocyte as well as leukocyte/endothelial cell interactions (36). Collectively, our data as well as the data of others have shown that mucins are important in a variety of aspects related to the trafficking of lymphocytes within the animal. Other mucins, such as CD45, are involved in cell functions likely separate from lymphocyte trafficking. Therefore, our demonstration of the sensitivity of WC1 and the GD3.5 antigen to O-sialoglycoprotease provides important information for future functional analyses of both molecules.

Earlier reports showed that treatment of neutrophils with low-dose chymotrypsin

leads to loss of L-selectin, inhibits neutrophil binding to high endothelial venules, and reduces the capacity of the cells to home to sights of inflammation (32). We have shown that GD3.5 antigen is very sensitive to low-dose chymotrypsin, exhibiting sensitivity greater than even L-selectin. Interestingly, P-selectin ligands also exhibit exquisite sensitivity to proteases (37-39). Based on our chymotrypsin and O-sialoglycoprotease results, we have pursued preliminary functional analyses of the GD3.5 antigen in the context of selectin binding. We have found that the low-dose chymotrypsin treatment that reduces GD3.5 antigen expression abrogates binding of γδ T cells to activated endothelial cells and platelets (see chapter three). In addition, the same concentration of chymotrypsin abrogates binding of human P-selectin chimera (40) to bovine γδ T cells by FACS analysis (data not shown). Thus, it is possible that the GD3.5 antigen represents a P-selectin ligand on γδ T cells. If GD3.5 antigen does represent a P-selectin ligand, then it is somewhat unique from PSGL-1 since it does not demonstrate a shift of 110kD under reducing conditions like the myeloid molecule (41,42).

Even though WC1 was identified approximately 10 years ago, a specific function for the molecule has not been conclusively defined. Some reports have suggested that it may also be involved in the trafficking of γδ T cells (4,23,26,28). Cloning of cDNAs encoding the various WC1 species has not cleared up the issue of function. Here we show that WC1 is quite sensitive to O-sialoglycoprotease treatment. Using the same line of reasoning as used for GD3.5 antigen, we support the hypothesis that WC1 is involved in trafficking, but that the molecular interaction will be distinct from GD3.5 antigen and its potential receptor. This latter conclusion is based on the clear molecular differences in the 2 molecules and that WC1 is not affected by low-dose chymotrypsin treatment. It is possible that WC1 could bind other selectins, such as E-selectin, which we have previously shown is bound by γδ T cells via a low-dose chymotrypsin-resistant surface antigen on the lymphocyte (29), or it could mediate binding to other lectin-like

molecules.

In summary, GD3.5 antigen is clearly distinct from IL-A29-reactive WC1 as described using the various techniques presented above, thus, revealing a second lineage-specific γδ T cell marker. Interestingly, GD3.5 and WC1 are sensitive to O-sialoglycoprotease treatment suggesting that these molecules possess sialomucin-like characteristics similar to other selectin ligands. In Chapter 3, I will present data that will address the regulation and potential function of GD3.5Ag.

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CHAPTER 3

REGULATION AND FUNCTIONAL CHARACTERIZATION OF A NOVEL γδ T CELL-SPECIFIC SURFACE ANTIGEN (GD3.5)

Introduction

A specific function of γδ T cells has continued to elude researchers for over a decade now. Understanding the tissue distribution of γδ T cells may lend some clues to their function. In ruminants, γδ T cells comprise 60-80% of the peripheral blood lymphocyte (PBL) population (1,2), and they do not appreciably accumulate in the organized lymphoid tissues, such as the lymph node (1,3-5). However, studies have demonstrated considerable homing of bovine γδ T cells into epithelial tissues (1,5,6). In addition, many reports have demonstrated homing of γδ T cells into nonlymphatic tissues in the mouse. In these reports, γδ T cells have been found in association with mucosal epithelia of the gut, tongue, and vaginal areas, as well as the skin (7-10). Interestingly, tissue localization seems to correlate with TCR gene usage in some cases (9-12). For example, skin γδ T cells appear to present limited γδ TCR Vγ and Vδ genes almost to the point where the population is oligoclonal or even monoclonal (9,12). Therefore, localization may either be linked to or correlate with TCR gene usage (9,12). Since TCR gene usage seems to "direct" specific γδ T cell subpopulations to various epithelial tissues where invading pathogens might first be encountered, it has been

hypothesized that γδ T cells play a major role in "the first line of defense" against these pathogens.

It has been shown that γδ T cells can respond and migrate into sites of inflammation (13-18). For example, γδ T cells accumulate in the intestinal mucosa upon infection with *Cryptosporidium parvum* (13). Using function-blocking mAb to E-selectin, migration of γδ T cells into sites of inflammation caused by PPD, TNF, LPS, and PHA can be inhibited indicating that selectins are involved in transmigration of γδ T cells into sites of inflammation (14, M. A. Jutila, unpublished observations). Furthermore, in vitro studies have shown that rolling of γδ T cells on activated endothelial cells and platelets can be blocked by anti-E- and P-selectin mAbs, indicating that initial adhesion of γδ T cells in sites of inflammation can be mediated by selectins (19). Finally, it has been shown that γδ T cells interact with chronically activated endothelium independent of E- or P-selectin, suggesting the use of alternative or novel cell adhesion pairs in chronic inflammation (20,21).

Previously, we biochemically defined a novel γδ T cell lineage-specific cell-surface molecule termed GD3.5Ag (24). Here, we describe the regulation and potential function of this molecule as an adhesion protein. Stimulatory compounds and various enzymes have been used to study the biochemical and regulatory characteristics of cell-surface adhesion molecules. For example, a known leukocyte adhesion molecule, L-selectin, is downregulated upon treatment with phorbol esters (14). In addition, L-selectin and P-selectin glycoprotein ligand-1 (PSGL-1) are sensitive to low-dose protease treatment (22,23). We showed that GD3.5Ag expression is downregulated upon mitogen activation and expression returns upon loss of stimulation. Also, a large population of γδ T cells in the spleen express lower levels of GD3.5Ag and WC1 when compared to peripheral blood γδ T cells. In addition, chymotrypsin treatment of γδ T cells results in a dose-dependent loss of their ability to roll on 24hr activated endothelium. Lastly, pretreatment of

bovine γδ T cells with anti-GD3.5Ag polyclonal serum inhibits accumulation of these cells on 24hr activated endothelium.

Materials and Methods

Lymphocyte purification, γδ T cell isolation, and biotin labeling

Blood was obtained by jugular venipuncture from Holstein calves housed at the Montana State University Large Animal Facility. Lymphocytes were purified by Histopaque gradient centrifugation (Sigma Chemical Co., St. Louis, MO). Lymphocytes were resuspended in HBSS and platelets were removed by Percoll gradient centrifugation (Sigma Chemical Co., St. Louis, MO) with subsequent incubation of lymphocytes in T175 flasks for 30min at 37^{o}C to allow monocytes to adhere and be removed from suspension. γδ T cell lymphocytes were purified from whole lymphocytes by incubation for 30min at room temperature (RT) in T175 flasks coated with 7 x 10^6 adherent E-selectin L-cell transfectants. Remaining unbound cells were discarded and bound γδ T cells lifted with 2mM EDTA (14). This purification protocol provided >90% pure γδ T cells (14). In some experiments, γδ T cells were then surface labeled with biotin. Briefly, 1 x 10^8 cells were washed 3 times with sterile PBS and resuspended in 1ml of NHS-LC-Biotin/PBS (550ug/ml Pierce, Rockford, IL). After a 30min incubation at RT, cells were washed 3 times with PBS and lysed for 30min on ice with NP-40 lysis buffer (2% NP-40, 100mM NaCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 5mM NaN_3, 10mM Hepes, and protease inhibitors: pepstatin A, 1,10-phenanthroline, phenylmethylsulfonyl fluoride, benzamidine, antipain, leupeptin, and chymostatin (Sigma Chemical Co., St. Louis, MO). Rabbit serum (5% v/v) was added to all lysates for 1hr at RT and then precleared with protein G beads (Boehringer Mannheim Biochemicals, Indianapolis, IN) overnight at 4^{o}C with constant rotation.

Beads were subsequently removed and lysates were either used immediately or frozen at -80°C.

mAbs, polyclonal antibodies, and FACS analysis

Monoclonal antibodies included IL-A29 (anti-WC1), GD3.5, and GD3.8. GD3.5 is a non-TCR, lineage-specific, γδ T cell antibody that recognizes a 220-240kD glycoprotein on the cell surface (24). GD3.8 is against the γδ TCR and is a pan γδ T cell marker (Wilson, E., et al., manuscript in preparation). Both antibodies were generated, as described previously (24,25). The GD3.5 antigen was purified by affinity column chromatography. Briefly, GD3.5 mAb was conjugated to protein G beads per manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN). Bovine lymphocyte lysates were incubated with the column matrix, washed, and bound GD3.5Ag was eluted with 0.5M NaCl, 0.1M glycine, pH 2.5 elution buffer and immediately neutralized with 10% 1.5M pH 8.8 Tris buffer. Purified antigen was concentrated and anti-GD3.5 polyclonal antibody was generated in BALB/C mice. Briefly, mice were first injected i.p. with GD3.5Ag mixed with Gerbu Adjuvant (Biotech, Poway, CA). Two weeks later, the mice were injected a second time with purified GD3.5Ag alone and one week after the second injection, the mice were bled and their serum analyzed by flow cytometry. Single-color FACS was performed on ice as follows: cells were incubated with IL-A29, GD3.5 or GD3.8 for 30min, washed, incubated with appropriate PE- or FITC conjugated goat α-mouse secondary antibody (Jackson ImmunoResearch, West Grove, PA), washed again, and analyzed on a BD FACscan or FACScalibur (Becton Dickinson). Two-color FACS analysis was performed on ice as follows: cells were incubated with anti-GD3.5 polyclonal serum (1/100) for 30min, washed, and incubated with appropriate PE-conjugated goat α-mouse secondary Ab (Jackson ImmunoResearch). Cells were blocked for 10min with 10% mouse serum and

subsequently washed, followed by incubation with FITC-labeled GD3.8 mAb for 30min, washed again, and analyzed on a FACscan or FACScalibur (Becton Dickinson). Control cells were incubated with preimmune mouse serum for 30min and treated, as stated above.

Tissue distribution of bovine γδ T cells

Tissues were obtained from control Holstein calves housed at the Montana State University Large Animal Facility. Prior to sacrifice, 15mls of peripheral blood were obtained, as described above. Immediately postmortem, a portion of spleen, thymus, mesenteric lymph node (MesLN), prescapular lymph node (PreLN), and flanking lymph node (FlaLN) were removed and placed in cold HBSS. Tissues were cut into small pieces after removal of excess connective tissues. Several pieces were placed into tissue grinders and single cell suspensions were made by gently moving the plunger up and down inside the glass cylinder. Cells were passed through Nitex filters to remove large particulates, resulting in single cell suspensions, which were used in single-color FACS analyses, as described above.

Immunoprecipitation/Crossprecipitation

Crossprecipitation experiments were performed as follows. Separate biotinylated lysates were precleared of Workshop Cluster 1 (WC1) or GD3.5 antigen by incubation with 20ug of either IL-A29 or GD3.5 mAb for 1hr at room temperature and then immunoprecipitated with protein G beads at 4^{o}C overnight . After bead removal, WC1 and GD3.5 antigen cleared lysates were incubated a second time with 15ug of IL-A29 or GD3.5 mAb, respectively, and immunoprecipitated a second time, as described above. Three subsequent clearing steps with protein G beads were performed on the lysates to ensure removal of all Ab/Ag complexes. Anti-GD3.5 polyclonal serum was used to

immunoprecipitate lysates cleared of either GD3.5 antigen or WC1. Polyclonal Ab/Ag complexes were precipitated with protein G beads, as described above. The beads were washed 3 times with wash buffer (0.1% NP-40, 100mM NaCl, 1mM $CaCl_2$, 1mM $MgCl_2$, 5mM sodium azide, and 10mM Hepes), mixed with nonreducing loading buffer and loaded onto 8% SDS-polyacrylamide gels (24). Gels were electrophoresed and transferred to PVDF membranes (Bio-Rad, Hercules, CA). After transfer, membranes were washed briefly with 1x TBST (10mM Tris pH 8.0, 150mM NaCl, and 0.05% Tween 20) and then blocked with 7% BSA/TBST for 2hrs at RT. Membranes were then washed with several changes of 1x TBST for 1.75hrs. Streptavidin horseradish peroxidase conjugate (Amersham Life Science, Buckinghamshire, England) was diluted 1/5000 in sterile PBS and incubated with the membrane for 40min and then washed for 1.75hrs with several changes of 1x TBST. ECL detection reagents were used according to manufacturer's recommendations (Amersham Life Science, Buckinghamshire, England) and incubated with the membranes for 1 min. Membranes were then covered with cellophane, exposed to X-OMAT film for 1-10 minutes, and the film was developed (Kodak, Rochester, New York).

Mitogen stimulation of total lymphocyte populations

Bovine lymphocytes were prepared by Histopaque (Sigma Chemical Co., St. Louis, MO) separation and incubation on plastic, as described earlier. Cells (3×10^7) were incubated in 15mls 10% cRPMI in T75 flasks at 37°C. Cells were either sham-treated (control) or treated with 2ug/ml Concanavalin A (ConA) or 2ug/ml Phytohemagglutanin (PHA) (Sigma Chemical Co.). Cells were removed at 0, 24, 48, 72, and 144hr time points, and single-color FACS analysis was performed, as described earlier.

In vitro low-shear adhesion assay

Bovine umbilical vein endothelial cells (BUVECs) and human umbilical vein endothelial cells (HUVECs) were plated in 8-well Lab-Tek slides (Nunc Inc., Napperville, IL) and allowed to adhere overnight. Subconfluent BUVEC monolayers or HUVEC monolayers were then activated with 500U/ml TNF or 10ng/ml IL-1B, respectively, for four hours at 37°C. Purified bovine lymphocytes (1×10^6) were either untreated or pretreated with GD3.5 mAb supernatant fluid for 30min at room temperature. Treated or untreated lymphocytes were added to each well and allowed to adhere for 30min with gentle shaking. After incubation, the slides were gently washed in PBS and the cells were fixed in glutaraldehyde/HBSS. The number of lymphocytes bound/BUVEC or HUVEC were counted in three separate fields/well.

In vitro capillary tube shear-dependent rolling assay

The shear-dependent assay has been described previously (19,26-28). Briefly, BUVECs, HUVECs, or E-selectin L-cell transfectants were grown to confluency on the inside surface of 1.4-mm (i.d.) capillary tubes (Drummond Scientific, Broomall, PA). BUVECs were treated with bovine IFN-γ for 20hrs with subsequent incubation using bovine TNF-α for 4hrs. In addition, BUVECs were sometimes activated with 100ng/ml LPS for 24hrs (Sigma Chemical Co., St. Louis, MO). HUVECs were treated with either 10ng/ml rhIL-1β or 100ng/ml LPS for 24hrs. Platelets were adhered to the glass capillary tubes as follows: Capillary tubes were pretreated with Cel-Tak (Collaborative Biomedical Products) for 30min at 37°C to facilitate platelet binding to the inside surface of the tube. Purified human platelets (1.5×10^9/ml) were activated with 1U/ml bovine thrombin, injected into the capillary tube, and allowed to adhere for 10min.

The capillary tubes were integrated into the closed recirculating loop system as described previously (19,26-28). Purified bovine lymphocytes or γδ T cells were rolled on endothelial cells or platelets at 2-3 dynes/cm^2 as predicted by the Hagen-Poisueille equation. Video microscopy was used to record endothelial cell/lymphocyte or platelet/lymphocyte interactions allowing individual frame analysis every 60 seconds using macro-driven National Institutes of Health Image software and a Macintosh 660 AV computer.

Various antibodies and anti-GD3.5 polyclonal sera were tested to determine their effect on cell/cell interactions. Purified γδ T cells were pretreated with anti-GD3.5 polyclonal antibody for 1hr at room temperature and infused into the loop assay system. Monoclonal and polyclonal antibody effects were analyzed, as describe above, to determine the effects on existing as well as new cell/cell interactions.

Results

Distribution of γδ T cells in organized lymphatic tissue

FACs analysis was performed on single cell suspensions from lymphoid tissues and peripheral blood to determine γδ T cell distribution and expression of WC1 and GD3.5Ag (Figure 8). As expected, γδ T cells represented a large percentage (46 +/- 7.1) of the total peripheral blood lymphocyte population. GD3.5 and IL-A29 mAbs stained approximately 95% and 60% of the total γδ T cell population, respectively, as reported earlier (24). γδ T cells represented approximately 10% of the total lymphocyte population in peripheral and mesenteric lymph nodes. In contrast, spleen lymphocyte populations were comprised of approximately 55% γδ T cells. Expression of GD3.5Ag and WC1 was markedly reduced on the splenic γδ T cells when compared to peripheral

blood; GD3.5 and WC1 mAbs stained 40% and 27% of the splenic γδ T cell population, respectively. Finally, approximately 10% of the lymphocytes in the thymus were γδ T cells and approximately 20% of these cells expressed GD3.5Ag and WC1.

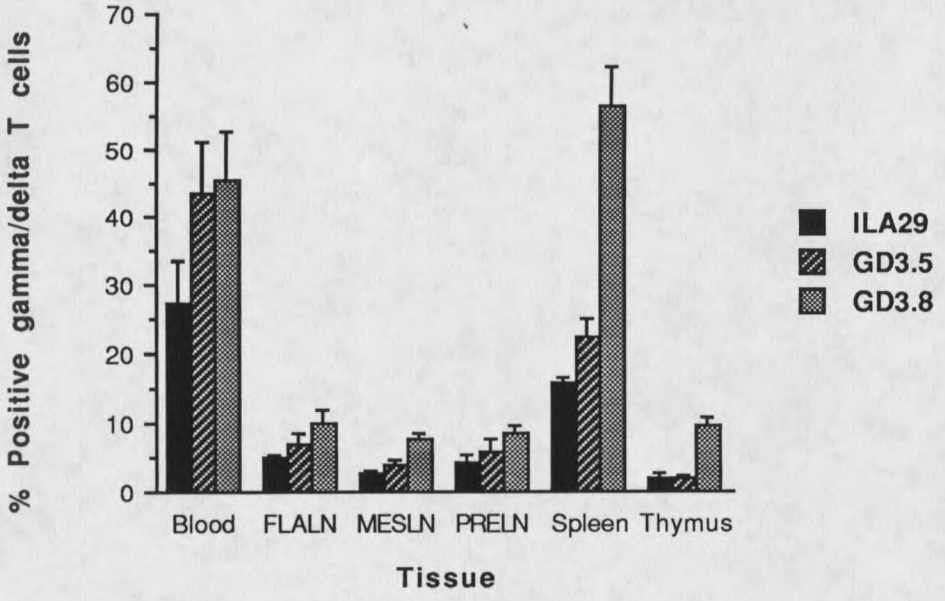

Figure 8. γδ T cells found in organized lymphatic tissues express varying levels of GD3.5Ag. Blood, thymus, spleen, and lymph nodes were obtained from bovine calves and single cell suspensions analyzed for WC1, GD3.5Ag, and γδ TCR expression by flow cytometry, as described in Materials and Methods. Solid bars, hatched bars, and crosshatched bars represent percent of lymphocytes expressing WC1, GD3.5, or TCR, respectively in the indicated tissue. Percentages are an average of four or five experiments +/-SEM.

Mitogen stimulation of bovine lymphocyte populations

Since GD3.5Ag and WC1 were downregulated in the spleen, we tried to reproduce this change in vitro. Specifically, we wanted to determine if overt signalling events, initiated by phorbol esters, mitogens, or calcium ionophores, would affect expression of these antigens in vitro. In addition, we wanted to know if these surface expressed molecules were regulated similarly to adhesion molecules, such as L-selectin, when

treated with these compounds. When lymphocytes were activated with mitogens, they increase in size forming blasts, which could be seen when analyzing their distinctive forward light-scatter profiles. Of interest, by 144hrs of incubation with mitogens, the lymphocytes diminished in size, indicating that the stimulus had subsided (data not shown). In Figure 9a, activation of bovine lymphocytes with ConA resulted in a continual increase in the percentage of the γδ T cell subset from 0-144hrs. GD3.5Ag expression dropped dramatically at 48hrs and was lowest at 72hrs. In contrast, WC1 did not exhibit a precipitous drop in expression, as seen with GD3.5Ag. Of interest, the ConA-treated cells began to return to a resting state by 144hrs, which resulted in a recovery of GD3.5Ag expression. Figure 9b shows that treatment of bovine lymphocytes with PHA resulted in the downregulation of both GD3.5Ag and WC1. Interestingly, WC1 was downregulated more rapidly (48hrs) than GD3.5Ag (72hrs), further distinguishing these antigens. As with ConA, the percentage of the γδ T cell subset increased from 0-144hrs of culture. Expression of WC1 and GD3.5Ag recovered at different rates when the stimulus subsided. Specifically, WC1 expression recovered more rapidly than GD3.5Ag expression. Treatment of lymphocytes with ionomycin, PMA or a combination of ionomycin and PMA did not result in any detectable changes in GD3.5Ag or WC1 expression.

Effect of GD3.5 mAb on established lymphocyte rolling or tight adhesion to 4hr activated endothelium

Since GD3.5Ag possesses biochemical and regulatory characteristics that are consistent with other known adhesion molecules, we focused our functional analyses on cell adhesion. First, we tested the ability of GD3.5 mAb to block lymphocyte adhesion to activated endothelial cells in a low-shear stress assay. Rolling and tight adhesion are indistinguishable in these assays. Thus, blocking could occur during initial adhesive

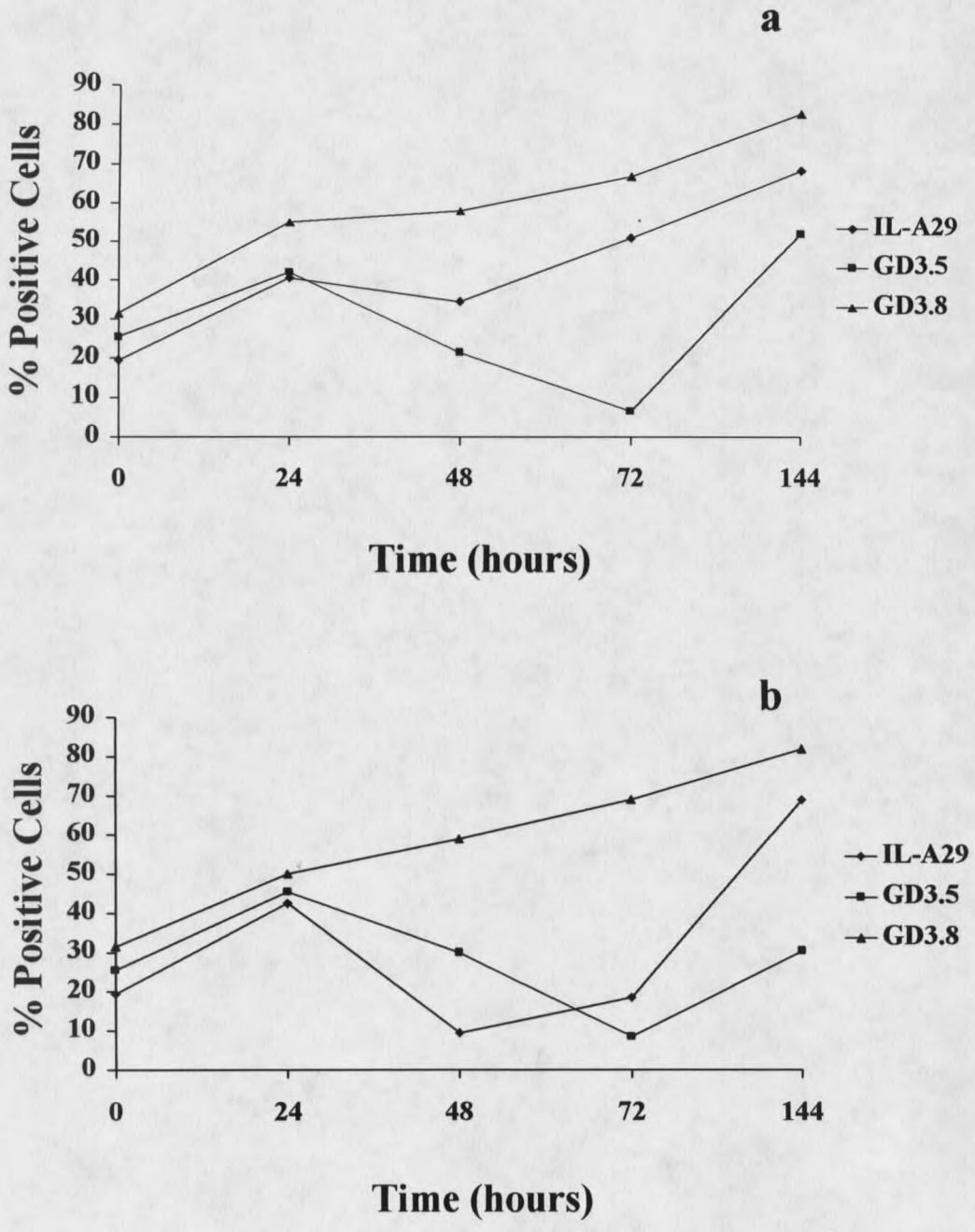

Figure 9. Mitogen stimulation differentially downregulates cell surface expression of GD3.5Ag and WC1. Purified bovine lymphocytes were activated with (a) 2ug/ml ConA or (b) 2ug/ml PHA. Cells were analyzed by flow cytometry for WC1, GD3.5Ag, and TCR expression at 0, 24, 48, 72, and 144hrs. Figure 9 is representative of at least three separate experiments.

events or tight adhesion. BUVECs and HUVECs were adhered to slides and activated for 4hrs, as described in Materials and Methods. Bovine lymphocytes were purified and either untreated or pretreated for 30min with GD3.5 mAb supernatant fluid. As shown in Figure 10a and 10b, GD3.5 mAb did not block adhesion to BUVECs or HUVECs.

In the past, we have used activated platelets and E-selectin-expressing mouse L-cell transfectants to study E- and P-selectin interactions (28). Here, we decided to test whether GD3.5 mAb would specifically block bovine lymphocyte interactions with E- or P-selectin. For P-selectin assays, platelets were activated for 10min and adhered to the inside surface of glass capillary tubes and integrated into the loop assay system. For E-selectin assays, E-selectin transfectants were adhered to the inside surface of glass capillary tubes and integrated into the loop assay system. After allowing the bovine lymphocytes to establish an interaction with either E- or P-selectin, 100ug/ml of control and GD3.5 mAbs were sequentially injected. As seen in Figure 11a and b, injection of control and GD3.5 mAb did not inhibit lymphocyte interactions with P- or E-selectin.

Effect of pretreating bovine lymphocytes with GD3.5 mAb on their interaction with 24hr activated endothelium

Since GD3.5 mAb did not inhibit lymphocyte adhesion to 4hr activated endothelium or to E- or P-selectin, we decided to pursue alternative adhesion assays. Lymphocytes have been shown to adhere to 24hr activated endothelium independent of E- and P-selectin (20,21). Therefore, we allowed bovine lymphocytes to interact with 24hr activated BUVECs (as described in Materials and Methods), and then sequentially injected 100ug/ml of control and GD3.5 mAbs to determine their affect on lymphocyte/endothelial cell interactions. As seen in Figure 12, injection of control or GD3.5 mAb did not inhibit lymphocyte/endothelial interactions.

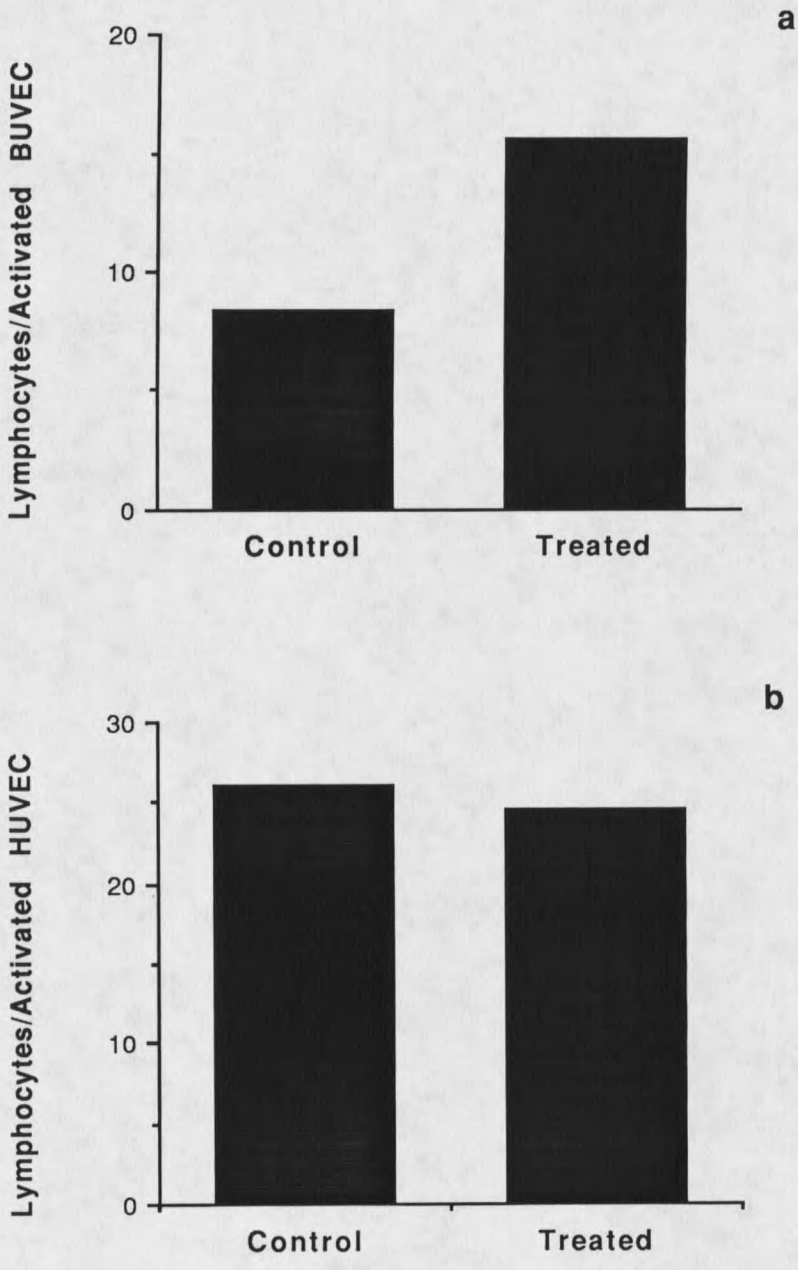

Figure 10. GD3.5 mAb does not block lymphocyte adhesion to 4hr activated BUVECs or HUVECs. a) Lymphocytes were untreated or pretreated with GD3.5 mAb supernatant fluid and allowed to adhere to 4hr activated BUVECs. b) Lymphocytes were untreated or pretreated with GD3.5 mAb supernatant fluid and allowed to adhere to 4hr activated HUVECs. The number of adherent cells/BUVEC or HUVEC were counted in three separate microscopic fields/well and the average was recorded.

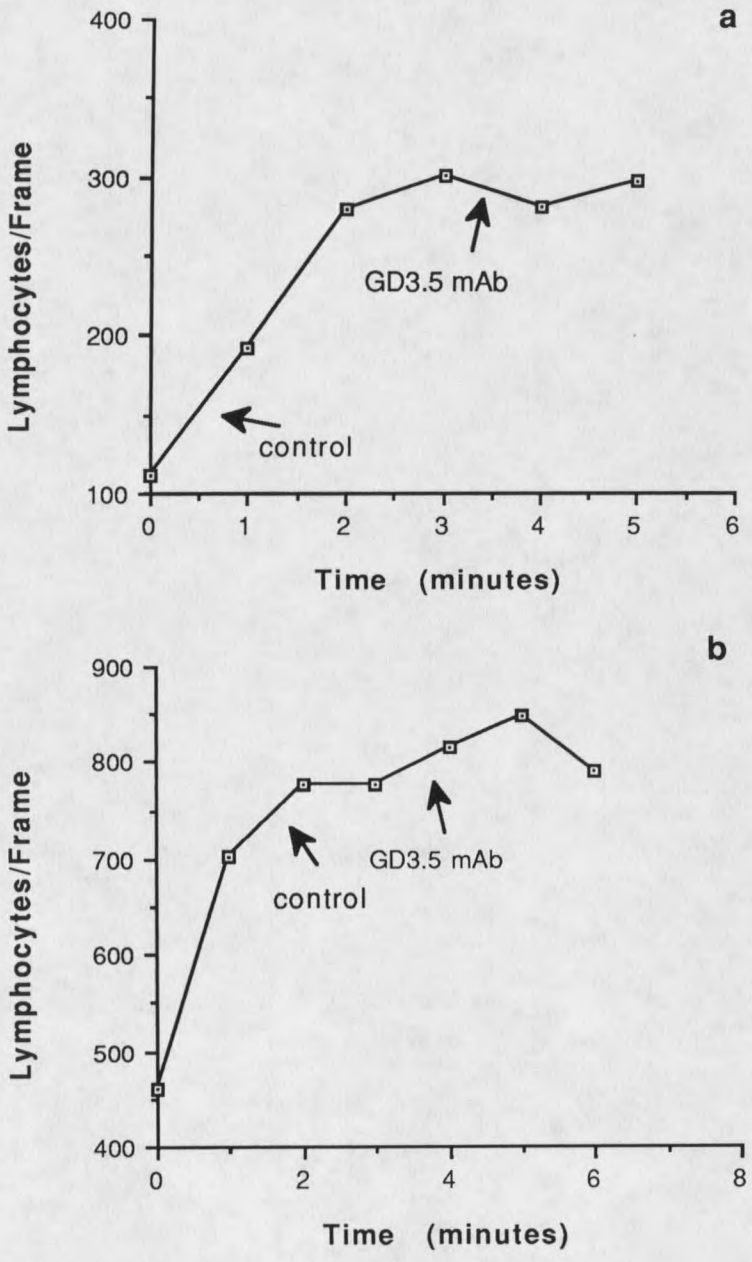

Figure 11. GD3.5 mAb does not block lymphocyte binding to E- or P-selectin. a) Bovine lymphocytes were allowed to interact with activated platelets and then control and GD3.5 mAbs were injected to determine their effect on lymphocyte/P-selectin interactions. b) Bovine lymphocytes were allowed to interact with E-selectin mouse L-cell transfectants and then control and GD3.5 mAbs were injected to determine their effect on lymphocyte/E-selectin interactions. The time of antibody injection is indicated by the arrows.

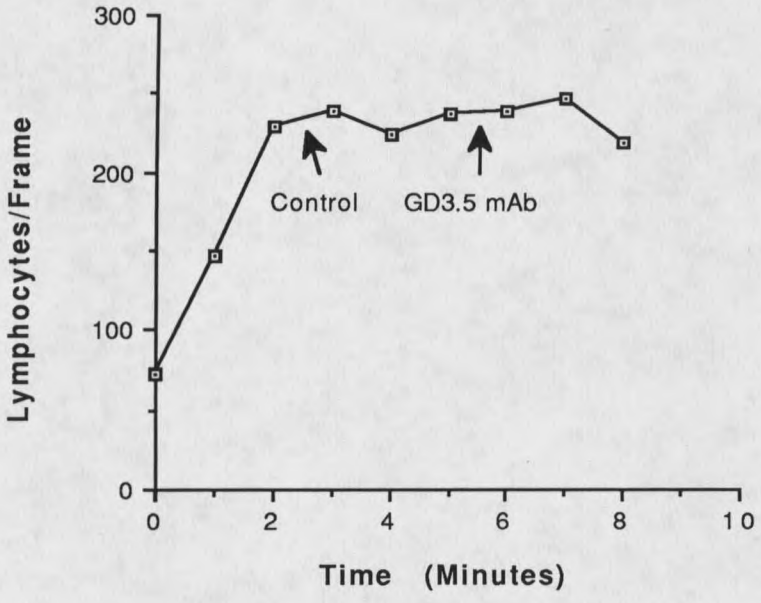

Figure 12. Injection of GD3.5 mAb does not block lymphocyte binding to 24hr activated endothelium. Bovine lymphocytes were allowed to interact with activated BUVECs and then control and GD3.5 mAbs were injected to determine their effect on lymphocyte/endothelial cell interactions. The time of antibody injection is indicated by the arrows.

Next, we determined if pretreatment of the lymphocytes with antibody would affect their ability to interact with 24hr activated endothelium. Bovine lymphocytes were pretreated with either 150ug/ml of GD3.8 or GD3.5 mAbs and integrated into the loop assay, as described above. As seen in Figure 13, lymphocyte treatment with GD3.5 mAb inhibited lymphocyte interactions with 24hr activated endothelium when compared to the GD3.8-treated controls. Thus, GD3.5 antibody appeared to inhibit $\gamma\delta$ T cell/endothelial cell interactions. Of interest, in some experiments, untreated cells were not significantly different than GD3.5 mAb-treated cells.

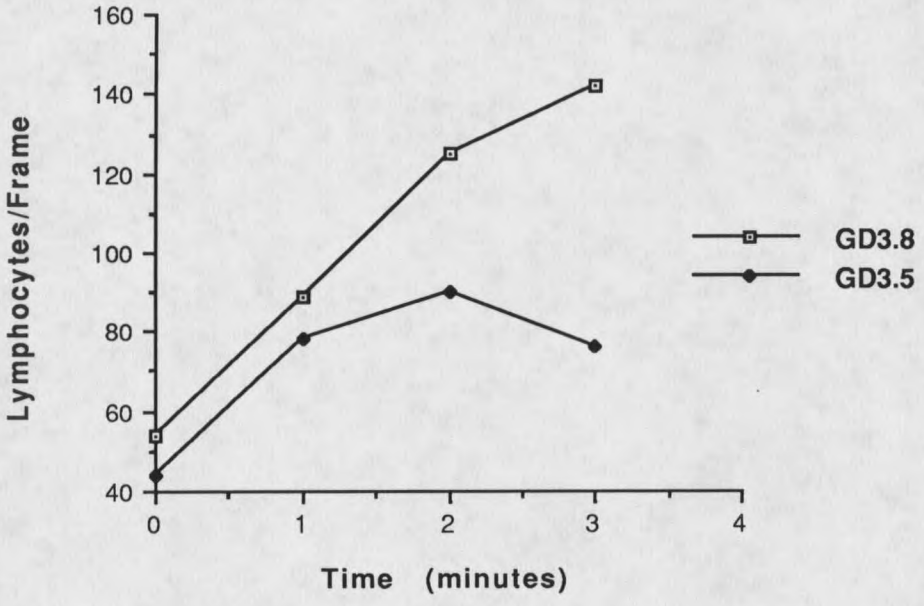

Figure 13. Pretreatment of lymphocytes with GD3.5 mAb blocks lymphocyte interactions with 24hr activated endothelium. Lymphocytes were pretreated with 150ug/ml of either GD3.8 or GD3.5 mAb and then infused into the in vitro shear-dependent assay to assess lymphocyte interactions with 24hr activated BUVEC monolayers. Data were analyzed, as described above, and recorded as the number of cells/frame at one minute intervals. Cells treated with GD3.8 or GD3.5 mAb are as marked.

Chymotrypsin-treated bovine lymphocyte interactions in adhesion assays

Previously, we reported that treatment of bovine γδ T cells with increasing concentrations of α-chymotrypsin resulted in a concomitant loss of GD3.5Ag on the cell surface (24). Therefore, we tested whether chymotrypsin-treated bovine lymphocytes would interact in any of our adhesion assays. Since protease treatment results in the loss of GD3.5 expression on the cell surface, we surmised that if GD3.5Ag represented an E- or P-selectin ligand, then loss of expression would inhibit lymphocyte rolling on E- or P-selectin. For E-selectin assays, E-selectin transfectants were grown on the inside

surface of glass capillary tubes and integrated into the loop assay. For P-selectin assays, platelets were activated for 10min and adhered to the inside surface of glass capillary tubes and integrated into the loop assay system. Bovine lymphocytes were treated with 1U/ml α-chymotrypsin or sham treated for 1hr at 37°C, resuspended in DMEM/20mM Hepes and infused into the assay; cell/cell interactions were assessed, as described above. As shown in Figure 14a, chymotrypsin-treated lymphocytes rolled as well or better on E-selectin, suggesting that lymphocyte/E-selectin interactions were not dependent upon chymotrypsin-sensitive molecules, such as GD3.5Ag. In addition, we have previously shown in the low-shear assay that chymotrypsin treatment of bovine lymphocytes does not block binding of cells to E-selectin transfectants (14). In Figure 14b, we show that lymphocyte rolling on P-selectin was inhibited by chymotrypsin treatment when compared to control. Bovine lymphocyte E- and P-selectin ligands are further characterized in Chapter 4.

Next, we wanted to determine if chymotrypsin removal of GD3.5Ag from the cell surface would affect lymphocyte rolling on 24hr activated endothelium. Cells ($1x10^7$) were resuspended in HBSS with Ca^{2+} and Mg^{2+} and 0, 62.5, 125, or 250 mU α-chymotrypsin/ml and incubated for 1hr at 37°C. After completion of incubation, cells were washed and resuspended in DMEM/20mM Hepes for use in the rolling assays. In addition, treated and control cells were analyzed by single-color, indirect FACS analysis for GD3.5 antigen expression. FACS analysis of the chymotrypsin-treated cells demonstrated that, in fact, GD3.5Ag expression decreased with increasing concentrations of α-chymotrypsin (Figure 15a, see also Figure 7). Figure 15b shows a representative experiment demonstrating rolling of chymotrypsin-treated γδ T cells on 24hr activated BUVECs. As γδ T cells were treated with increasing concentrations of chymotrypsin (0-250mU/ml), they exhibited a stepwise loss in ability to interact with the activated

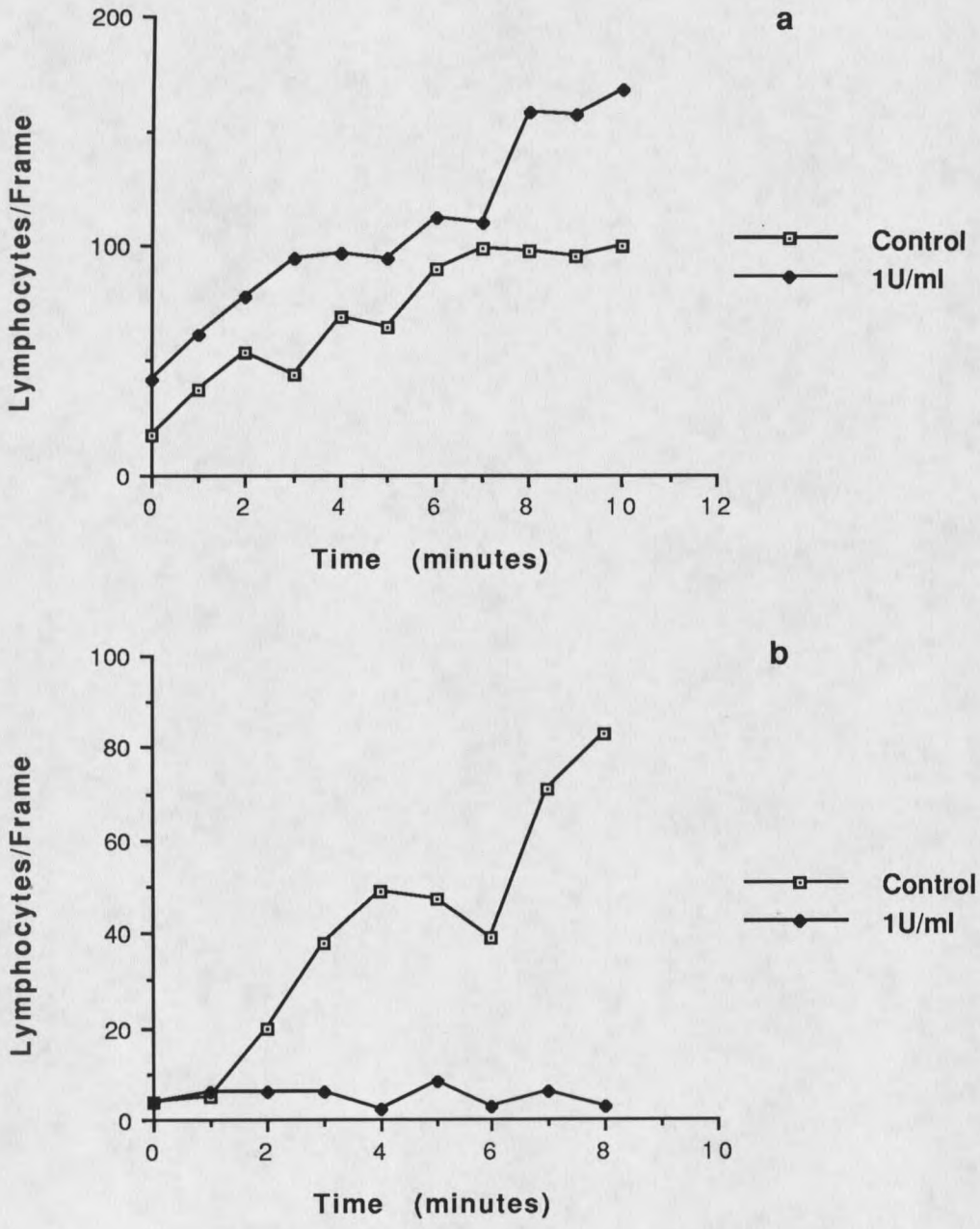

Figure 14. Loss of GD3.5Ag expression inhibits lymphocyte rolling on P-selectin, but not E-selectin. Chymotrypsin-treated or mock-treated bovine lymphocytes were allowed to adhere to E-selectin transfectants (a) or activated platelets (b) in the shear-dependent adhesion assay. The number of cells interacting with E- or P-selectin was assessed, as described in Materials and Methods. Control and treated are as marked.

endothelium, which correlated well with loss of expression on the cell surface. Importantly, bovine lymphocyte rolling on 24hr activated HUVEC monolayers revealed similar results (data no shown).

Characterization of polyclonal antibodies against GD3.5Ag

To further investigate GD3.5 function, we raised polyclonal sera against purified GD3.5Ag (see Materials and Methods). Mice were injected two separate times with purified GD3.5Ag and then bled for screening of antibody titers against bovine γδ T cells. To test serum titers, mouse serum was serially diluted (1/10, 1/100, and 1/1000) and tested by FACS analysis. Dilution of the mouse serum (1/100) resulted in intense staining of bovine γδ T cells (Figure 16). Clearly, purification of GD3.5Ag and injection into the mice, as described in the Materials and Methods, resulted in polyclonal antibodies that specifically stained bovine γδ T cells, indicating that our antigen purification procedure worked well. Also, the polyclonal serum, which contains antibodies against multiple GD3.5 epitopes, specifically stained γδ T cells, further supporting the idea that GD3.5Ag is a lineage-specific γδ T cell marker.

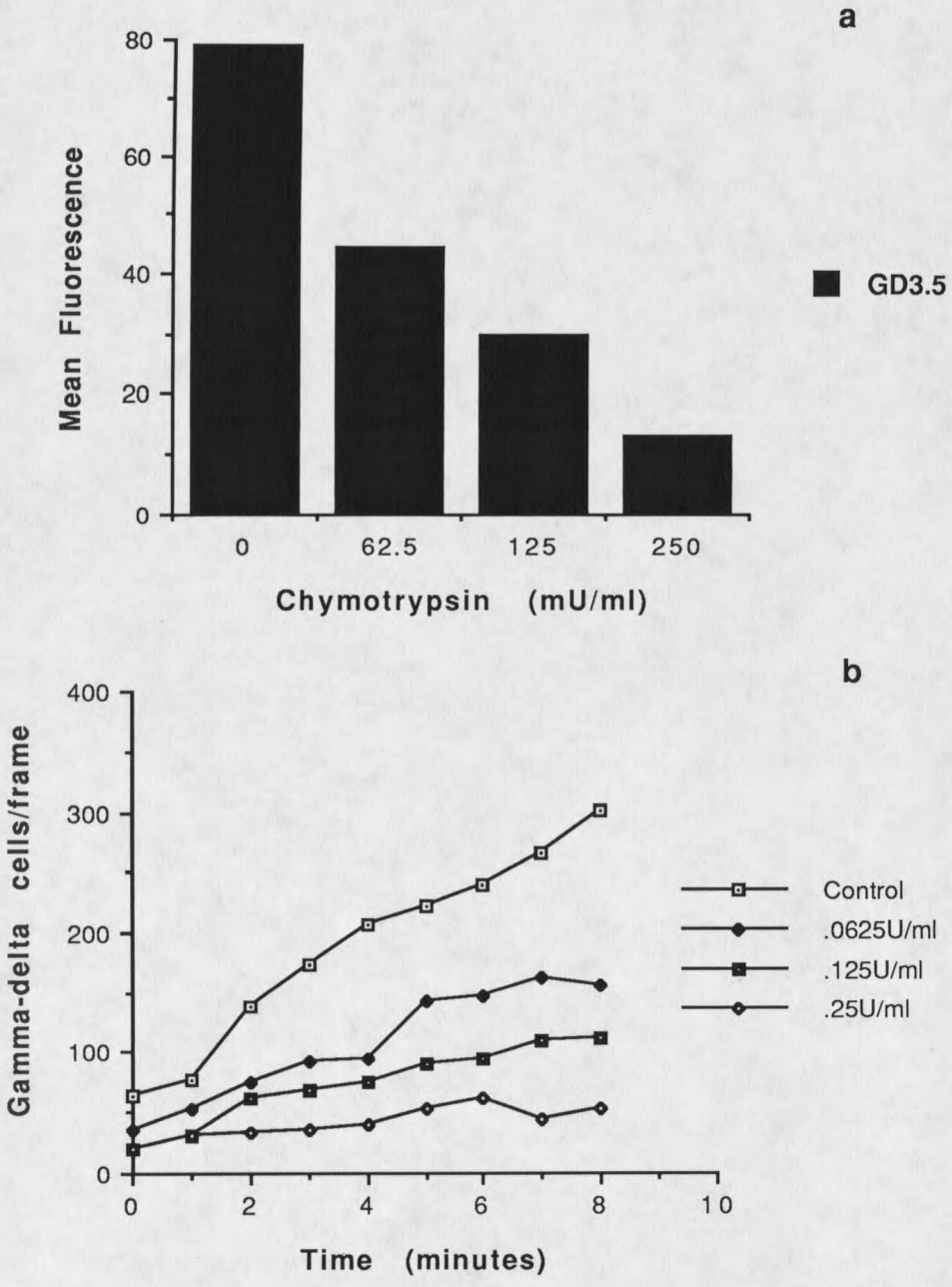

Figure 15. γδ T cell rolling on 24hr activated endothelium is inhibited by low-dose chymotrypsin treatment of the rolling cells. 15a) Purified γδ T cells were treated with 0-250 mU α-chymotrypsin/1x10^7 cells for 1hr at 37°C and analyzed by FACS for GD3.5 antigen expression. 15b) Cells were then resuspended in media and infused into the loop assay system, as described in Materials and Methods. Figure 15 is representative of at least three separate experiments.

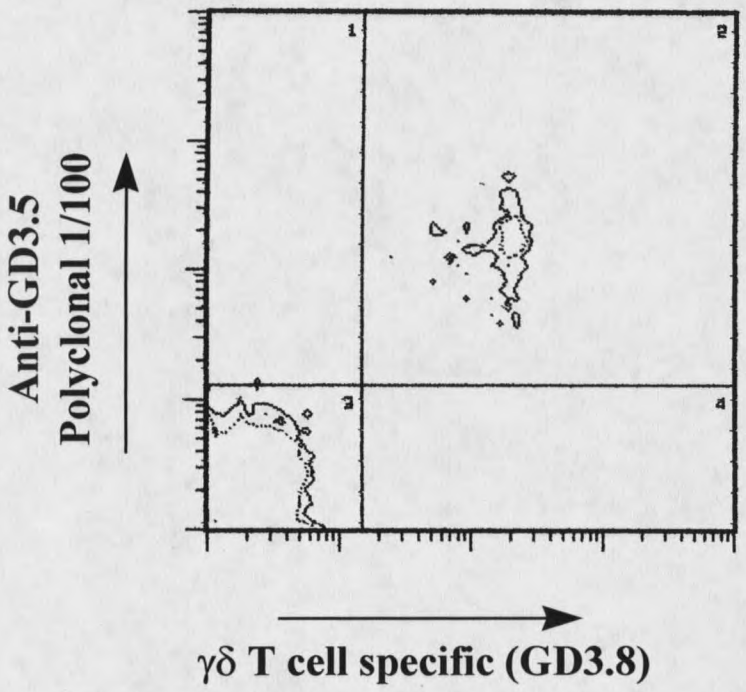

Figure 16. Anti-GD3.5 polyclonal sera stains bovine γδ T cells. Polyclonal antibody was raised against GD3.5Ag, as described in Materials and Methods. Two color FACS analysis illustrating polyclonal staining vs pan-γδ T cell antibody (GD3.8). Figure 16 is representative of at least three assays.

Next, we wanted to see if GD3.5 polyclonal antibodies specifically immunoprecipitated GD3.5Ag. Biotin-labeled γδ T cell lysates were immunoprecipitated with GD3.5 polyclonal serum, control serum, and GD3.5 mAb (Figure 17, lanes 1,2, and 3, respectively). As expected, the polyclonal serum immunoprecipitated a 230kD band which was precleared by GD3.5 mAb (data not shown and see Chapter 5). Therefore, the polyclonal antibodies recognized GD3.5 antigen.

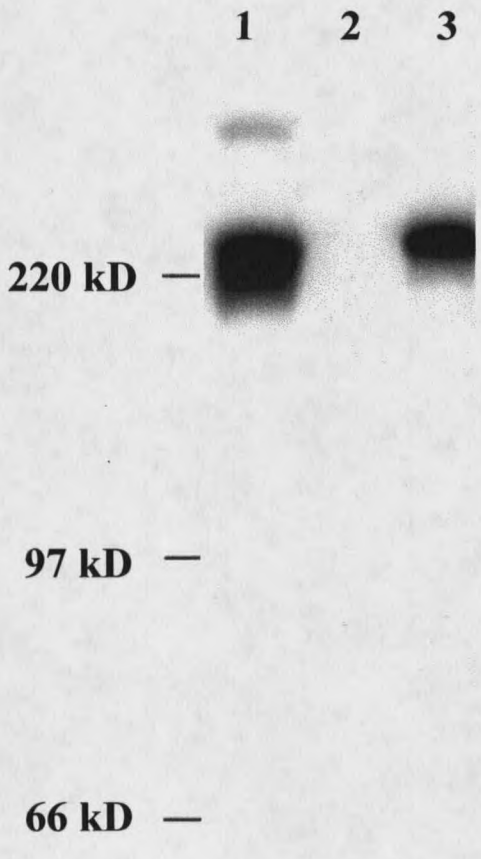

Figure 17. Anti-GD3.5 polyclonal sera immunoprecipitates GD3.5Ag. Biotinylated bovine γδ T cell lysates were immunoprecipitated with anti-GD3.5 polyclonal antibody (lane 1), control serum (lane 2), or GD3.5 mAb (lane 3), as described in Materials and Methods. Figure 17 is representative of two experiments.

Since GD3.5 mAb appeared to inhibit lymphocyte accumulation on 24hr activated BUVECs, we wanted to determine if the polyclonal serum could block γδ T cell interactions under the same conditions. Purified γδ T cells were treated for 1hr at room

temperature with immune serum (1/100 dilution) or preimmune serum (1/100 dilution). Cells were then suspended in DMEM/20mM Hepes and infused into the loop assay system, as described in Materials and Methods. As seen in Figure 18, pretreatment of the cells with GD3.5 polyclonal serum resulted in the partial blocking of γδ T cell accumulation on 24hr activated BUVECs. Thus, GD3.5Ag may be involved in adhesion of γδ T cells to chronically activated endothelium.

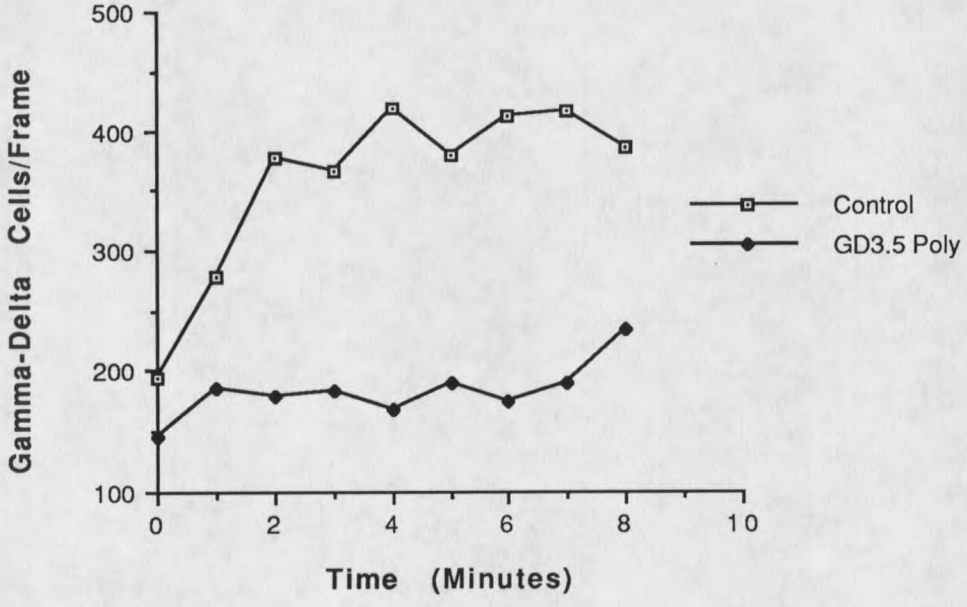

Figure 18. Anti-GD3.5 polyclonal sera specifically inhibits accumulation of bovine γδ T cells on 24hr activated endothelial cells. Purified bovine γδ T cells were pretreated with control or anti-GD3.5 polyclonal antibody and infused into the in vitro shear-dependent adhesion assay, as described in Materials and Methods. Figure 18 is representative of at least three separate experiments using polyclonal antibodies from two different mice.

Discussion

The identification and characterization of γδ T cell-specific surface molecules will be useful in understanding the biology and function of this cell type. In this report, we have characterized the regulation and potential function of a novel γδ T cell-specific marker (GD3.5 antigen).

Bovine γδ T cells are a unique subset of lymphocytes that are associated with epithelial tissues (1,5). γδ T cells do not appreciably accumulate in the organized lymphoid tissues, such as the lymph node (1,4,5); however, several reports have demonstrated that larger populations of γδ T cells are found in the spleens of ruminants when compared to lymph nodes (2,3). We confirmed that in fact γδ T cells do not appreciably accumulate in the lymph nodes. The few γδ T cells which are found in the lymph nodes possibly enter the node through the afferent lymph from the surrounding tissue on their way back to the blood (5). In addition, we showed that large populations of γδ T cells are found in the spleen. Interestingly, the number of γδ T cells in the spleen compared to the blood is not significantly different; however, the expression of GD3.5Ag and WC1 is significantly lower in the spleen population than in the blood. Perhaps the spleen population represents cells that were partially activated, or older, dying cells.

It has been shown that adhesion molecules are shed upon activation with artificial and natural compounds. For example, L-selectin is downregulated on murine T cells upon treatment with ConA and L-selectin expression returns when mitogen stimulation is removed (29). Thus, we wanted to determine if GD3.5Ag expression could be downregulated in vitro by treatment with various artificial activating compounds. We showed that γδ T cells downregulate GD3.5Ag when treated with both ConA and PHA. In

addition, PHA downregulates WC1 expression. Incidentally, others have reported that higher concentrations of ConA result in the downregulation of WC1 (30), indicating that 2uM ConA treatment is suboptimal for WC1 downregulation under these conditions. Interestingly, after four days of culture, the lymphocytes begin to return to a resting state which coincides with a return of both GD3.5Ag and WC1 expression by the sixth day. These results indicate that GD3.5 and WC1 expression correlate with the mitogen activation profile. Since GD3.5Ag and WC1 demonstrate similar regulation upon mitogen activation when compared to L-selectin, we initially hypothesized that these molecules may represent adhesion ligands on γδ T cells. Therefore, we tested whether or not GD3.5 mAb would block bovine lymphocyte adhesion in our panel of functional assays.

Initially, we tested whether GD3.5 mAb would block adhesion of lymphocytes to 4hr activated BUVECs and HUVECs. Leukocyte adhesion and transmigration through endothelial layers involves a multistep process requiring many adhesion molecules. Using the low-shear assay, initial and tight adhesion events can occur. Therefore, if GD3.5 mAb blocked binding of lymphocytes in this assay, it could be due to blocking initial or tight adhesion events; however, GD3.5 mAb does not have any effect in these assays.

In an attempt to look more closely at binding to E- or P-selectin, we used our in vitro shear-dependent assay to address whether or not GD3.5 mAb specifically inhibits binding to E- or P-selectin. As shown above, lymphocytes were allowed to adhere to E- or P-selectin and then control and GD3.5 mAb were injected (100ug/ml) to determine if it would block adhesion to these selectins. As shown above, GD3.5 mAb does not inhibit lymphocyte rolling on E- or P-selectin.

Since GD3.5 mAb did not block adhesion to E- or P-selectin or inhibit tight adhesion to 4hr activated endothelium, we decided to study its effect in other adhesion assays.

Recent reports have indicated that lymphocytes roll on 24hr activated endothelium independent of E- and P-selectin (20). More recently, we determined that γδ T cells interact with chronically activated endothelium in an E- and P-selectin-independent manner (21). Thus, we tested whether GD3.5 mAb injected into the shear-dependent adhesion assay would inhibit bovine lymphocyte interactions with 24hr activated endothelium. GD3.5 mAb, when compared to control, does not inhibit established lymphocyte interactions with the 24hr activated endothelium. In contrast, when bovine lymphocytes are pretreated with GD3.5 mAb vs. another γδ T cell-specific antibody (GD3.8), only GD3.5 has an inhibitory effect on lymphocyte interactions with 24hr activated endothelium. However, since considerable variability was seen in these assays, we pursued other approaches to further address this issue (see below).

Earlier reports showed that treatment of neutrophils with low-dose chymotrypsin leads to loss of L-selectin, inhibits neutrophil binding to high endothelial venules, and reduces the capacity of the cells to home to sites of inflammation (22). We have previously shown that GD3.5 antigen is very sensitive to low-dose chymotrypsin, exhibiting sensitivity greater than even L-selectin (24). Based on this information, we tested whether chymotrypsin-treated cells would interact with E- and/or P-selectin. We showed that γδ T cell rolling on E-selectin is unaffected by low-dose chymotrypsin treatment. Thus, consistent with our previous report (14), we showed that γδ T cell E-selectin ligands are resistant to low-dose chymotrypsin treatment.

Several investigators have described a glycoprotein ligand for P-selectin on neutrophils and lymphocytes (31,32). This ligand, termed P-selectin glycoprotein ligand-1 (PSGL-1), is sensitive to protease treatment (31,32). Since GD3.5Ag is sensitive to chymotrypsin treatment, we proposed that perhaps this antigen was a P-selectin ligand. In fact, chymotrypsin-treated lymphocytes do not roll efficiently on P-selectin, suggesting that perhaps GD3.5Ag is the ligand. However, further investigation

into this possibility suggests that GD3.5Ag is not a P-selectin ligand (see Chapter 4).

Next, we tested whether chymotrypsin-treated γδ T cells would interact with 24hr activated endothelium. In fact, rolling of chymotrypsin-treated γδ T cells on 24hr activated endothelium is inhibited in a dose-dependent manner, which correlates well with the loss of GD3.5Ag expression as measured by FACS analysis. Therefore, we proposed that perhaps GD3.5Ag was mediating the adhesion of γδ T cells to the 24hr activated endothelium; although, it is possible that other adhesion ligands could share similar chymotrypsin sensitivity.

Since pretreatment of lymphocytes with GD3.5 mAb appeared to partially, though variably, inhibit their interactions with 24hr activated endothelium, and the loss of GD3.5Ag expression correlated with the loss in ability to roll on 24hr activated endothelium, we generated polyclonal anti-serum against GD3.5Ag to further study this interaction. Anti-GD3.5 polyclonal antibody stains only bovine γδ T cells indicating that this molecule is a lineage-specific γδ T cell-surface antigen. Due to the specific staining of γδ T cells by the anti-GD3.5 polyclonal antibodies, we decided to determine if it would block lymphocyte adhesion to 24hr activated endothelium. Three mice exhibited GD3.5Ag serum titers, two of which provided serum that partially blocked γδ T cell interactions with 24hr activated endothelium. Importantly, the polyclonal antibody inhibits bovine lymphocyte interactions with 24hr activated endothelium similarly to GD3.5 mAb.

We have recently identified an adhesion molecule expressed on 24hr activated endothelium (21). In these experiments, we discovered that platelets secrete a soluble form of the antigen that specifically binds γδ T cells. Preliminary results indicate that polyclonal serum does not block this interaction (data not shown). Recent results in our lab indicate that bovine umbilical vein endothelial cells express E-selectin, and since GD3.5Ag does not appear to be an E-selectin ligand, it is possible that it binds a different

ligand on the endothelial cell surface. Possibly, GD3.5 mediates binding to the endothelium independent of E-selectin, but shares an overlapping function with E-selectin similar to the apparent overlapping functions of E- and P-selectin.

Another possibility regarding function could involve downstream adhesion events. Leukocyte adhesion and transmigration through endothelial layers requires a multistep process including rolling, tight adhesion and transmigration. It is possible that GD3.5Ag is involved in these types of adhesion events; however, preliminary results suggest that GD3.5 polyclonal antibodies do not inhibit tight adhesion or transmigration (data not shown).

Finally, it is possible that engagement of GD3.5Ag with antibody doesn't directly block lymphocyte/endothelial interactions but may provide a signal that alters the expression of the directly involved lymphocyte adhesion ligand. In fact, some adhesion molecules are expressed in a less active form until the cell is activated. For example, P-selectin glycoprotein ligand-1 (PSGL-1), which binds to P-selectin, is expressed in a less functional form on lymphocytes. However, once the lymphocyte is activated, a more functional form is expressed (32). Perhaps pretreatment of the lymphocytes with GD3.5 antibodies inhibits the expression of higher avidity adhesion ligands, such as PSGL-1. If GD3.5 is a signalling molecule then we could begin to explore the involvement of various signalling pathways. For instance, the inhibitory effects of GD3.5 mAb treatment may be overcome by treating cells with activating compounds, such as phorbol esters and calcium ionophores. In addition, cell signalling inhibitors, such as kinase inhibitors, could be used to determine if the engagement of GD3.5 requires a signal to elicit the inhibitory effect.

In summary, GD3.5Ag expression is downregulated in vivo and this can be mimicked in vitro. In addition, chymotrypsin removal of GD3.5Ag from the cell surface correlates well with the loss in ability of $\gamma\delta$ T cells to roll on 24hr activated

endothelium. Finally, pretreatment of bovine γδ T cells with monoclonal antibodies or polyclonal antibodies against GD3.5Ag inhibits their accumulation on 24hr activated endothelium.

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CHAPTER 4

COMPARISON OF E-SELECTIN BINDING GLYCOPROTEIN LIGANDS ON HUMAN LYMPHOCYTES, NEUTROPHILS, AND BOVINE γδ T CELLS: IDENTIFICATION OF NOVEL E-SELECTIN LIGANDS ON HUMAN LYMPHOCYTES AND BOVINE γδ T CELLS

Introduction

Many of the initial adhesion events between leukocytes and endothelial cells require a unique group of molecules called selectins (1-4). To date, three proteins have been identified and classified as selectins. These include E-selectin, P-selectin, and L-selectin (CD62E, CD62P, and CD62L, respectively). The selectins are defined by an NH_2-terminal, Ca^{2+}-dependent, C-type lectin domain, an EGF (epidermal growth factor-like) region, several short consensus repeat (SCR) units depending upon the selectin, and a transmembrane region with a cytoplasmic tail (1,2,5). E-selectin is expressed on endothelial cells while P-selectin is expressed on endothelium and platelets. L-selectin is expressed on leukocytes and is characterized by a different molecular mass depending upon the expressing cell type. The two vascular selectins, E- and P-selectin, support interactions of myeloid cells and unique subsets of T cells, including memory cells and γδ T cells (6-11). Because of their critical role in the initial leukocyte tethering to endothelium, many investigators have devoted a great deal of time to defining the ligands for each selectin on various target cells.

All three selectins bind to both sialyl Lewis a (SLe^a) and sialyl Lewis x (SLe^x)

tetrasaccharide structures, which are expressed by many different cell types (12-18). Specific high molecular mass glycoproteins present these carbohydrates in appropriate fashion to support cell/cell adhesion (17,19). In the context of the vascular selectins, E- and P-selectin, high molecular mass ligands on neutrophils have been well defined (20-28). For example, P-selectin glycoprotein ligand-1 (PSGL-1) on neutrophils serves as a ligand for P-selectin (20,21), and, perhaps, E-selectin as well (22-26). E-selectin ligand-1 (ESL-1) is another neutrophil glycoprotein that has been shown to bind E-selectin (27,28). Finally, some studies have shown that human neutrophil L-selectin is decorated by SLe^X and supports E-selectin binding (29-32).

In contrast to neutrophils, far less is known about the glycoprotein ligands for the vascular selectins expressed by lymphocytes. An appropriate post-translationally modified form of PSGL-1 is likely to be the lymphocyte ligand for P-selectin (8,33); however, the lymphocyte ligands for E-selectin are unknown. Lymphocyte PSGL-1 does not appear to interact appreciably with E-selectin (6,34,35), as has been shown with neutrophil PSGL-1 (22-26). Indeed, O-sialoglycoprotease treatment of lymphocytes, which hydrolyzes PSGL-1, blocks their binding to P-selectin, but not E-selectin (6,34,35). Interestingly, the mAb HECA-452 recognizes the E-selectin binding human lymphocytes, but HECA-452 staining does not correlate with the P-selectin binding subset, further distinguishing the two (34,36,37). The HECA-452 epitope is a carbohydrate structure called cutaneous lymphocyte-associated antigen (CLA) that decorates many different cell surface ligands (9) and directly interacts with E-selectin (36,37). Which of the different CLA glycoproteins serve as the predominant E-selectin-binding molecule is unknown at this time.

Recently, we showed that virtually all bovine $\gamma\delta$ T cells in newborn animals have the capacity to bind E-selectin (11). In contrast, $\alpha\beta$ T cells acquire this binding

capacity only after conversion to a memory phenotype (9,10,37). We isolated a 250kD E-selectin binding molecule from γδ T cells using an affinity isolation procedure (11) that involved using purified, recombinant E-selectin immobilized on Sepharose beads by a nonblocking anti-E-selectin mAb. Until now, we were unable to fully confirm the 250kD molecule as the first defined lymphocyte E-selectin ligand.

A powerful tool to study selectin ligands are selectin/immunoglobulin chimeras that in effect can be used as an antibody. These constructs are comprised of the adhesion binding portion of the selectins (lectin domain, and sometimes EGF and SCR domains) attached to the constant domain of Ig. We have had success in the past using a P-selectin/Ig chimera to study γδ T cells (7). In this report, we describe new studies using an E-selectin/immunoglobulin chimera containing a lectin domain from porcine E-selectin, which apparently has a much higher affinity for E-selectin ligand (38). The E-selectin chimera was used to analyze γδ T cell ligands for E-selectin and to compare and contrast these ligands with those expressed by human neutrophils and lymphocytes.

Materials and Methods

Human and bovine leukocyte purification and biotin labeling

Bovine blood was obtained, and γδ T cells were purified as described previously (39). Briefly, lymphocytes were purified from Holstein calf blood by Histopaque (Sigma Chemical Co., St. Louis, MO) separation and then incubated in T175 flasks to allow monocyte removal. γδ T cells were purified from whole lymphocyte preparations by incubation in T175 flasks coated with 7×10^6 adherent E-selectin cDNA L-cell transfectants. Unbound cells were discarded and bound γδ T cells removed by treatment with 2mM EDTA/PBS providing >90% pure γδ T cells (11). Human lymphocytes were

prepared by Histopaque separation with subsequent incubation in T175 flasks to remove monocytes, resulting in >95% pure lymphocyte populations by scatter profile analysis. Human neutrophils were purified by 1% dextran separation for 1hr at 4°C, and then the top layer was removed and neutrophils were pelleted by Histopaque separation. γδ T cells, human neutrophils, and human lymphocytes were separately biotinylated as described below. 1 x 10^8 cells were washed 3 times with sterile PBS and resuspended in 1ml of NHS-LC-Biotin/PBS (550ug/ml Pierce, Rockford, IL). After 30min incubation at room temperature (RT), cells were washed 3 times with PBS and lysed for 30min on ice with Nonidet P-40 (NP-40 lysis buffer) (39). 5% rabbit serum was added to lysates for 1hr at RT and then precleared with protein G beads (Boehringer Mannheim Biochemicals, Indianapolis, IN) overnight at 4°C with constant rotation. Beads were subsequently removed and lysates were either used immediately or frozen at -80°C.

mAbs, E- and P-selectin chimeras, and FACS analysis

Monoclonal antibodies used included IL-A29 (anti-WC1), DREG-56 (anti-L-selectin), HECA-452 (anti-CLA), YT11.1 (anti-pig E-selectin) (40,41), anti-ESL-1 polyclonal antibody (42), and two in-house bovine γδ T cell-specific mAbs GD3.5 and GD3.8 (pan γδ TCR) (39, Wilson, E., and M. A. Jutila, manuscript in preparation). Human E- and P-selectin chimeras were kindly provided by S. Watson. Pig E-selectin chimera (P11.4) was kindly provided by M. Robinson. Pig chimera was used to immunoprecipitate relevant selectin ligands on human neutrophils, lymphocytes, and bovine γδ T cells.

Antibody/Chimera	Antigen/Specificity	Species
IL-A29	WC1	Cattle
GD3.5	GD3.5Ag	Cattle
GD3.8	TCR	Cattle
DREG-56	L-selectin	Cattle/Human
HECA-452	CLA	Human
ESL-1 polyclonal	ESL-1	Mouse/Human/Cattle
YT11.1	anti-E-selectin chimera	Pig
P11.4 (E-selectin chimera)	E-selectin ligands	Human/Cattle

Table 1. List of reagents used for E-selectin ligand analysis. The antibody and chimera reagents used in these studies are listed above describing antigen specificity in the indicated species.

FACS analysis was performed on ice as follows: cells were incubated with pig E-selectin chimera supernatant for 30min ($4^{o}C$). Staining of human lymphocytes with P11.4 required incubation for 90min at RT. Cells were washed and incubated with PE-conjugated α-human secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 30min, washed again, and analyzed. In blocking experiments, pig E-selectin chimera was incubated with excess blocking antibody (YT11.1) for 30min and then added to cells. Two-color analysis was performed using PE-conjugated secondary antibody followed by a 10min block with 10% mouse serum and subsequent 30min incubation with FITC-conjugated second stage antibody. Staining of bovine lymphocytes with GD3.8 and anti-ESL-1 antibodies was performed by incubation with GD3.8 mAb and anti-mouse PE-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) and blocked as described above. Cells were then stained with anti-ESL-1 polyclonal antibody, washed, and incubated with FITC-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch). HECA-452 positive human lymphocytes were gated for analysis to reduce background resulting from B-cells stained with anti-human PE-conjugated secondary antibody. Irrelevant rabbit polyclonal anti-serum

was used as a negative control to ensure specificity of ESL-1 FACS analyses. All analyses were performed using a FACScan flow cytometer (Becton Dickinson).

Western Blot

Purified γδ T cell lysates were run under nonreducing conditions through a SDS-8% polyacrylamide gel and then transferred to PVDF membrane overnight (Bio Rad, Hercules, CA). After transfer, the membrane was blocked for 30 minutes with 50% calf serum, 5% goat serum, in HBSS containing Ca^{2+} and Mg^{2+} at 4°C. The membrane was then placed in a miniblotter apparatus and selected lanes were incubated with E-selectin chimera (P11.4) at 4°C for 1 hour. Chimera was gently removed by hand and washed by aspiration with blocking buffer. The lanes were filled with clean blocking buffer and incubated for 5-10 minutes at room temperature. After aspiration of blocking buffer, anti-human alkaline phosphatase second stage (Jackson ImmunoResearch Laboratory) was diluted 1:100 in the blocking buffer and added to appropriate lanes for 15 minutes a 4°C and then incubated for 15 minutes at room temperature. Second stage antibody was aspirated off and lanes were gently aspirated up and down by hand using blocking buffer. A second wash with blocking buffer was performed followed by two additional washes with Hepes buffered (10 mM) HBSS containing Ca^{2+} and Mg^{2+}. Membranes were removed from the miniblotter and gently rinsed with PBS before developing to desired intensity using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemical Co., St. Louis, MO) in AP buffer (100mM Tris, pH 9.5, 100mM NaCl, and 5mM $MgCl_2$).

Immunoprecipitation/Crossprecipitation

Precleared biotin-labeled lysates were incubated with 10-20ug GD3.5, IL-A29, DREG-56, control, or E-selectin chimera for 1hr at RT. Ab/Ag or chimera/ligand

complexes were precipitated with protein G beads at 4°C overnight under constant rotation. The beads were washed 3 times with wash buffer (0.1% NP-40, 100mM NaCl, 1mM $CaCl_2$, 1mM $MgCl_2$, 5mM sodium azide, and 10mM Hepes) and mixed with nonreducing loading buffer and loaded onto 8% SDS-polyacrylamide gels (39). Gels were electrophoresed and transferred to PVDF membranes (Bio-Rad, Hercules, CA). After transfer, membranes were washed briefly with 1x TBST and then blocked with 7% BSA/TBST for 2hrs at RT. Membranes were then washed with several changes of 1x TBST for 1.75hrs. Streptavidin horseradish peroxidase conjugate (Amersham Life Science, Buckinghamshire, England) was diluted 1/5000 in sterile PBS, incubated with the membrane for 40min, and then washed for 1.75hrs with several changes of 1x TBST. ECL detection reagents were used according to manufacturer's recommendations (Amersham Life Science, Buckinghamshire, England) and incubated with the membranes for 1 min. Membranes were then covered with cellophane, exposed to X-OMAT film for 1-10 minutes, and the film was developed (Kodak, Rochester, New York). YT11.1 mAb was used to block E-selectin chimera by first incubating mAb with chimera for 15min at RT, then adding it to the appropriate lysate.

Crossprecipitation experiments were performed as follows. Separate biotinylated lysates were cleared of Workshop Cluster 1 (WC1) or GD3.5 antigen by incubation with 20ug of either IL-A29 or GD3.5 mAbs, respectively, and immunoprecipitated with protein G beads, as described above. After bead removal, WC1 and GD3.5 antigen cleared lysates were incubated a second time with 15ug of IL-A29 or GD3.5 mAbs, respectively, and immunoprecipitated. Three subsequent clearing steps with protein G beads were performed on the lysates to ensure removal of all Ab/Ag complexes. E-selectin chimera or P-selectin chimera was used to immunoprecipitate lysates cleared of either GD3.5 antigen or WC1. Chimera/ligand complexes were precipitated with protein G beads and mixed with standard 2x nonreducing loading buffer, boiled for two

minutes, and loaded onto 8% SDS-polyacrylamide gels. Gels were electrophoresed, transferred, and developed, as described above.

Crossprecipitation of human neutrophil lysates was performed as follows: Separate biotinylated lysates were cleared with 20ug control or DREG-56 mAb (anti-L-selectin) and immunoprecipitated with protein G beads, as described above. After bead removal, lysates were cleared a second time with 15ug of control or DREG-56 mAb, respectively, and immunoprecipitated a second time. Three subsequent clearing steps with protein G beads were performed on the lysates to ensure removal of all Ab/Ag complexes. E-selectin chimera was used to immunoprecipitate lysates cleared with either control or DREG-56 mAb. Chimera/ligand complexes were precipitated with protein G beads and mixed with standard 2x nonreducing loading buffer, boiled for two minutes, and loaded onto 8% SDS-polyacrylamide gels. Gels were electrophoresed, transferred, and developed, as described above. Developed gels were scanned into Adobe Photoshop using a Hewlett Packard ScanJet 4c and images were imported into Microsoft PowerPoint and printed on high grade laser copier paper.

Results

Flow cytometric analysis of bovine leukocytes

First, we determined if the pig E-selectin chimera could be used in flow cytometric analysis to confirm our earlier results that γδ T cells in newborn animals have the capacity to bind E-selectin. Figure 19a shows a two-color analysis comparing P11.4 staining versus staining with a bovine pan-γδ T cell mAb. Virtually all of the lymphocytes that were stained by P11.4 were γδ T cells and most of the γδ T cells were stained by the chimera. Figure 19b demonstrates a histogram overlay showing control, P11.4, and P11.4 plus E-selectin blocking YT11.1 mAb staining profiles.

Preincubation of the chimera with anti-E-selectin mAb YT11.1 completely blocked binding of the chimera, indicating that binding of P11.4 to bovine lymphocytes was specific. These results are exactly the same as those described previously using cell/cell binding assays, and they confirm the specificity of the P11.4 chimera (7, 11).

Western Blot and immunoprecipitation of bovine E-selectin ligands

Due to the intense staining of bovine γδ T cells with P11.4 by flow cytometry, we evaluated whether the chimera could be used to probe E-selectin ligands expressed by γδ T cells. In our first experiment, we tested whether P11.4 reacted with affinity-purified 250kD ligand isolated, as described in our earlier study (11). Affinity-purified 250kD ligand was dotted onto PVDF membrane and then probed with P11.4. P11.4 reacted intensely with the 250kD fraction (data not shown). To determine if other γδ T cell antigens react with P11.4, total γδ T cell lysate was probed with P11.4. In Figure 20, lanes 2 and 4, we show that P11.4 reacted with a 250kD species as well as three other molecules at 180kD, 200kD, and 300kD. These results confirm that the 250kD ligand described in our earlier report does indeed bind E-selectin; however, E-selectin also appears to have the potential to interact with three additional molecules.

Our next series of analyses was intended to determine if any of the ligands detected by the Western blot procedure are expressed on the cell surface. Cell surface antigens on γδ T cells were labeled with biotin, detergent extracts made, immunoprecipitation procedures performed, and the immunoprecipitated fractions run on an 8% PAGE gel. The gel was transferred to PVDF membrane and biotin-labeled proteins were detected by streptavidin horseradish peroxidase conjugate, as described in Materials and Methods. In Figure 21, lane 1, two distinct bovine γδ T cell E-selectin ligands of 180-200kD and 250kD were precipitated by this procedure (indicated by arrows). The

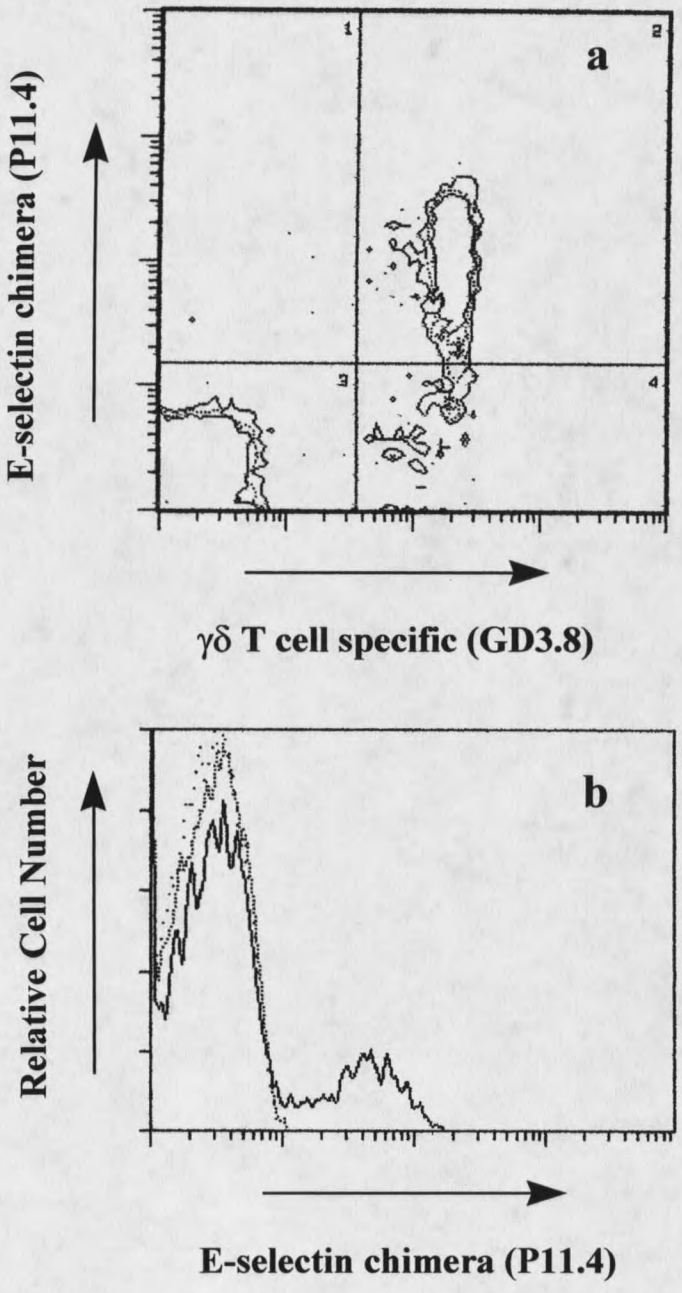

Figure 19. Bovine γδ T cells are specifically stained by E-selectin chimera. 19a) Two-color analysis of bovine lymphocytes stained with P11.4 and GD3.8 (pan γδ T cell marker). 19b) Single-color histogram overlay illustrating second-stage control (hashed line), cells stained with P11.4 (solid line), and cells stained with P11.4 that was incubated with function-blocking mAb YT11.1 (dotted line), as described in Materials and Methods. Figure 19 is representative of at least three separate experiments.

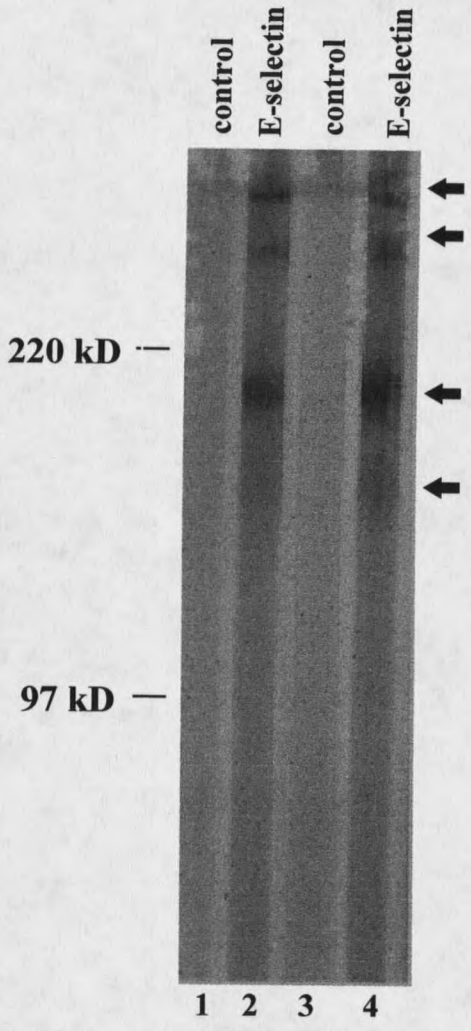

Figure 20. γδ T cell lysates contain four E-selectin-reactive molecules by Western Blot. Lanes 1 and 3 are negative controls (media alone), while lanes 2 and 4 represent duplicate lanes probed with P11.4. Arrows indicate E-selectin chimera-reactive bands. Western blots were analyzed, as described in Materials and Methods. Molecular standards are as marked. Figure 20 is representative of at least three separate experiments.

interaction of P11.4 with these two ligands was inhibited by preincubation of the chimera with the YT11.1 E-selectin blocking mAb (Figure 21, lane 2), once again illustrating specificity of the interaction. Although the 250kD ligand is completely blocked, a small percentage of the 180-200kD molecule is not blocked indicating the blockage was incomplete or a second 200kD nonspecific contaminant exists. In addition,

pretreatment with EDTA blocked binding of P11.4 to these ligands indicating that the chimera/ligand interaction was divalent cation-dependent (data not shown). In some experiments, we immunoprecipitated a 300kD ligand, but it was not consistently detected (data not shown). Therefore, we have confirmed that one of the E-selectin ligands expressed by γδ T cells is a 250kD molecule, as previously reported. In addition, P11.4 chimera immunoprecipitation provided evidence for additional ligands of 200kD and, inconsistently, 300kD.

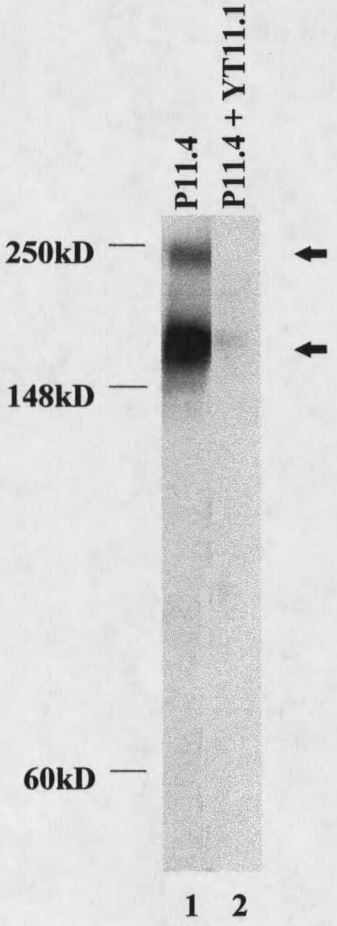

Figure 21. Two distinct E-selectin ligands are immunoprecipitated from surface biotinylated γδ T cell lysates using E-selectin chimera. γδ T cell lysates were immunoprecipitated with E-selectin chimera (lane 1) or functionally blocked E-selectin chimera (YT11.1) (lane 2) and analyzed by SDS-PAGE, as described in Materials and Methods. Arrows indicate relevant E-selectin ligands on γδ T cells. Molecular standards are indicated on the left. Figure 21 is representative of at least three separate experiments using blocking antibody YT11.1 or EDTA.

The above data suggest that GD3.5Ag (230kD) is electrophoretically distinct from the immunoprecipitated E-selectin ligands (200kD and 250kD). In addition, as shown in Chapter 3, GD3.5 mAb does not block lymphocyte adhesion to E- or P-selectin. However, it is possible that GD3.5 antibody is not a function blocker. Also, the chymotrypsin data in Chapter 3 showed that E-selectin ligands were chymotrypsin resistant and P-selectin ligands were chymotrypsin sensitive, characteristics of WC1 and GD3.5Ag, respectively. Therefore, we decided to use crossprecipitation experiments to determine if GD3.5Ag and WC1 represented E- or P-selectin ligands. Biotinylated γδ T cell lysates were precleared of GD3.5Ag or WC1 and then crossprecipitated with E- or P-selectin chimera. In Figure 22a and 22b, lanes 1 and 2 show the first round of lysates cleared with 20ug of IL-A29 or GD3.5 mAbs, respectively. Lanes 3 and 4 show lysates cleared of all IL-A29/WC1 and GD3.5Ag/GD3.5Ab complexes, respectively. In Figure 22a, lanes 5 and 6, both the 180-200kD and 250kD E-selectin ligands were precipitated from WC1 and GD3.5Ag cleared lysates, respectively. In addition, P-selectin chimera precipitated ligands from WC1 and GD3.5Ag cleared lysates, respectively (Figure 22b, lanes 5 and 6). Importantly, crossprecipitated E- or P-selectin ligands were not diminished in the WC1 or GD3.5 cleared lysates, indicating that GD3.5Ag and WC1 do not represent E- or P-selectin ligands.

Flow cytometric analysis of human leukocytes

Next, we used P11.4 to examine E-selectin ligands on human neutrophils and lymphocytes. Importantly, previous reports indicate that P11.4 binds human HL-60 cells, suggesting that it reacts with human E-selectin ligands as well (38). To confirm the binding specificity of P11.4 on human leukocytes, we performed two-color FACS analyses on human neutrophils and lymphocytes. As shown in Figure 23a, all human neutrophils stained with P11.4 and HECA-452, and P11.4 staining was inhibited by

220 kD
97 kD
66 kD
1 2 3 4 5 6
IL-A29 (1st)
GD3.5 (1st)
IL-A29 (5th)
GD3.5 (5th)
E-select/IL-A29
E-select/GD3.5
a
220 kD
1 2 3 4 5 6
IL-A29 (1st)
GD3.5 (1st)
IL-A29 (5th)
GD3.5 (5th)
P-select/IL-A29
P-select/GD3.5
b

Figure 22. E- and P-selectin chimeras do not immunoprecipitate GD3.5Ag or WC1. Biotinylated bovine γδ T cell lysates were cleared of WC1 or GD3.5Ag and crossprecipitated with E- or P-selectin chimera. a) lanes 1 and 2 represent the first preclearing step of WC1 and GD3.5Ag lysates. Lanes 3 and 4 show the complete removal of WC1/IL-A29 and GD3.5Ag/GD3.5 mAb complexes. Lanes 5 and 6 represent the crossprecipitation of WC1- and GD3.5Ag-cleared lysates crossprecipitated with E-selectin chimera. b) lanes 1 and 2 represent the first preclearing step of WC1 and GD3.5Ag lysates. Lanes 3 and 4 show the complete removal of WC1/IL-A29 and GD3.5Ag/GD3.5 mAb complexes. Lanes 5 and 6 represent the crossprecipitation of WC1- and GD3.5Ag-cleared lysates crossprecipitated with P-selectin chimera. Figure 22 is the result of two experiments.

blocking mAb YT11.1 (Figure 23b). Upon initial analysis of lymphocytes, we found that a subset of HECA-452^{high} lymphocytes consistently stained with P11.4. Figure 23c shows staining of gated HECA-452^{high} human lymphocytes with P11.4. In addition, P11.4 staining was blocked by YT11.1 mAb (Figure 23d). HECA-452^{low} lymphocytes displayed variable staining with P11.4 that was only partially blocked by YT11.1 mAb (data not shown). As stated above, HECA-452 staining correlates with E-selectin binding cell populations; however, the correlation is not necessarily absolute. In any case, P11.4 E-selectin chimera specifically stains both human neutrophils and a subset of lymphocytes.

Immunoprecipitation of human leukocyte E-selectin ligands

To identify potential human E-selectin binding ligands, we attempted to immunoprecipitate ligands from biotin-labeled detergent extracts of human neutrophil and lymphocyte membrane preparations. First, neutrophil lysates were precipitated under nonreducing conditions with the chimera which resulted in at least three specific high molecular mass glycoproteins of 80-90kD, 130kD, and 230kD, (Figure 24, lane 1 indicated by arrows) whose interaction with the chimera could be blocked by YT11.1 (Figure 24, lane 2) or EDTA (data not shown). Bands at 150kD and 60kD were only partially reduced in their intensity following the mAb block. The molecular mobility of