Role of the Intestinal Immune System in the Pathogenesis of Autoimmune Diabetes in the BB Rat Model of Type 1 Diabetes Mellitus

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A Dissertation Presented

By

DERRICK JAMES TODD

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ROLE OF THE INTESTINAL IMMUNE SYSTEM IN
THE PATHOGENESIS OF AUTOIMMUNE DIABETES IN
THE BB RAT MODEL OF TYPE 1 DIABETES MELLITUS

A Dissertation Proposal

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This dissertation is more than the culmination of just my effort:

None of this would have been possible without the overwhelming generosity of my mentor, Dr. Aldo Rossini, who, when I was a second year medical student, gave me the opportunity to join his laboratory in the summer of 1997. At the time, I had committed to just one year in the laboratory, and I was unsure of what role research would play in my career. Through the careful guidance and mentorship of Dr. Rossini, Dr. Mordes, Dale, Rita, and many others too numerous to mention, I soon realized that I loved the mysteries and challenges of science. I can only hope to model their examples in the future.

How can I fairly thank my parents, James and Deanna, and my brother, Benjamin, who have been an inspiration to me for as long as I can remember? It was upon the advice of my father, himself a Ph.D., that I was convinced to pursue this degree. I also thank Nonni and the memory of my other grandparents: Papa Derrico, Grandma Todd, and Grandpa Todd. I will always keep a bit of each of you with me, and I know that you're proud of me. Along this journey, I've also picked up another family, and I sincerely thank Jack, Mary, and Heather Elias for their love and support. I am, of course, most indebted to Tina: my wife, my lover, my friend, and my colleague. Her spirit has been and always will be the light of my life. Finally, I thank God for His guidance, wisdom, and love through these times both joyous and difficult.
Following my dissertation defense, Dr. Rossini asked me what I had learned from my experience in his laboratory. After some reflection, I told him that my answer was dependent upon when in my training I was asked that question. My first year in the laboratory was spent simply learning the procedural technicalities of individual experiments. During my second year, I gained a greater appreciation for experimental controls such that logical conclusions could be drawn from my data. In my third year, I learned how to weave individual experiments into a complete manuscript for publication. At that point, I mistakenly felt that I understood the “golden thread” about which Dr. Rossini preached, and I believed I was done.

This year, however, my eyes were opened to what it truly means to be a scientist. Although individual experiments and even manuscripts have a defined beginning and end, the story that they tell is never complete. As scientists, we can only hope to “thread” our golden pieces into the infinitely large puzzle called nature. Those individuals with whom I have had the pleasure of working have shared with me their vision of the puzzle. Thank you all, for I have benefited from your gifts more than you can possibly imagine. This dissertation is my gift to you.
The intestine is the largest lymphoid organ in the body, challenged constantly by an enormous quantity and diversity of antigens. Distinct from peripheral lymphocytes, intestinal lymphocytes have evolved unique mechanisms of tolerance and appear to govern mucosal processes such as “chronic physiologic inflammation” and oral tolerance. Failure of mucosal tolerance has been implicated in the pathogenesis of several diseases, including inflammatory bowel disease, celiac disease, and even autoimmune diabetes. One population of intestinal lymphocytes, intraepithelial lymphocytes (IELs), exists within the intestinal epithelium itself and remains poorly characterized. IELs respond to unique activation signals and appear to be in part responsible for the maintenance of epithelial integrity and mucosal tolerance.

Type 1 diabetes is one of the most common chronic childhood illnesses and causes significant morbidity and mortality. Type 1 diabetes mellitus is an autoimmune disease that results from immune-mediated destruction of insulin-producing pancreatic beta cells and is characterized by an absolute insulin deficiency. Several animal models are used to study the immunopathogenesis of type 1 diabetes, including the BB rat and NOD mouse. BBDP rats spontaneously develop autoimmune diabetes mellitus and are severely deficient in peripheral T cells. BBDR rats do not spontaneously develop autoimmune diabetes, have normal numbers of peripheral T cells, and can be induced to become diabetic by injections of a cytotoxic anti-ART2a mAb and low doses of poly I:C. The cause of autoimmune diabetes in BB rats and humans is still unknown, but both
genetic and environmental factors appear to participate. I hypothesize that one important class of environmental factors—diet and enteromicrobial agents—participates in this pathogenic process through the mediation of the gut immune system.

In this dissertation, I report a new method for the isolation of rat IELs that is based on the selective removal of intestinal epithelial cells under conditions that leave the basement membrane undisturbed. The yield of rat IELs using this method is 5-10 fold greater than that reported for other methods. Morphological and phenotypic analyses demonstrate that the purified cell population is comprised of IELs and is not contaminated with lamina propria or Peyer's patch lymphocytes. Phenotypic analysis reveals 5 major subsets of IELs, including populations of γδ T and natural killer (NK) cells present at levels not previously detected.

I also report that rat intraepithelial NK (IENK) and peripheral NK cells are similar in morphology, in their ability to lyse NK-sensitive targets, and in their ability to suppress a one-way mixed lymphocyte culture. In contrast, IENK cells differ from splenic NK cells phenotypically, and a substantial fraction of IENK cells appear to spontaneously secrete IL-4 and/or IFN-γ. I conclude that rat IELs harbor a large population of NKR-P1A+ CD3- cells that function as NK cells but display an activated phenotype and unusual cytokine profile that clearly distinguish them from splenic NK cells. Their phenotypic and functional characteristics suggest that these distinctive intraepithelial NK cells may participate in the regulation of mucosal immunity.

I next demonstrate that, prior to diabetes, both BBDP and ART2a-depleted BBDR rats have a reduced total number of IELs and exhibit a selective deficiency of IENK cell
number and function as compared to control BBDR rats. The deficiency of BBDP rat IELs can be corrected by engraftment of bone marrow from histocompatible WF donors. These results suggest 1) that the peripheral lymphopenia in BBDP rats extends to the IEL compartment, particularly to IENK cells, 2) that in BBDR rats the diabetes-inducing treatment depletes IELs, particularly IENK cells, and 3) that the defect in BBDP rat IELs is intrinsic to hematopoietic cells, not intestinal stromal cells.

I also establish that, unlike BBDR and WF rats, BBDP rats are also deficient in γδTCR⁺ IELs, a population of T cells that may play a role in normal mucosal tolerance. In addition, I report preliminary data supporting the hypothesis that systemic autoreactivity may be initiated in the intestine; peripheral autoreactive lymphocyte populations appear to emanate first from mesenteric lymph nodes that drain the intestine, and such cells may initiate a type 2 autoimmune phenomenon driven by IL-4.

Collectively, my findings support the hypothesis that a failure of mucosal tolerance in BBDP rats, perhaps secondary to deficiencies in one or more IEL subpopulations, participates in the pathogenesis of autoimmune diabetes in these animals by activating peripheral autoreactive T cells. The nature of the autoimmune response in BB rats (driven by IL-4) appears to be distinct from that of NOD mice. Despite the differences between these two well-accepted animal models of autoimmune diabetes, until more is known about the pathogenesis of type 1 DM in humans, lessons learned from both the BB rat and NOD mouse continue to be of tremendous benefit to our understanding of human disease.
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ABBREVIATIONS

APC(s), antigen presenting cell(s); ART, ADP-ribosyltransferase; β2m, β2-microglobulin; BB, BioBreeding; BBDP, BioBreeding Diabetes Prone; BBDR BioBreeding Diabetes Resistant; BMT, bone marrow transplantation; BSA, bovine serum albumin; CD, cluster of differentiation; CFSE, carboxyfluorescein succinimidy ester; CLN(s), cervical lymph node(s); cpm, counts per minute; CTL(s), cytotoxic T lymphocyte(s); DAF, decay accelerans factor; DC(s), dendritic cell(s); DM, diabetes mellitus; DP, Diabetes Prone; DR, Diabetes Resistant; DTE, dithioerythritol; EAE, experimental autoimmune encephalomyelitis; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ELISpot, enzyme-linked immunospot assay; FACS, fluorescence activated cell sorter; FITC, fluorescein isothiocyanate; GAD, glutamate decarboxylase; GALT, gut-associated lymphoid tissue; GPI, glycosylphosphatidylinositol; HLA, human histocompatibility leukocyte antigen; HRP, horse radish peroxidase; IA-2, insulinoma-associated antigen-2; IBD, inflammatory bowel disease; ICA, islet cell antibodies; IDDM, insulin-dependent diabetes mellitus; IE, intraepithelial; IEC(s), intestinal epithelial cell(s); IEL(s), intraepithelial lymphocyte(s); IENK, intraepithelial natural killer; IFN, interferon; IL, interleukin; IL-2R, interleukin-2 receptor; Ig, immunoglobulin; KGF, keratinocyte growth factor; KRV, Kilham’s rat virus; LAT, linker of activated T cells; LEFA, low essential fatty acids; LNC(s), lymph node cell(s); LP, lamina propria; LPL(s), lamina propria lymphocyte(s); LPS, lipopolysaccharide; mAb, monoclonal antibody; MALT, mucosa-associated lymphoid
tissue; MHC, major histocompatibility complex; MLC, mixed lymphocyte culture; MLN(s), mesenteric lymph node(s); mRNA, messenger ribonucleic acid; NK, natural killer; NOD, non-obese diabetic; PBS, phosphate buffered saline; PE, phycoerythrin; PMA, phorbol myristate acetate; poly I:C, polyinosinic-polycytidylic acid; PP, Peyer's patch; PPL(s), Peyer's patch lymphocyte(s); SCF, stem cell factor; SPC(s) splenocyte(s); TCR, T cell receptor; TGF, transformation growth factor; Th, T helper; TNF, tumor necrosis factor; TNP, trinitrophenol; Tr, T regulatory; VAF, viral-antibody free; WF, Wistar Furth;
Chapter I

COMPREHENSIVE INTRODUCTION

Chapter I, Section 1 - General Background to Introduction

Diabetes mellitus: Definitions and classifications

Diabetes mellitus (DM), the most common endocrine disease, afflicts approximately 5% of Americans (1). It is a leading cause of morbidity and mortality in the United States and accounts for a disproportionately high fraction of U.S. health care expenses, costing $105 billion dollars (14.6% of health care expenses) in 1992 (2). The term diabetes mellitus actually includes several endocrine disorders. Type 2 diabetes mellitus, by far the most prevalent type of DM, is a collection of disease states that have in common the feature of "hyperglycemia resulting from defects in insulin secretion, insulin action, or both" (3). Type 1 DM, the focus of this dissertation, is an autoimmune disease that results from immune-mediated destruction of insulin-producing pancreatic beta cells and is characterized by an absolute insulin deficiency (1;3;4). Type 1 DM typically presents in children and young adults and accounts for approximately 10% of cases of DM (1). Type 1 DM has been known formerly as insulin-dependent DM (IDDM) and juvenile-onset diabetes mellitus (3).
Clinical rationale: Why study pathogenesis of type 1 diabetes mellitus?

The absolute insulin deficiency associated with untreated type 1 DM results in defects of carbohydrate, lipid, and protein metabolism that contribute to hyperglycemia, electrolyte abnormalities, and ketoacidosis (5). Acutely, hyperglycemia promotes an osmotic diuresis that depletes the body of water and electrolytes. Untreated, these events culminate in coma and death. Given appropriate insulin treatment, patients with type 1 DM can avoid the metabolic disturbances associated with an absolute insulin deficiency. Unless blood sugar is strictly controlled, however, patients are still at risk for complications of chronic hyperglycemia such as blindness, renal failure, amputations, infection, and cardiovascular disease (5).

Individuals afflicted with type 1 DM face a lifetime of insulin injections, and children therefore experience a prolonged period over which morbidity may develop. A cure for type 1 DM is clearly necessary. Currently, restoration of beta cell function through transplantation of functional pancreatic tissue is being investigated as a curative therapy for type 1 DM. This modality is limited, however, by a lack of donor organs and immunologic barriers of transplantation and recurrent autoimmunity (6). Such considerations underscore the importance of preventing in addition to curing type 1 DM. Before we can successfully attempt to prevent this devastating disease in humans, however, a greater understanding of the immunopathogenesis of type 1 DM is necessary.
Experimental tools: Animal models in the study of type 1 diabetes

Several limitations hinder one's ability to study the immunopathogenesis of type 1 DM in humans. These include the relative inaccessibility of the pancreas, the genetic heterogeneity of human beings, and ethical considerations regarding experimentation in humans (4;7). The use of animal models overcomes these limitations, and fortunately there exist several animal models of human type 1 DM. Two rodent models, the BioBreeding Diabetes-Prone (BBDP) rat and the Non-Obese Diabetic (NOD) mouse, spontaneously develop autoimmune diabetes that in many ways resembles human disease. In addition, autoimmune diabetes can be induced in BioBreeding Diabetes-Resistant (BBDR) rats and in streptozotocin-treated rats. The immunopathogenesis of autoimmune diabetes has been studied extensively in all of these models and has provided much insight into human disease (7).

Overview of this dissertation

The comprehensive goal of this dissertation is to increase our understanding of the immunopathogenesis of autoimmune diabetes in BioBreeding (BB) rats, with the hope that information uncovered by this work may eventually contribute to the prevention of type 1 DM in humans. Specifically, these studies focus on intestinal intraepithelial lymphocytes (IELs), a large yet poorly understood compartment of the gut-associated lymphoid tissue (GALT) that lines the entire gastrointestinal tract. As will be discussed in greater detail below, environmental factors clearly influence the expression of autoimmune diabetes in humans and rodents, and intestinal lymphocytes are among the first
cells of the immune system that encounter potential environmental triggers of autoimmunity (8;9). This dissertation characterizes the phenotype and function of IELs in normal and diabetic rats and postulates a mechanism whereby deficiencies in one or more IEL populations contribute to the earliest immunopathogenesis of autoimmune diabetes.

Overview of Chapter I

The objective of this introductory chapter is to acquaint the reader to background material as it pertains to the remainder of this dissertation. Section 2 of this chapter provides an overview of the immune system and describes characteristics and functions of peripheral T and natural killer (NK) lymphocytes as they relate to this dissertation. Section 3 presents evidence supporting the concept that type 1 DM in humans is an autoimmune disorder. Section 4 describes in detail our current understanding of the immunopathogenesis of autoimmune diabetes in the BB rat. Section 5 introduces the intestinal immune system, particularly intraepithelial lymphocytes. Finally, Section 6 states the aims and goals of this dissertation.

Chapter I, Section 2 - The Immune System

To study the autoimmune pathogenesis of diabetes mellitus in humans or animal models, a working knowledge of the immune system is necessary. This section of the introduction presents a general overview of the immune system and then details two populations of peripheral lymphocytes, T and NK cells, as they pertain to this dissertation.
General overview of the immune system

Mammals and other multi-cellular organisms have evolved an elaborate immune system to identify and eliminate pathogenic agents such as microorganisms, viruses, toxins, and neoplasms. An immune response must therefore be selective for the offending pathogen yet limit damage to healthy tissue. The immune system is often divided functionally into innate (natural) and acquired (adaptive) immunity, and each of these divisions is comprised of humoral (plasma-borne) and cellular elements (10). Cells of the immune system (immunocytes) exist in peripheral blood, lymph, and lymphoid organs such as the spleen, lymph nodes, and intestine (10).

The innate immune system

The innate immune system responds rapidly to injury or infection. Stimuli for innate immunity include highly conserved and distinctly foreign molecules like lipopolysaccharide (LPS), a component in the outer cell wall of gram-negative bacteria (11). Limitations of the innate immune system include 1) a lack of pathogen specificity thus allowing for bystander damage, 2) a restricted repertoire of triggering antigens, and 3) a failure to generate memory, as described below. Humoral components of innate immunity include proteolytic cascades (e.g. complement), locally acting lytic enzymes (e.g. defensin), and secreted pattern-recognition receptors (e.g. mannan-binding lectin) (11). Cellular components of innate immunity include NK cells, phagocytes (neutrophils, eosinophils and macrophages), and antigen presenting cells (APCs) such as dendritic cells (DCs), activated macrophages, and activated B cells (10;11). Recognition of LPS by cells
of the innate immune system is mediated in part by a family of surface-bound pattern-recognition receptors termed toll-like receptors (11).

The acquired immune system

Directed by B and T cells, the acquired immune system evolved to compensate for limitations of innate immunity. Acquired immunity generates an antigen-specific immune response, responds to a diversity of antigens, and allows for memory through the creation of memory cells. The clonal selection theory states that antigen-specific immune responses are mediated by selective proliferation and differentiation of antigen-specific lymphocytes that recognize antigen by cell surface receptors (12). In a primary immune response, however, this process takes time (days to weeks), and an organism is therefore dependent upon innate immunity to contain pathogens in the interim. Diversity of the acquired immune response is accomplished through random rearrangement of genes coding for antigen-specific receptors (13;14). Memory is the phenomenon whereby, following a primary encounter with an antigen, memory cells are generated that allow for a rapid response by the acquired immune system in the event of subsequent exposures to the same pathogen. The principle humoral component of acquired immunity is immunoglobulin (Ig) secreted by plasma cells (differentiated B cells). Cellular components of acquired immunity include both B and T cells.

Primary and secondary lymphoid organs

Lymphocytes (B, T, and NK cells) are of hematopoietic lineage and derived from pluripotent stem cells in the fetal liver and bone marrow. The majority of T cells undergo
additional maturation in the thymus (described below). These organs in which lymphocytes originate and mature are referred to collectively as primary lymphoid organs (10).

Mature lymphocytes are found in peripheral blood, lymph, and designated secondary lymphoid organs (spleen, lymph nodes, and mucosal surfaces). The spleen and lymph nodes contain large numbers of peripheral (circulating) lymphocytes (10). Mucosal surfaces such as the gastrointestinal, respiratory, urinary, and reproductive tracts harbor vast numbers of lymphocytes that are distinct from peripheral lymphocytes and referred to collectively as mucosa-associated lymphoid tissue (MALT) (15;16). One mucosal compartment, the gut-associated lymphoid tissue, is comprised of three lymphoid populations: Peyer's patch lymphocytes (PPLs), lamina propria lymphocytes (LPLs) and intraepithelial lymphocytes. The GALT, particularly IELs, is a focus of this dissertation and is discussed in Section 5 of this chapter. Figure 1 illustrates the various pathways by which T lymphocytes migrate to secondary lymphoid organs.

Overview of peripheral T cells

T cell receptor

The antigen receptor clonally expressed by mature T cells is termed T cell receptor (TCR). TCR includes either α and β or γ and δ chains and is the product of a successful genomic rearrangement of TCR-encoding genes (14). The vast majority of peripheral T cells express TCR comprised of α and β chains (αβTCR). Antigens specifically recognized by αβTCR are almost always short peptides presented in the peptide-binding groove of class I or class II major histocompatibility complex (MHC) molecules ex-
Figure 1: Normal T cell lymphopoiesis
**Figure 1: Normal T cell lymphopoiesis.** All T lymphocytes are of hematopoietic lineage and in adults are derived from pluripotent stem cells in the bone marrow (*steps 1 and 2*). A majority of T cells mature further in the thymus before entering circulation and seeding secondary lymphoid tissues like the spleen, lymph nodes, and mucosal surfaces (*step 3*). In normal circumstances, a small percentage of recent thymic emigrants undergo apoptosis in the liver. T cells that localize to the intestine contribute to the Gut-Associated Lymphoid Tissue (GALT) that includes Peyer’s patch lymphocytes (PPLs), lamina propria lymphocytes (LPLs), and intraepithelial lymphocytes (IELs). Although the majority of PPLs and LPLs are thymus-derived (*green*), IELs appear to be of both thymus-dependent (*green*) and thymus-independent (*blue*) ontogeny.
pressed on the surface of target cells or APCs, respectively (17). Only a low percentage of peripheral T cells express TCR comprised of γ and δ chains (γδTCR). These γδ T cells for the most part localize to mucosal surfaces (18;19) and are discussed further in Section 5 of this chapter.

**Phenotype of peripheral T cells**

In addition to TCR, T cells express many other surface molecules that allow them to interact with their environment. Most of these molecules have been assigned to the cluster of differentiation (CD) nomenclature. Such nomenclature will be followed throughout this dissertation unless another more common name prevails in the literature.

All mature T cells co-express CD3 with TCR to form the TCR-CD3 complex (20). Unlike TCR, the CD3 complex has substantial intracellular components that allow for interactions with intracellular signaling molecules (20). Thus, upon specific engagement of TCR by peptide plus MHC, CD3 initiates a signal that can activate the T cell (20). Activated T cells synthesize interleukin- (IL-) 2 and IL-2 receptor (IL-2R) that drive T cell proliferation and differentiation in an autocrine fashion (20).

Mature peripheral αβ T cells also express either CD8 or CD4 co-receptors (10). CD8, expressed on peripheral T cells as a heterodimer of α and β chains (21), enhances T cell interaction with class I MHC, which is expressed by all nucleated cells and presents endogenously synthesized peptides (17). In contrast, CD4 stabilizes T cell interaction with class II MHC, which is expressed constitutively only by APCs and presents exogenously derived peptides (17).
The majority of mature peripheral T cells are naïve, meaning that they have yet to encounter specific antigen. Most naïve T cells express CD2 and CD28 but not CD25 (22). CD2, an accessory molecule to the TCR complex, adheres to CD58 on target cells or APCs to strengthen cell-cell interaction (23). CD28 binds B7.1 (CD80) or B7.2 (CD86) molecules on APCs to provide a co-stimulatory signal to T cells that synergizes with the CD3-driven activation signal (24). CD25, the high affinity α chain of IL-2R, is associated mostly with T cell activation and therefore is not expressed by the majority of peripheral T cells (20). In rats, a majority of mature peripheral T cells also express the glycosyl-phosphatidylinositol- (GPI-) linked cell surface protein ADP-ribosyltransferase- (ART-) 2 (4;25;26). ART2 is addressed in greater detail in Section 4 of this chapter.

Function of peripheral T cells

Circulating through blood, lymphatics, and secondary lymphoid organs, naïve peripheral T cells scour MHC-peptide complexes expressed on the surface of APCs or target cells (27). Successful engagement of TCR by a MHC-peptide complex signals the T cell that it has encountered its specific antigen (20). In response to this CD3-driven signal and other co-stimulatory signals, the T cell may proliferate and differentiate into an effector T cell (28). Activated CD8+ T cells differentiate into 1) memory cells; 2) Tc1 or Tc2 cells that, analogous to Th1 and Th2 T helper cells, secrete type 1 or type 2 cytokines, respectively (see below); or 3) cytotoxic T lymphocytes (CTLs) that kill target cells via secretion of membrane-disrupting molecules like perforins and granzymes (28). Acti-

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1 A new nomenclature for the rat has been adopted. ART2 now replaces RT6; ART2a and ART2b, isoforms of ART2, now replace RT6.1 and RT6.2, respectively (150).
vated CD4+ T cells differentiate into either memory T or T helper (Th) cells (28). It is widely believed that the CD4+ Th cell is responsible for coordinating an immune response (29). Th cells are critical for optimal antibody function, as isotype switching, somatic hypermutation, and B cell memory are absent in humans and rodents lacking T cells (22;30). Th cells also support other immunocytes, including CTLs, macrophages, and NK cells (28).

The Th1/Th2 model of immune responses

Based on numerous experiments, mostly conducted in mice, immune responses can be divided into one of two major themes, termed type 1 and type 2 immunity (Figure 2) (29;31). Upon successful engagement of TCR by a specific peptide-MHC complex – plus a costimulation signal (often through CD28) – naïve CD4+ T cells undergo IL-2 driven proliferation and differentiation into effector Th1 or Th2 lymphocytes. Th1 lymphocytes direct type 1 immune responses by recruiting CTLs, NK cells, macrophages, and antibody-producing B cells (IgG2a) (29). Th2 lymphocytes direct type 2 responses by recruiting eosinophils, mast cells, and antibody-producing B cells (IgG1, IgA, and IgE) (29). Type 1 responses tend to be more effective against intracellular pathogens (viruses, neoplasms, intracellular bacteria, and intracellular protozoa), whereas type 2 responses tend to be more effective against extracellular pathogens (bacterial toxins and helminths) (29;31).

The nature of an immune response is determined by CD4+ Th cells through their differential expression of cytokines. Th1 cells secrete IL-12 and interferon- (IFN-) γ to
Figure 2: Th1/Th2 model of immune responses

- Naïve
  - IL-2
  - IFN-γ, IL-12
  - IL-4, IL-10

Th0

Type 1 Cell Mediated
- CTL, NK
- Mϕ, IgG2a

Type 2 Humoral Mediated
- Eos, MC
- IgG1, IgA, IgE
Figure 2: Th1/Th2 model of immune responses. Upon successful engagement of TCR by a specific peptide-MHC complex – plus a costimulation signal (often through CD28) – naïve CD4⁺ T cells undergo IL-2 driven proliferation and differentiation into effector Th1 or Th2 lymphocytes. Prior to full differentiation, proliferating CD4⁺ Th cells pass through a transient phase (termed Th0) in which they can commit to either Th1 or Th2 lineage and theoretically secrete both IFN-γ and IL-4. By secreting IL-12 and IFN-γ, Th1 lymphocytes direct type 1 immune responses and recruit CTLs, NK cells, macrophages, and antibody-producing B cells (IgG2a). By secreting IL-4 and IL-10, Th2 cells direct type 2 responses and recruit eosinophils, mast cells, and antibody-producing B cells (IgG1, IgA, and IgE).
promote a type 1 response, whereas Th2 cells secrete IL-4 and IL-10 to promote a type 2 response (29;31). Before fully differentiating, CD4\(^+\) Th cells pass through a transient phase (termed Th0) during which they commit to either a Th1 or Th2 lineage. Th0 cells theoretically secrete both IFN-\(\gamma\) and IL-4 (29). The exact mechanism that commits a Th0 cell to become either a Th1 or a Th2 cell is unknown, but it may be influenced by 1) the nature of the antigen itself, 2) the type of activation signal received by the T cell upon antigen engagement, 3) properties of the Th cell itself, and 4) the cytokine milieu of the T cell at the time of activation (29).

It has been postulated that, in normal circumstances, a balance exists between Th1 and Th2 immunity, and autoimmune diseases may result from overactive type 1 or type 2 immunity (29;31). In NOD mice, diabetes is associated with high levels of type 1 cytokines and can be prevented by IL-4 driven restoration of the Th1/Th2 balance, a process termed immune deviation (32-34). Other Th1-dominated diseases include experimental autoimmune encephalomyelitis (EAE), autoimmune arthritis, and Crohn’s disease (35). Diseases associated with an overactive type 2 response include food allergy, asthma, and ulcerative colitis (35). Applicability of this Th1/Th2 model to autoimmune diabetes in rats and humans remains unclear and is addressed in Section 4 of this chapter.

Central tolerance

Genomic rearrangement of TCR genes generates tremendous diversity (14) but also allows for the creation of non-functional or self-reactive T cells (35). During T cell ontogeny, selection processes exist that delete non-functional or self-reactive T cells, a
process termed central tolerance (35). T cell progenitors originate in the bone marrow and mature in the thymus, where they undergo two major checkpoints, positive and negative selection (10;35). Positive selection deletes T cells that fail to recognize self-MHC, thus eliminating non-functional TCR rearrangements (35). Negative selection deletes T cells that recognize self-peptide in the context of self-MHC, thus eliminating self-reactive (autoreactive) TCR rearrangements (35). Therefore, for a T cell to survive both positive and negative selection, it must express TCR that recognizes foreign antigen (peptide) in the context of self-MHC (35). Thymic selection is not a perfect process, however, as T cells that express autoreactive TCR recognizing antigens synthesized extra-thymically may escape negative selection and central tolerance (35). In these instances, additional tolerance mechanisms are required peripherally to prevent autoreactivity (35).

Peripheral tolerance

Despite the highly selective nature of T cell ontogeny, autoreactive T cells escape the thymus and can be found in the periphery of disease-free individuals (36;37). Most individuals do not manifest autoimmune disease, however, so mechanisms must exist peripherally to prevent the aberrant activation of autoreactive cells (35). These mechanisms are referred to collectively as peripheral tolerance, and failure of peripheral tolerance appears to contribute to autoimmunity. Immunologic ignorance is a mechanism of peripheral tolerance whereby autoantigens sequestered in immunologically privileged sites (e.g. the central nervous system) are "invisible" to circulating T cells (38). Deletion, analogous to thymic central tolerance, appears also to be a mechanism for peripheral tolerance (39;40). Anergy, a hyporesponsiveness of T cells to TCR stimulation (41-43), may be an
additional mechanism of peripheral tolerance, but anergy is currently only an in vitro phenomenon and has yet to be convincingly demonstrated in vivo (35). A final mechanism of peripheral tolerance may involve the down-regulation of autoreactive T cell responses by other lymphocytes, termed regulatory cells, which act to deviate an aberrant immune response away from a destructive phenotype (35). Multiple lines of study have demonstrated that manipulation of peripheral tolerance in animal models of autoimmunity modulates disease expression, and such modalities are currently being investigated for the prevention and/or cure of human autoimmune diseases (35). Peripheral tolerance mechanisms seem to be particularly relevant at mucosal surfaces and are discussed in Section 5 of this chapter.

NK T cells

A candidate regulatory cell described recently is the NK T cell that, unlike most peripheral T cells, also expresses the NKR-P1 (CD161, NK1.1) cell-surface antigen typically expressed by NK cells (see below) (44;45). Mouse NK T cells are unique T cells in that most express a TCR with a highly restricted Vα14-Jα281 variable region that recognizes non-peptide antigens presented by non-classical MHC molecules like CD1 (46). Functionally, NK T cells have gained attention because of their differential secretion of either IL-4 or IFN-γ upon TCR or NKR-P1 engagement, respectively (46). IL-4 secretion is associated with regulatory activity in vivo (31), and NOD mice are deficient in NK T cells (47;48). NK T cells have also been described in humans (49) and rats (50;51), but their immunoregulatory role in these instances is unclear.
Overview of peripheral NK cells

Origin and phenotype of NK cells

Natural killer cells, a component of innate immunity, are large granular lymphocytes derived from bone marrow precursors. They are abundant in the peripheral blood and spleen but are rarely observed in lymph nodes (52). In rats, most peripheral NK cells express CD2 and CD8 but not TCR or CD3 (51;53;54), thus distinguishing them phenotypically from NK T cells (46). NK cells in the rat express a homodimeric CD8αα (21). NK cells also express a number of distinct receptor families (e.g. NKR-P1 and Ly-49) that intimately relate to their cytotoxic function (44;45;55).

Function of NK cells in an immune response

NK cells kill virus-infected or neoplastic cells, an activity mediated by membrane-disrupting perforin molecules, serine proteases, and IFN-γ (52;56). Antibody-mediated cross-linking of NKR-P1 (or NK1.1 in the mouse) triggers phosphoinositide turnover and arachnidonic acid production in NK cells that induces a rise of intracellular Ca++, provides an activation signal, and stimulates natural cytotoxicity (55;57-59). Recombinant NKR-P1 binds synthetic carbohydrate, although in vivo ligands of NKR-P1 have yet to be identified (55;60;61). A second class of NK cell receptors, members of the Ly49 family, binds to class I MHC on potential target cells and (along with so-called killer inhibitory receptors) provides an inhibitory signal that prevents NK cytotoxicity (55;62;63). Healthy cells, which theoretically express ligands for both NKR-P1 and Ly-
49, avoid NK cytotoxicity, whereas virus-infected or neoplastic cells, which have often down-regulated class I expression, are killed (55).

Function of NK cells in immune regulation

NK cells are traditionally considered a component of type 1 immunity. A growing pool of evidence, however, has also suggested that NK cells may act as regulatory cells in vitro and in vivo (64-71). Mechanisms of NK cell regulation are still unclear, but they may involve secretion of inhibitory cytokines like transformation growth factor- (TGF-) β (66) or direct killing of activated T cells or APCs (65).

Chapter I, Section 3 - Immunopathogenesis of Type 1 Diabetes

Multiple lines of study in human subjects and animal models have suggested that type 1 DM results from an immune-mediated destruction of insulin-producing pancreatic beta cells (1;72). Both genetics and environment clearly contribute to disease immunopathogenesis, a process whereby unknown environmental factors trigger an autoimmune destruction of beta cells in genetically susceptible individuals (1). Much of the mechanism whereby genetics and environment precipitate disease expression remains poorly characterized. This section of the introduction describes our current understanding of the autoimmune pathogenesis of type 1 DM in humans and reviews genetic and environmental factors that may contribute to disease expression.
Type 1 diabetes mellitus as an autoimmune disease

Patients with type 1 diabetes mellitus have a number of immunologic disturbances suggesting that the pathogenesis of this disease involves an autoimmune process (Table 1) (1). These include the presence of mononuclear insulitis, the detection of islet cell autoantibodies, an association of type 1 DM with other autoimmune diseases, and the recurrence of autoimmunity (memory). In addition, type 1 DM is associated with human histocompatibility leukocyte antigen (HLA) genes, and pharmacological immunosuppression is effective in delaying the onset of overt diabetes in high-risk patients (1;4;7).

Insulitis in human type 1 diabetes mellitus

In patients with recent-onset type 1 DM, histologic analysis of pancreatic tissue demonstrates in the islets a markedly reduced number of insulin-producing beta cells and a striking mononuclear infiltrate (1;73). Many of the infiltrating mononuclear cells are CD4+ or CD8+ T cells, and similar findings have been observed in pre-diabetic BB rats and NOD mice (74). Class I MHC is hyperexpressed on beta cells in the lesion (75).

Autoantibodies in human type 1 diabetes mellitus

Antibodies specific for islet self-antigens are found in the serum of at least 70% of patients with newly diagnosed type 1 DM (76-78). Antigens recognized by these so-called islet cell antibodies (ICA) include the 65 KDa glutamate decarboxylase (GAD) (79), the tyrosine phosphatase insulinoma-associated antigen-2 (IA-2) (80), and insulin itself (81). Although the contribution of autoantibodies to the pathogenesis of type 1 DM
## TABLE 1

EVIDENCE FOR AUTOIMMUNITY IN TYPE 1 DIABETES MELLITUS

<table>
<thead>
<tr>
<th>Evidence for autoimmunity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. HLA associated</td>
</tr>
</tbody>
</table>
| 2. Preventable by immuno-
  prophylaxis               |
| 3. Pancreatic insulititis |
| 4. Islet cell antibodies  |
| 5. Association with other
  autoimmune diseases       |
| 6. Recurrence of autoim-
  munity                   |

*Legend to Table 1:* Listed are attributes of type 1 diabetes mellitus in humans that suggest that selective β cell destruction results from an autoimmune process.
remains unclear, ICA have been measured in at-risk individuals and used as a diagnostic predictor for patients most likely to progress to clinical disease (1;72).

Association of human type 1 diabetes mellitus with other autoimmune diseases

Type 1 DM is associated with a number of other organ-specific diseases that are also believed to result from autoimmune disturbances. Approximately 10% of patients with type 1 DM have such afflictions, which may include thyroiditis, Grave’s disease, Addison’s disease, or pernicious anemia (82-85). In addition, upwards of 5% of patients with type 1 DM exhibit evidence of celiac disease (86), an intestinal disorder characterized by hypersensitivity to dietary gluten (87;88).

Recurrence of autoimmunity

Sporadic case reports in humans provide perhaps the most compelling evidence that type 1 DM results from immunologic disturbances. There is a recurrence of autoimmunity in patients with type 1 DM that receive a pancreas transplant from a discordant monozygotic twin; despite identical MHC loci, donor β cells evoke a memory immune response in the recipient and are selectively destroyed (89). In other cases, patients undergoing bone marrow transplantation (BMT) for a hematopoietic disorder that receive bone marrow from a donor with type 1 DM have an increased risk of developing type 1 DM themselves (90).
Genetics and type 1 diabetes mellitus

Genetics clearly influence the expression of type 1 DM in humans. First-degree relatives of patients with type 1 DM have a 10-fold greater risk of developing the disease versus persons with no family history (lifelong risks of ~5% and ~0.5%, respectively) (1;72). Additional evidence for the role of genetics in disease expression comes from twin studies, which have shown a disease concordance of 30-50% in monozygotic twins versus 5% in dizygotic twins (72;91-93). Genetic studies in humans have mapped at least four genes that confer susceptibility to type 1 DM (94). Underscoring the role of the immune system in the pathogenesis of type 1 DM, the strongest genetic association is in the HLA locus that encodes for class II MHC molecules (94;95). For example, more than 90% of patients with type 1 DM carry genes for HLA-DR3, DQB1*0201 or -DR4, DQB1*0302 (96). Such genotypes are only permissive for disease expression, however, since the majority of people that carry these genes do not develop type 1 DM (97). Class II genes that confer protection from type 1 DM have also been identified (98-100).

Environment and type 1 diabetes mellitus

The fact that the concordance rate for type 1 DM between monozygotic twins is less than 100% (72;91-93) strongly suggests that environmental factors also contribute to disease expression (1). Indeed, both viruses and diet have been implicated in the pathogenesis of type 1 DM in humans (Table 2) (1;101). Epidemiological studies have identified an association between type 1 DM and Coxsackie B4, mumps, and rubella viruses
TABLE 2
EVIDENCE FOR ENVIRONMENTAL ROLE IN PATHOGENESIS OF TYPE 1 DIABETES MELLITUS

<table>
<thead>
<tr>
<th>Environment and type 1 diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Discordance between monozygous twins</td>
</tr>
<tr>
<td>2. Most genetically predisposed individuals do not develop type 1 diabetes mellitus</td>
</tr>
<tr>
<td>3. Viruses?</td>
</tr>
<tr>
<td>4. Diet?</td>
</tr>
</tbody>
</table>

Legend to Table 2: Listed is evidence for the role of environmental factors in the immunopathogenesis of type 1 diabetes mellitus in humans.
(1;102-105), but the importance of viral infection as a precipitating event for disease expression remains uncertain (106;107).

The role of dietary proteins and toxins in the expression of type 1 DM has been debated for many years and remains unclear. Several groups have suggested that dietary cow’s milk, when administered to genetically susceptible infants, is an environmental risk factor for type 1 DM (108-110). The immunogenic component of cow’s milk may be bovine albumin (110), and antibodies to fragments of bovine albumin are found in greater abundance in the serum of diabetic versus non-diabetic children (109). In contrast to these findings, however, other studies have found little if any association between dietary cow’s milk proteins and type 1 DM in humans (111;112). Of importance to this dissertation, it has recently been established that a correlation exists between type 1 DM and celiac disease (86), an intestinal disorder characterized by hypersensitivity to dietary gluten (87;88). In addition, several studies document a relationship between smoked mutton (which contains high levels of N-nitroso compounds) and expression of type 1 DM (113-115). Clearly, the question of how environmental factors like viruses and diet contribute to the expression of type 1 DM in humans requires further study.

**Chapter I, Section 4 - The BB Rat Model of Type 1 Diabetes**

Due to ethical and moral considerations regarding experimentation in humans, animal systems have been used extensively to model human diseases, including type 1 diabetes mellitus (7). One well-characterized animal model of human type 1 DM, the BB rat, is a major focus of this dissertation. This section 1) presents a general overview of the
BB rat; 2) describes clinical diabetes and immune cell defects in diabetes-prone BB rats; 3) describes diabetes induction in diabetes-resistant BB rats; 4) reviews evidence that diabetes in BB rats is an autoimmune disease; 5) explains the potential roles of ART2-expressing T cells in the pathogenesis of diabetes in BB rats; 6) illustrates the “Balance Hypothesis” of diabetes in BB rats; 7) reviews environmental factors that influence diabetes expression in BB rats; and 8) summarizes the applicability of BB rats as a model of human type 1 DM.

**General overview of the BB rat**

Originally discovered in 1974 at the BioBreeding Laboratories in Ottawa, Canada, the spontaneously diabetic BB rat has received much attention as one of the few animal models of human type 1 diabetes mellitus (4). Many subsequent breeding colonies of the original diabetes prone BB rats have been established, including several inbred colonies in Worcester, MA (4). Two of these inbred BB rat colonies have been maintained for >70 generations and are referred to extensively in this dissertation: BBDP rats, which have been bred for expression of spontaneous diabetes; and BBDR rats, which were derived from BBDP forebears but have been bred for resistance to spontaneous diabetes (4). It is important to note that, due to a combination of genetic drift and environmental variability, individual colonies of BB rats have diverged from one another over time, giving rise to significant genotypic and phenotypic heterogeneity among different colonies of BBDP or BBDR rats (4).
Diabetes Prone BB rats: A model of spontaneous type 1 diabetes

Clinical characteristics of diabetes in BBDP rats

Greater than 85% of male and female BBDP rats spontaneously develop diabetes between 55 and 120 days of age (rat adolescence) (4). Diabetes in these rats is similar to human disease in that there is a period of insulitis, followed rapidly by selective beta cell destruction, absolute insulin deficiency, hyperglycemia, and ketoacidosis if left untreated by exogenous insulin (116). A number of environmental perturbants (discussed below) influence diabetes expression in BBDP rats.

Peripheral lymphocyte populations in BBDP rats

*T cell lymphopenia in BBDP rats*

Unlike humans with type 1 DM, BBDP rats exhibit a marked T cell lymphopenia, particularly of CD8$^+$ (117) and ART2$^+$ (118) peripheral T cells (116). The lymphopenia maps to an autosomal recessive locus on chromosome 4 (*iddml*) (119) and is the result of decreased thymic export of T cells (120) and increased frequency of intrahepatic T cell apoptosis (121). The mechanism whereby homozygosity at *iddml* causes lymphopenia in BBDP rats is not yet known. Discussed in more detail below, diabetes can be prevented in BBDP rats via infusion of splenocytes from non-diabetic histocompatible rats provided that ART2$^+$ T cells engraft (122;123).
NK cells, B cells, and NK T cells in BBDP rats

The relative number and cytotoxicity of splenic NK cells is enhanced in BBDP rats (53). Although the relative number of B cells is also increased in BBDP rats (124), humoral immune responses to T cell dependent antigens appear to be defective in these rats (4). In addition to the peripheral T cell lymphopenia, BBDP rats are also deficient in splenic and intrahepatic NK T cell populations (51), although the significance of this observation is not yet known.

Diabetes Resistant BB rats: A model of inducible type 1 diabetes

BBDR rats never develop spontaneous diabetes in a viral antibody free (VAF) facility (4). Diabetes can be induced in these rats, however, by co-administration of a cytotoxic anti-ART2a monoclonal antibody (mAb) plus an immune stimulant like polyinosinic-polycytidylic acid (poly I:C) (125). This induction protocol depletes BBDR rats of ART2a+ T cells and induces diabetes in >90% of animals after ~14 days of treatment provided the animal is less than 30 days of age prior to initiation of the induction protocol (4). Other immune stimulants such as Kilham’s rat virus (KRV), a rat parvovirus, can substitute for poly I:C (125;126). Clinically, the diabetes induced in BBDR rats is similar to that of BBDP rats in that it is characterized by insulitis, selective beta cell destruction, an absolute insulin deficiency, hyperglycemia, and ketoacidosis if left untreated by exogenous insulin (4).
Diabetes in BB rats as an autoimmune disease

Like human type 1 DM, numerous lines of evidence suggest that diabetes in both spontaneously diabetic BBDP and diabetes-induced BBDR rats results from an immunological disturbance, mostly likely an autoimmune disease. Evidence for autoimmunity listed in Table 1 are equally applicable to BB rats. (4).

Immunoprophylaxis

Immunosuppressive agents such as radiation (4), cyclosporine (4), fusidic acid (127), tacrolimus (128), and anti-lymphocyte serum (4) prevent spontaneous diabetes in BBDP rats. Administration of anti-CD8 but not anti-NKR-P1 cytotoxic mAbs also prevents spontaneous diabetes in BBDP rats, presumably by deleting effector cells (4;129).

HLA association

All diabetes-prone BB rats share the class II RT1^u rat MHC (4), which colocalizes with the iddm2 diabetes-susceptibility gene to chromosome 20 of the rat (4;116). Other RT1^u-expressing rat strains, such as RT1^u PVG and RT1^u LEW.WR1 rats, are also susceptible to diabetes induction (130;131). RT1^u is only permissive and not required for diabetes expression, however, as RT1^u Wistar Furth (WF) rats never get diabetes (130;131), and RT1^s PVG rats can be induced to become diabetic via a thymectomy and irradiation protocol (132;133).
Insulitis

Insulitis resembling that of human type 1 DM is present in newly diabetic BBDP and BBDR rats (4;116;134). This lesion is characterized by mononuclear infiltrates of CD4+ and CD8+ T cells, macrophages (135), NK cells, dendritic cells (136), and B cells (4). Cells infiltrating the islet express messenger ribonucleic acid (mRNA) for inflammatory cytokines such as tumor necrosis factor- (TNF-) α, IL-1, and IL-6 (137), and mRNA for Th1-associated cytokines like IFN-γ and IL-12 (p40 chain) are also detected in acutely inflamed islets (138). Hyperexpression of class I MHC (139) but not class II MHC (140) also occurs on beta cells in diabetic BB rats.

Autoantibodies

Antibodies reactive against lymphocytes, gastric parietal cells, smooth muscle, and thyroid colloid have been detected in the serum of BBDP rats (4). In contrast, the islet cell antibodies, including those specific for GAD (141), IA-2 (142;143), and insulin (144), have not been convincingly documented in diabetic BBDP rats. The lack of ICA in diabetic BBDP rats may reflect an impaired humoral response to T cell dependent antigens (4).

Association with other autoimmune diseases

Both BBDP and diabetes-induced BBDR rats also show histologic evidence of autoimmune thyroiditis (122;145) and sialadenitis (4). Thyroiditis in these animals is similar to diabetes in that it can be prevented in BBDP rats by infusion of histocompatible lymphocytes (122) and induced in BBDR rats (125).
Recurrence of autoimmunity

Both BBDP and BBDR rats harbor autoreactive T cells as shown using a number of adoptive transfer models (4). Splenocytes isolated from spontaneously diabetic BBDP rats can adoptively transfer diabetes to non-diabetic rats (146). Lymphocytes from diabetes-induced BBDR rats can likewise transfer diabetes to athymic recipients (147;148). Adoptive transfer of thymocytes from BBDR rats also induces diabetes in athymic rats, but only if recipient rats are depleted of ART2+ T cells and administered poly I:C (4). These findings suggest the presence of a defect in thymic selection in BB rats that allows for the production of autoreactive T cells (4).

ART2: Surface protein expressed by regulatory cells in rats

Genetics and expression of ART2

First identified in the early 1970s, ART2 is expressed as a GPI-linked protein on the surface of most mature, resting peripheral T cells in the rat. At least two ART2 alleles (ART2a and ART2b) that encode for ART2a and ART2b proteins, respectively, have been reported in the rat (4). BB rats carry the ART2a allele, but BBDP rats are particularly deficient in peripheral ART2a+ T cells. Other lymphocyte populations in addition to resting peripheral T cells also express ART2. Peripheral NK cells and activated T cells express ART2, but only at very low levels, and IELs express ~10 times more ART2 than peripheral T cells (149;150). The significance of these observations is not yet known.
Adoptive transfer of ART2+ T cells protects BB rats from diabetes

Evidence for the existence of a regulatory population of lymphocytes that prevent diabetes in BB rats has been shown in numerous adoptive transfer experiments (122;123;146;148;151). Diabetes can be prevented in BBDP rats by administration of ART2+ CD4+ T cells from non-diabetic histocompatible rats (116;122). In addition, the diabetes induced in athymic rats that receive T lymphocytes from diabetes-induced BBDR rats is prevented by co-administration of ART2+ T cells (148). T cells with an ART2+ CD4+ phenotype have also been implicated as regulatory cells in another model of autoimmune diabetes whereby diabetes is induced in PVG-RT1u rats via thymectomy and irradiation (133).

Mechanisms of regulation by ART2+ T cells

Several possible mechanisms may explain how ART2+ T cells prevent diabetes in BB rats (4). First, ART2 may simply be a marker of a regulatory T cell population that acts to deviate the immune response away from a pro-destructive inflammatory phenotype, perhaps through differential expression of regulatory cytokines (31;152). Second, the cell-signaling properties of ART2 (153) may allow ART2+ T cells to acquire regulatory activity. Third, the ability of ART2 to metabolize nicotinamide adenine dinucleotide (154) may have immunoregulatory roles or allow ART2+ T cells to acquire regulatory activity.
The "Balance Hypothesis" of autoimmunity in BB rats

The observation that both autoreactive and regulatory T cell populations can be found in BB rats has led to the "Balance Hypothesis" that diabetes expression in BB rats depends on a balance that exists between ART2⁻ autoreactive T cells and ART2⁺ regulatory T cells (Figure 3) (4;155). In unmanipulated BBDR rats and in BBDP rats that have received infusion of ART2⁺ regulatory T cells, a proper balance exists between regulatory and autoreactive T cells; diabetes is avoided (4). In contrast, spontaneously diabetic BBDP rats and diabetes-induced BBDR rats are deficient in ART2⁺ regulatory T cells secondary to iddm1 or ART2-depletion, respectively; the balance is tipped in favor of autoreactive T cells and diabetes expression (4). It should be noted that, in BBDR rats, depletion of ART2⁺ T cells alone is insufficient to induce diabetes (116;156), as some sort of immune stimulation (poly I:C, KRV, etc.) is necessary for disease expression. Whether a similar stimulation of the immune system is necessary for spontaneous diabetes in BBDP rats is unknown, however, as even gnotobiotic BBDP rats spontaneously develop diabetes (157).

Environmental factors and diabetes expression in BB rats

Environmental factors clearly influence the expression of autoimmune diabetes in BB rats. In BBDP rats, only ~90% of animals ever become spontaneously diabetic (<100% disease concordance). Diabetes is modified in BB rats by cytokine-directed therapy, viruses, and diet.
Figure 3: Balance hypothesis of diabetes in BB rats

BBDR Rat

ART2+ T Cell Depletion
Plus Poly I:C

BBDP Rat

ART2+ T Cell Transfusion

R

β

A
Figure 3: Balance hypothesis of autoimmune diabetes in BB rats. Our laboratory has proposed a teeter-totter model to illustrate the balance that exists in BB rats between ART2− autoreactive cells and ART2+ regulatory cells. In unmanipulated BBDR rats (upper-left) and in BBDP rats that have received infusions of ART2+ regulatory T cells (upper-right), a proper balance exists between regulatory and autoreactive T cells; β cell mass (yellow) is preserved and diabetes is avoided. In contrast, spontaneously diabetic BBDP rats (lower-right) and diabetes-induced BBDR rats (lower-left) are deficient in ART2+ regulatory T cells secondary to iddm1 or ART2-depletion, respectively; the balance is tipped in favor of activated autoreactive T cells, β cell destruction, and diabetes expression.
Cytokine-directed therapy

Current dogma states that autoimmune diabetes results from an overactive Th1 immune response to beta cell antigens, and restoration of the Th1/Th2 equilibrium prevents diabetes (32;33). Data for this paradigm comes mostly from NOD mice, however, and is not necessarily applicable to BB rats. Administration of antibody specific for IFN-\(\gamma\) prevents or delays spontaneous diabetes in BBDP rats (158), but so does treatment with recombinant IFN-\(\gamma\) (159;160). Spontaneous diabetes in BBDP rats is also prevented or delayed by recombinant TNF-\(\alpha\) (161) and IFN-\(\alpha\) (162), but not IL-2 (163).

Viruses

As mentioned previously, viral infection with KRV induces diabetes in BBDR rats (125;126), particularly when combined with depletion of ART2\(^+\) T cells (125;164). KRV does not accelerate diabetes expression in BBDP rats (126), and lymphocytic choriomeningitis virus actually prevents spontaneous diabetes in these animals (165). Poly I:C, a synthetic double-stranded polyribonucleotide that mimics viral ribonucleic acid (4) induces diabetes in BBDR rats when used at high doses (10 \(\mu\)g/g) alone (166) or at low doses (5 \(\mu\)g/g) in conjunction with ART2 T cell-depletion (125). In BBDP rats, spontaneous diabetes is accelerated by high doses of poly I:C (167;168) but actually prevented by low doses of poly I:C (169).
Diet

Modification of dietary protein sources is associated with changes in spontaneous diabetes in BBDP but not BBDR rats (4;170). Diets in which proteins sources are comprised strictly of elemental l-amino acids, casein, or hydrolyzed casein are diabetes-protective in BBDP rats (170-174), and dietary wheat gluten or soy meal abrogate this effect (170;175). In diabetes-induced BBDR rats, however, diets of hydrolyzed casein do not decrease disease incidence (172).

The effect of dietary cow’s milk protein (bovine albumin) on diabetes expression in BBDP rats is still debated (4). Although some investigators have found variably-diabetogenic effects of dietary cow’s milk in BBDP rats (170), the elimination of dietary cow’s milk does not protect BBDP rats from spontaneous diabetes (172).

The mechanism whereby diabetogenic diets influence diabetes expression in BBDP rats may involve 1) changes in the beta cell itself and 2) deviation of the cytokine profile of autoreactive cells (170). Neither the up-regulation of class I by beta cells nor the dominance of IFN-γ in the acutely inflamed islet are observed in BBDP rats fed a diabetes-protective hydrolyzed casein diet (176;177).

Changes in dietary carbohydrate or fat sources do not normally modify the incidence of spontaneous diabetes in BBDP rats (170). An exception to this is the low essential fatty acids (LEFA) diet, which is diabetes-protective (178). The mechanism of protection for the LEFA diet may involve decreased macrophage migration and function (4). It has also been claimed in a single study that oral nicotinamide delayed and prevented spontaneous diabetes in BBDP rats, but this observation has yet to be confirmed (179).
**BB rats as a model of type 1 diabetes mellitus**

The pathogenesis of autoimmune diabetes in BB rats resembles that of human type 1 DM in that both diseases are associated with insulitis, immune-mediated destruction of beta cell, absolute insulin deficiency, and hyperglycemia. Both diseases are also associated with MHC genes, autoantibodies, bone marrow-derived cells, and other autoimmune disorders. Additionally, environmental factors like infection and diet that clearly influence diabetes in BB rats may also contribute to the expression of type 1 DM in humans. Limitations of using the inbred BB rat as a model of human type 1 DM are apparent when one is reminded of the lymphopenia of BBDP rats and the genetic heterogeneity of human beings. These similarities and differences between rat and human autoimmune diabetes must therefore be considered whenever the BB rat is used to model human type 1 DM (7).

**Chapter I, Section 5 - The Intestine as an Immune Organ**

An introduction to the intestinal immune system is necessary in order to address the role that environmental triggers may play in the immunopathogenesis of diabetes in BB rats. The intestinal immune system is considered distinct from the peripheral immune system; much of the dogma surrounding peripheral lymphocytes is inapplicable to intestinal lymphocytes, which are constantly confronted with foreign antigens from sources that are not typically pathogenic (e.g. the diet and commensal flora). This section of the introduction 1) reviews the structure/function relationships in the intestine, 2) addresses barrier function of the intestinal epithelium, 3) describes the different compartments of
the gut-associated lymphoid tissue, particularly intraepithelial lymphocytes, and 4) defines current concepts of mucosal tolerance, the state whereby intestinal lymphocytes remain tolerant to foreign antigens from sources that are not typically pathogenic.

**Anatomy of the small intestine: A structure/function relationship**

The gastrointestinal tract is a collection of organs primarily concerned with digestion and absorption of dietary nutrients. One of these organs, the small intestine, has evolved anatomically from a relatively simple tube in primitive organisms into a highly sophisticated tube in mammals. The terms “outer” or “superficial” refer to lumenal aspects of the intestine, and this nomenclature will be used here.

From superficial to deep, the small intestine is divided histologically into four layers: the mucosa, the submucosa, the muscularis, and the adventitia. The mucosa, which consists of many fingerlike projections called villi, is further subdivided into epithelial and lamina propria layers (Figure 4). The epithelium, which lines the entire lumenal surface of the small intestine, is primarily responsible for nutrient absorption and is just a single cell-layer thick. It is separated from the lamina propria by a basement membrane composed mostly of type IV collagen, laminin (a glycoprotein), and heparin sulfate (a proteoglycan) (180).

**Barrier function of intestinal epithelial cells**

The primary function of intestinal epithelial cells (IECs) is nutrient absorption, but IECs also subserve several functions in the intestinal immune system. First, the epithelium presents a physical barrier to potential pathogens. The apical surface of IECs is
Figure 4: Normal intestinal histology
Figure 4: Histology of intestine. Shown is a hematoxylin- and eosin-stained section of paraffin-embedded rat small intestine (10x), with lumenal aspect labeled. From superficial to deep, the small intestine is divided into four layers: the mucosa, the submucosa (difficult to visualize in this section), the muscularis, and the adventitia (not visible in this section). The mucosa is further divided into epithelial and lamina propria layers that are separated by a basement membrane (represented schematically by a black curve). Also indicated is a Peyer’s patch.
coated with a mucus layer that, besides acting as a physical barrier to microorganisms, contains secreted IgA immunoglobulins (discussed below) and lytic enzymes like defensins (181). Any microorganism successfully penetrating the mucus layer is then confronted with the epithelial surface itself, a continuous sheet of epithelial cells firmly adhered to one another by tight junctions. When intact, these tight junctions prevent the paracellular transit of lumenal material and force gut antigens to be endocytosed and broken down in a transcellular fashion (Figure 5). To cross the epithelium, pathogenic agents must either 1) break down the integrity of these tight junctions or 2) bind to and penetrate the IEC itself. Epithelial permeability is increased by cytokines like TNF-α, IFN-γ, and IL-4 (182-184), and chronically increased epithelial permeability (due to leaky tight junctions) is a hallmark of inflammatory bowel disease (185), celiac disease (88), and food allergy (181).

**Gut-associated lymphoid tissue**

Mucosal villi greatly increase the surface area for absorption in the small intestine. Such an advantage comes at significant risk, however, as many microorganisms seek to exploit the intestinal mucosa as a means of invading a host. An additional complication is the presence in the intestine of many non-pathogenic yet foreign antigens derived from dietary sources or commensal bacteria. Not surprisingly, a specialized intestinal immune system has evolved to provide a first line of defense against infectious agents yet maintain tolerance to antigens from typically non-pathogenic sources. Although poorly understood, by no means is the intestinal immune system insignificant; it is estimated that up-
Figure 5: Trafficking of gut antigens through epithelium

Transcellular

Paracellular
Figure 5: Trafficking of lumenal antigens across the intestinal epithelium. Maintenance of epithelial integrity is critical to normal absorptive and immune function of intestinal epithelial cells (IECs). When intact, tight junctions (*shown schematically as white bulges in epithelial cell membrane*) prevent the paracellular transit of luminal material (*right*) and force gut antigens to be endocytosed and broken down in a transcellular fashion (*left*). Chronically increased epithelial permeability (due to leaky tight junctions) is a hallmark of inflammatory bowel disease, celiac disease, and intestinal hypersensitivity.
wards of $10^{12}$ lymphocytes inhabit the gut, more than any other organ (185). Based on location, intestinal lymphocytes are classified as Peyer’s patch lymphocytes, lamina propria lymphocytes, and intraepithelial lymphocytes.

**Peyer’s patches: Inductive sites for mucosal immunity and tolerance**

Peyer’s patches (PP), discretely organized lymphoid follicles scattered throughout the length of the small intestine, are a major inductive site in the GALT for both active mucosal immune responses and mucosal tolerance (185;186). Cellular components of PPs include M cells, macrophages, dendritic cells, and PPLs, which are comprised mostly of naïve B and CD4+ T cells (187;188). M cells are specialized epithelial cells that blanket the luminal surface of PPs and sample gut material, especially particulate antigens (185;189). M cells transport such material basolaterally where it is taken up by PP macrophages that then migrate into the lymphocyte-rich zones of the Peyer’s patch and present antigen to local T cells (190).

In the Peyer’s patch, activated CD4+ T cells differentiate into TGF-β secreting helper cells (Th3 cells) that promote IgA class switching by PP B cells (191-193). Activated PPLs also upregulate expression of α4β7, an integrin that directs homing of cells to the lamina propria (194;195). Lymphocytes activated in this manner leave the Peyer’s patch, pass through draining (mesenteric) lymph nodes and the thoracic duct, enter the circulation, and eventually seed the lamina propria along the length of the GALT (185;196). The role of Peyer’s patches, IgA, and Th3 cells in mucosal tolerance is addressed later in this chapter.
Effector lymphocytes and dendritic cells of the lamina propria

The lamina propria (LP), which lies just below the basement membrane along the entire GALT, is believed to act primarily as an effector site for normal intestinal immune responses to gut pathogens (185;186). Intestinal LPLs include both B and T cells, the majority of which are differentiated effector cells that have an activated phenotype and express the α4β7 integrin associated with mucosal surfaces (185;197). Dendritic cells also exist in the lamina propria and appear to participate in both mucosal immunity and tolerance (198).

Lamina propria B cells and IgA production

Of the B cells present in the LP, many exist as IgA-secreting plasma cells (185;188). IgA is the predominant antibody type secreted at mucosal surfaces (185;188). In secretions, IgA exists as an immunoglobulin dimer coupled by a protein called J chain and surrounded by a glycoprotein called secretory component. By binding to specific receptors on the basolateral surface of intestinal epithelial cells, secretory component allows IgA to be transported across the intestinal epithelium in a retrograde fashion and secreted into the intestinal lumen. Within the lumen, IgA effectively occupies the docking sites of many microorganisms, thus preventing them from binding to and penetrating the epithelium.

Lamina propria regulatory cells

Recent reports suggest that lamina propria T cells that secrete primarily IL-10 may function as regulators of intestinal immune responses and mucosal tolerance (199).
Mice genetically deficient in IL-10 lack these so-called T regulatory (Tr) 1 cells and exhibit marked inflammatory bowel disease (199;200) that likely represents an overactive immune response to gut antigens from typically non-pathogenic sources (199;201). Likewise, Tr1 clones have been shown to be immunosuppressive in vitro (202).

**Lamina propria dendritic cells**

The lamina propria harbors numerous DCs poised to receive antigens that traverse the epithelium (181;203;204), but the function of these DCs remains debated. Intravenous administration of LPS to mice mobilizes dendritic cells from the intestine to mesenteric lymph nodes (205), where they can activate peripheral T cells (206). Activated mucosal lymphocytes acquire an effector phenotype, home to the lamina propria, and initiate active intestinal immune responses (186). In apparent contrast to these studies, however, lamina propria DCs are associated with regulatory immune responses in the intestine (207), and expansion of dendritic cells with Flt3 ligand actually enhances the induction of oral tolerance (198).

**Intraepithelial lymphocytes**

The presence of lymphocytes in the intestinal epithelium has been recognized for over a century (208), but the origins and functions of this unique GALT population are still under intense investigation (15;209). Intraepithelial lymphocytes are mostly granular lymphocytes, have a distinct phenotype as compared to peripheral lymphocytes, include thymus-dependent and thymus-independent T cell populations, and exhibit both traditional and novel lymphocyte functions.
**Morphology of intraepithelial lymphocytes**

The majority of IELs are granular lymphocytes (210-212), suggesting that IELs are effector cells (209). Granules in IELs contain perforin and granzymes but not histamine (209;212). Accordingly, IELs exhibit various cytotoxic activities (discussed below) (209).

**Phenotype of intraepithelial lymphocytes**

*Preponderance of γδ T cells in the IEL compartment.* In humans, rats, and mice, lymphocytes of the intraepithelial compartment differ phenotypically from those of the periphery. Most IELs are T cells, and cells expressing γδ TCR are much more frequent in IELs than in peripheral lymphocyte populations (15;18;209). In rats, the percentage of IELs expressing γδ TCR has been reported to be 5-20% (213-216), and greater relative numbers of γδ T cells in the IEL are associated with young rats and rats housed under germ-free conditions (217;218).

*Non-conventional CD8 and CD4 expression by IELs.* The majority of IELs are CD8⁺ and, unlike peripheral CD8⁺ T cells, express a homodimeric form of CD8 (CD8αα) (21). In rats, the majority of γδ TCR⁺ IELs express CD8αα (21). CD4⁺ T cells are present in the IEL at lower frequencies than in the periphery, and a percentage of CD4⁺ cells co-express CD8αα (21). Greater relative numbers of CD4⁺ T cells in the IEL are associated with older rats and conventional housing (217).

*Other cell surface markers expressed by IELs.* Intraepithelial and peripheral lymphocytes differ with respect to a number of other cell surface antigens. Most IELs are
CD2\(^{-}\) (213), and CD5 and CD28 tend to be expressed only on CD4\(^{+}\) or CD8\(\alpha\beta^{+}\) IEL sub-populations (217;219). Supporting the hypothesis that IELs represent an effector lymphocyte population, many IELs display markers typically associated with activated and memory T cells (209). In addition, the vast majority of IELs express a novel integrin (\(\alpha\epsilon\beta_{7}\)) that is associated with epithelial lymphocytes (220;221). Expression of \(\alpha\epsilon\beta_{7}\) may account for the observation that adoptively transferred IELs tend to home back to the intestinal epithelium (222;223), and mice genetically deficient in \(\alpha\epsilon\) (CD103) have a reduction in the number of IELs but not peripheral lymphocytes (224).

**Non-T cells in the IEL compartment.** Besides T cells, the IEL compartment is also comprised of NK. The cell surface molecules NKR-P1 and asialo GM1 are expressed by a subpopulation of IELs (54;225;226), and IELs kill NK-sensitive targets (227-229). B cells are not detected in the IEL compartment, and the presence of B cells in isolated populations of IELs is indicative of contamination by LPLs or PPLs (16).

**Ontogeny of intraepithelial lymphocytes**

**Thymus-dependent versus thymus-independent intraepithelial lymphocytes.** Although many IELs clearly arise in a thymus-dependent fashion, many lines of evidence suggest that some IEL subpopulations arise in a thymus-independent fashion (230;231). Neonatally thymectomy prevents the development of peripheral T cells but does not prevent the generation of IELs (232). IELs also develop in athymic mice and rats (233;234). In irradiated thymectomized mice given hematopoietic stem cells, peripheral T cell populations of donor origin are absent, whereas CD8\(\alpha\alpha^{+}\) IEL populations of donor origin
are detectable (235;236). Collections of lymphocytes found at epithelial crypts (so-called cryptopatches) may represent a location of extrathymic IEL ontogeny (237). Although no cell surface marker yet differentiates between thymus-dependent and thymus-independent IELs, CD4⁻ CD8αα⁺ IELs are most consistently of thymus-independent ontogeny (209).

**Growth factors for intraepithelial lymphocytes.** Several growth factors influence the ontogeny of IELs. Interleukin-7, which plays a major role in peripheral lymphocyte development, stimulates the growth and proliferation of IELs (238;239). Stem cell factor (SCF), critical for the development of hematopoietic cells and melanocytes, also influences IEL ontogeny (209). SCF-deficient mice are selectively deficient in γδTCR⁺ IELs and have expanded numbers of CD8⁺ CD4⁺ IELs (240;241).

**Selection processes in the intestinal epithelium.** The thymus-independent ontogeny of some IEL subpopulations poses the possibility that these cells may harbor autoreactive lymphocytes that escape negative selection (186;209;230). Indeed, autoreactive cells are found in the intraepithelial but not peripheral lymphocyte compartment of mice expressing a transgenic TCR specific for autoantigens (15;209;242-244). Supporting the argument that thymus-independent IELs are not subject to the same selection processes as thymus-dependent IELs is the observation that β2-microglobulin- (β2m-) deficient mice, which are unable to assemble a functional MHC class I for thymic selection of CD8⁺ T cells, have normal numbers of γδTCR⁺ but not αβTCR⁺ IELs (245). Non-classical antigens recognized by γδ T cells include mycobacterial glycoproteins, non-peptide phosphates, and the stress-related proteins MIC-A and MIC-B (8;246;247).
**Function of intraepithelial lymphocytes**

**Cytotoxic activity of intraepithelial lymphocytes.** Intraepithelial lymphocytes display several cytotoxic activities *in vitro*. Antigen-independent mechanisms of cytotoxicity include natural killer activity (227-229) and redirected cytotoxicity (248). Antigen-specific CTL activity by IELs against alloantigen (249) and virus-infected targets (250-252) has also been demonstrated.

**Cytokine secretion by intraepithelial lymphocytes.** Reflecting their activated state, mouse intraepithelial T lymphocytes spontaneously secrete cytokines including IFN-γ (Th1-type) and IL-5 (Th2-type) (253;254). Stimulation of their TCR shifts this profile to a predominantly Th1-type profile (255). The significance of these cytokine activities remain unknown, but it has been suggested that IELs modulate local immune responses through the differential secretion of Th1 or Th2 cytokines (15).

**Activation pathways of intraepithelial lymphocytes.** Due to their location, intraepithelial lymphocytes lay directly juxtaposed to enteric antigens, which may account for their activated phenotype (223). Surprisingly, however, IELs exhibit impaired IL-2 production and proliferation to traditional TCR stimuli such as anti-CD3 mAb, Concanavalin A, or phorbol ester plus calcium ionophore (223;256-259). Anti-CD2 and sheep erythrocytes stimulate IL-2 production by human IELs (260), but rat IELs are mostly CD2⁺ (213). In addition, although IELs are present in mice expressing an autoantigen-specific transgenic TCR, there is no histologic evidence of intestinal pathology in these mice suggesting that IELs are tolerized to self-antigen (15;186;209;244).
Interaction of intraepithelial lymphocytes and epithelial cells. Intraepithelial lymphocytes are surrounded by intestinal epithelial cells, and it is generally accepted that these two populations interact (19;185;209;261;262). As already mentioned, IELs are capable of IFN-γ secretion, and this cytokine increases epithelial permeability and up-regulates IEC expression of class I and class II MHC molecules (182;183;263). Communication is bi-directional, as intestinal epithelial cells express IL-7 and SCF, two growth factors that influence IEL ontogeny (262). Recent experiments have shown that γδTCR⁺ IELs recognize stress-related proteins expressed by IECs and may support epithelial integrity through the expression of keratinocyte growth factor (KGF) following epithelial damage (247;264;265). Indeed, mice genetically lacking γδ T cells exhibit increased epithelial permeability (265).

Intraepithelial lymphocytes and mucosal immunity. Although most IEL functions reviewed to this point describe in vitro activities, several in vivo observations strongly support the hypothesis that IELs participate in active intestinal immune responses. Intraepithelial lymphocytes, although present, are drastically reduced in number in germ free animals (266) and increased in parasitized mice (267). Adoptive transfer of IELs from Toxoplasma infected mice protects recipients from disease (268-270). Additionally, virus-specific T cells have been shown to migrate into the IEL compartment following active viral infection (271-274).
Conclusions regarding intraepithelial lymphocytes

Intraepithelial lymphocytes represent a unique and intriguing lymphocyte compartment for several reasons. Unlike peripheral lymphocytes, their physical location in the intestinal epithelium constitutively places them in direct or near-direct contact with foreign antigen. In addition, the preponderance of γδTCR+ and CD8αα+ cells that may respond to alternative antigen presentation and TCR stimuli differentiate them from peripheral lymphocytes. Finally, their function remains poorly characterized in part due to the fact that IELs have been notoriously difficult to isolate for in vitro analyses (275).

Chronic physiologic inflammation in the intestine

As already suggested, the intestinal mucosa is unique histologically because of the presence of large numbers of lymphocytes that appear activated phenotypically and morphologically in absence of overt pathology (185;276). Such an observation has led to the concept that a state of chronic yet “physiologic” inflammation exists in the intestine (185). Physiologic inflammation is not seen in germ free animals but rapidly ensues following microbial colonization of the intestine (277). In addition, active infection and inflammatory bowel diseases are associated with exacerbations of intestinal inflammation (185).

Mucosal tolerance

The vast number of lymphocytes and the huge antigenic load in the gut demands that intestinal lymphocyte populations have a heightened level of tolerance to foreign an-
tigens from sources that are not typically pathogenic (e.g. diet and commensal flora) (181;185). Termed mucosal tolerance, this variation of peripheral tolerance has been the subject of much investigation and appears to involve at least two inductive and three effector mechanisms (Figure 6). Disruption of mucosal tolerance is most likely responsible for a number of intestinal disorders including inflammatory bowel diseases (201) and celiac disease (87;88).

Inductive mechanisms of mucosal tolerance

Inductive mechanisms of mucosal tolerance include antigen uptake and processing by M cells of the Peyer’s patch and intestinal epithelial cells. Whether lumenal antigens in question are of particulate or soluble form may dictate the induction mechanism used. Particulate antigens tend to be processed by Peyer’s patch M cells, and soluble antigens tend to be processed by intestinal epithelial cells (278;279).

Effector mechanisms of mucosal tolerance

Regulatory and/or suppressive mechanisms are believed to maintain mucosal tolerance. Two effector mechanisms are responsible for mucosal tolerance of particulate antigens sampled by Peyer’s patches. First, secretory IgA that is synthesized by lamina propria plasma cells effectively adheres to and occupies the docking sites of many microorganisms and toxins, thus preventing them from binding to and penetrating the epithelium (187;188). Second, antigen processed by Peyer’s patch lymphocytes generates local and systemic CD4+ T cell populations that predominately secrete the immunoregulatory cytokines TGF-β (Th3 cells) or IL-10 (Tr1 cells) (199;280). These effector mechanisms ap-
Figure 6: Mechanisms of mucosal tolerance

1. Peyer's Patch
2. Soluble Antigens
3. ML
4. Particulate Antigens
5.
**Figure 6: Mechanisms of mucosal tolerance.** Mucosal tolerance appears to involve at least two inductive and three effector mechanisms. Inductive mechanisms include antigen uptake and processing by M cells of the Peyer’s patch (*step 1*) or intestinal epithelial cells (*step 2*). Particulate antigens tend to be processed by Peyer’s patch M cells, and soluble antigens tend to be processed by intestinal epithelial cells. Two effector mechanisms are responsible for mucosal tolerance to particulate antigens sampled by Peyer’s patches. First, B cells activated in the Peyer’s patch (*maroon*) isotype switch to IgA, migrate to draining mesenteric lymph nodes (MLN), and differentiate into IgA secreting plasma cells (*step 3*). Second, T cells activated in the Peyer’s patch (*green*) migrate to draining MLN and differentiate into TGF-β-secreting Th3 cells or IL-10-secreting Tr1 cells that operate as regulatory cells either locally (in the mucosa) or systemically. A third effector mechanism may be responsible for mucosal tolerance to soluble gut antigens processed by intestinal epithelial cells. Transcellular antigen processing by intestinal epithelial cells generates CD8+ T cells with immunosuppressive activity (*tan*), although their mechanism of action is currently unknown (*step 5*).
pear to contribute to oral tolerance, a specialized feature of mucosal tolerance (discussed below) (278;279).

An additional effector mechanism of mucosal tolerance is initiated by transcellular trafficking of lumenal antigens by intestinal epithelial cells. These antigens are processed and presented on CD1, which promotes the generation of CD8+ regulatory/suppressor cells in the intestinal mucosa (185;261;281). The relative contribution of this mechanism to oral tolerance is unknown, but oral tolerance is inducible in μMT mice that have a genetically engineered disruption of the transmembrane region of the IgM heavy chain and therefore lack B cells, M cells, and Peyer’s patches (282).

Oral tolerance

Originally described by Wells in 1911 (283), oral tolerance is the phenomenon whereby oral administration of an antigen induces a state of antigen-specific non-responsiveness by systemic lymphocytes (278-280;284;285). Mechanisms of oral tolerance depend on antigen dose: high dose oral antigen induces clonal deletion or anergy of antigen-specific lymphocytes, whereas low dose oral antigen generates regulatory Th2, Th3, or Tr1 lymphocytes locally and systemically (280;285).

Oral administration of candidate autoantigens prevents autoimmunity in several animal models. Oral myelin basic protein decreases exacerbations of induced EAE in mice (280) and rats (286). Oral administration of type II collagen inhibits the development of collagen-induced arthritis in mice (287). Oral insulin, which protects NOD mice from diabetes (288), fails to prevent (289) and may even exacerbate (290) spontaneous
diabetes in BBDP rats. Oral tolerance has been investigated as a potential therapeutic modality for autoimmune disorders including type 1 DM (278-280;284;285;291). Current attempts by researchers to prevent autoimmunity in human subjects by oral administration of candidate autoantigens have been largely unsuccessful, however, underscoring the necessity for a greater understanding of the mechanisms of oral tolerance (278).

Intraepithelial lymphocytes and mucosal tolerance

The role of IELs in mucosal tolerance is unclear (185). In addition to their hypo-responsiveness following TCR stimulation and their expression of immunomodulatory cytokines (15;209), IELs also exhibit suppressive activity in vitro (214;292). Paradoxically, adoptive transfer of IELs has been shown to abrogate oral tolerance (293).

Chapter I, Section 6 - Objectives, Aims, Rationale, and Results

Overall objective of this dissertation

Type 1 diabetes mellitus is a devastating disease that often afflicts children and is associated with significant morbidity and shortened lifespan. Historically, insulin injections have been the only therapeutic modality available for individuals with type 1 DM, but islet cell transplantation is becoming an increasingly viable alternative. Significant obstacles to transplantation still remain, however, so other lines of investigation have sought to decrease the incidence of type 1 DM through preventative measures. Although current attempts at preventing type 1 DM in humans have yet to be clinically efficacious,
animal models of type 1 DM have provided us a greater insight into disease pathogenesis that one day may allow for successful disease prevention.

There is clearly a major role for the immune system in the pathogenesis of type 1 DM in humans and animal models. Both genetics and environment contribute to initiation of disease, but much remains unknown regarding these earliest events that trigger the autoimmune disease process. One major lymphoid organ, the intestine, constantly encounters environmental antigens, some of which influence expression of autoimmunity in animal models. Intestinal intraepithelial lymphocytes are among the first cells of the immune system that encounter potential environmental triggers of autoimmunity. The overall objective of this dissertation is to investigate whether defects in IEL number or function may contribute to the expression of autoimmunity in the BB rat model of type 1 diabetes mellitus. Four specific aims address this objective.

**Specific aim #1 (Chapter II): Improve the methodology for isolation of rat intraepithelial lymphocytes**

Rationale and objectives for Aim #1

It is estimated that upwards of $100 \times 10^6$ intraepithelial lymphocytes inhabit the rat small intestine. Despite these numbers, current methodologies allow for the isolation of only $5 - 15 \times 10^6$ IELs per rat, and IEL preparations are often contaminated with significant numbers of intestinal epithelial cells. In addition, IEL yields generated by these methodologies tend to be inconsistent between similar animals, and the isolation procedure itself is quite time intensive. Moreover, some procedures utilize chemicals like eth-
ylenediaminetetraaceticacid (EDTA) or enzymes like collagenase for the isolation of IELs, and the affect of these perturbants on IEL phenotype or function is unknown. I first sought to develop an efficient new technique for the rapid isolation of large numbers of highly purified rat IELs.

Summary of results for Aim #1

The technique is based in part on the susceptibility of intestinal epithelial cells to hypoxic conditions that leave the basement membrane relatively undisturbed. The sloughed epithelium carries with it the IELs, effectively dissecting away the lamina propria and Peyer’s patches. Using this methodology, upwards of 50 million IELs are recoverable. These cells are of very high purity (>97%) and contain 5 major subsets, including populations of γδ T and NK cells at levels much higher than previously described.

Specific aim #2 (Chapter III): Determine phenotype and function of intraepithelial natural killer cells

Rationale and objectives for Aim #2

Experiments conducted for Aim #1 identified a population of NKR-P1A⁺ CD3⁻ intraepithelial natural killer (IENK) cells. Peripheral natural killer cells function in innate immunity and may have additional regulatory functions. The presence of a potential immunoregulatory population at the intestinal epithelium, site of first contact between environmental antigens and the immune system, prompted me to study further the phenotype and function of these IENK cells.
Summary of results for Aim #2

Using the new and improved protocol for the isolation of rat intraepithelial lymphocytes, I now report the phenotypic and functional characteristics of these IENK cells. The data suggest that the rat IEL compartment harbors a large population of NKR-P1A⁺ CD3⁻ cells that function as NK cells but display an activated phenotype and an unusual cytokine profile that clearly distinguish them from splenic NK cells. Unlike splenic NK cells, IENK cells are CD25⁺ ART2⁺ CD45RC⁻ and spontaneously secrete the cytokines IFN-γ and IL-4, suggesting that these distinctive intraepithelial NK cells may participate in the regulation of mucosal immunity.

Specific aim #3 (Chapter IV): Analyze intraepithelial natural killer cell number and function in BB rats

Rationale and objectives for Aim #3

Diabetes-prone BB rats lack peripheral ART2⁺ regulatory cells due to homozygosity at the lyp gene locus. Diabetes can be induced in diabetes-resistant BB rats by depletion of ART2⁺ regulatory cells and co-administration of an immune stimulant like poly I:C. Although peripheral ART2⁺ regulatory cells appear to influence disease expression in BB rats, less is known about IEL populations in these rats. I therefore investigated IEL number and function in BB rats, giving particular attention to intraepithelial natural killer cells.
Summary of results for Aim #3

I now demonstrate that, prior to diabetes in both BBDP rats and in BBDR rats that have been treated to become diabetic, reduced total number of IELs are present as compared to untreated BBDR and WF rats. Interestingly, there is a selective deficiency of IENK cell number and function in all BB rats as compared to control WF rats. After one week of diabetes-inducing treatments, the number and function of IENK cells in BBDR rats is further reduced. The deficiency of BBDP rat IENK cells can be corrected by engraftment of bone marrow from histocompatible WF donors.

Specific aim #4 (Chapter V): Provide preliminary evidence that defective mucosal tolerance in BB rats potentially contributes to the expression of autoimmune diabetes

Rationale and objectives for Aim #4

Genetics and environment both determine the expression of autoimmune diabetes in rats and humans. One important class of environmental factors – diet and enteromicrobial agents – may participate in this process through the intestinal immune system, which must possess unique mechanisms for dealing with the huge antigenic load of intestinal contents. For example, dietary manipulation influences the expression of autoimmune diabetes in BBDP rats, suggesting a role for mucosal tolerance and the intestinal immune system in the immunopathogenesis of this disease in BBDP rats. In this chapter, I provide preliminary evidence that inappropriate intestinal immune responses are translated into systemic autoimmunity in the BB rat.
Summary of results for Aim #4

I establish that, unlike BBDR and WF rats, BBDP rats are deficient in $\gamma\delta$TCR$^+$ IELs, a population of T cells that may play a role in normal mucosal tolerance mechanisms. I also report preliminary data showing that systemic manifestations of autoreactivity may initiate in the intestine; autoreactive cells appear in mesenteric lymph nodes that drain the intestine prior to cervical lymph nodes, and such cells may initiate a type 2 autoimmune phenomenon driven by IL-4. These findings support the argument that a failure in mucosal tolerance in BBDP rats, perhaps secondary to deficiencies in one or more IEL subpopulations, triggers activation of autoreactive T cells peripherally and contributes to the immunopathogenesis of spontaneous diabetes in BBDP rats.
Summary of Chapter II

Intraepithelial lymphocytes play critical roles in gut immunity. In mice, γδ T cells are a large component of the IEL population. In the rat, γδ IELs are reportedly much less common, but technical issues suggest that previous analyses should be interpreted cautiously. The study of IELs in rats has been impeded by isolation procedures that are lengthy and complex, leading to small cell yields. For this reason, it is possible that rat IELs analyzed in previous studies have not been representative of the entire IEL compartment. I report a new method for the isolation of rat IELs that is based on the selective removal of intestinal epithelial cells under conditions that leave the basement membrane undisturbed. The method is rapid and requires neither enzymatic digestion, nor surgical removal of Peyer’s patches, nor vigorous mechanical manipulation of the intestine. The yield of rat IELs using this method is 5-10 fold greater than that reported for other methods. Morphological and phenotypic analyses demonstrated that the purified cell population is comprised of IELs and is not contaminated with lamina propria or Peyer’s patch lymphocytes. Phenotypic analysis revealed 5 major subsets of IELs based on differential cell surface expression of CD4, CD8, and αβ T cell receptor. Among the αβ T cell re-
ceptor negative cells were populations of γδ T and NK cells present at levels not previ-
ously detected. The isolation of IEL subpopulations using this methodology should fa-
cilitate studies of the function of these cells in gut immunity.

**Introduction to Chapter II**

Intestinal lymphoid cells are present in three physically and functionally distinct
tissue compartments: the lamina propria, Peyer’s patches, and the intraepithelial space
(16). Analyses of cell populations in each of these compartments has been impeded by
isolation methods that yield small numbers of cells and do not reproducibly prevent
cross-contamination (16;216;294-297). This has been particularly true for analyses of rat
IELs, only 5-10 million of which have typically been isolated from one animal.

Different laboratories have reported variable cell yields and IEL phenotypes
(21;214;216;217;298-300). Nonetheless, based on available purification methods, a
working consensus has been reached on the phenotype of rat IELs (54;215-
217;294;301;302). The majority appear to be CD8⁺ (294;301). Many of the CD8⁺ IELs
express the CD8αα homodimer (21). The majority of rat IELs (>95%) also express the
ART2 surface differentiation alloantigen at high density (213;234;298;302-304). Most
investigators have reported that >90% of IELs in euthymic rats express the αβ T cell re-
ceptor (TCR) whereas <10% express the γδ TCR (21;54;213;216;300;302).

The original protocols developed for IEL isolation require dissection to remove
Peyer’s patches, and mincing of the tissue to physically disrupt the epithelium
(16;295;297;305). This method is limited by variable, sometimes extensive, contamina-
tion of the IEL preparation with lamina propria lymphocytes. To address this problem, the original protocols for IEL isolation (16;295) have required enzymatic digestion, chelation with agents like EDTA, panning, and/or magnetic bead separation (16;295;297;305). None of the modifications have proven entirely satisfactory.

An alternative method for IEL isolation everts, ligates, and distends the intestine, which is then incubated with dithioerythritol (DTE) and subjected to repeated rigorous vortexing (210). Using discontinuous Percoll gradient centrifugation, yields of ~5 x 10^6 IELs per rat that are ~90% pure have been obtained (216). Contamination of this IEL preparation with lymphocytes from lamina propria and Peyer’s patches is minimal. The low cell yield, however, makes it uncertain that phenotypic and functional characteristics of IELs prepared in this way are representative of the entire, much larger, IEL compartment (210;216).

To address these issues, I have developed an efficient new technique for the rapid isolation of large numbers of highly purified rat IELs. The technique is based in part on the susceptibility of intestinal epithelial cells to hypoxic conditions that leave the basement membrane relatively undisturbed. The sloughed epithelium carries with it the IELs, effectively dissecting away the lamina propria and Peyer’s patches. IELs prepared in this way are high in yield and purity. They contain 5 major subsets, including populations of γδ T and NK cells present at levels much higher than previously described.
Materials and Methods of Chapter II

Animals

Ten to 16 week old WF (RT1\textsuperscript{u}, ART2\textsuperscript{b}), BN (RT1\textsuperscript{u}, ART2\textsuperscript{b}), DA (RT1\textsuperscript{a}, ART2\textsuperscript{b}), and F344 (RT1\textsuperscript{v}, ART2\textsuperscript{b}) rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN). DR-BB/Wor (RT1\textsuperscript{u}, ART2\textsuperscript{b}) and PVG.RT1\textsuperscript{u} (RT1\textsuperscript{u}, ART2\textsuperscript{a}) rats were obtained from BMR, Inc. (Worcester, MA). Except where noted, all analyses were performed using 10-12 week old WF rats.

Rat tissue donors were killed in an atmosphere of 100% CO\textsubscript{2}. Animals in this study were continuously monitored for infection and were serologically free of Sendai virus, pneumonia virus of mice, sialodacryoadenitis virus, rat corona virus, Kilham rat virus, H1 (Toolan's virus), GD7, Reo-3, Mycoplasma pulmonis, lymphocytic choriomeningitis virus, mouse adenovirus, Hantaan virus, and encephalitozoon cuniculi. Animals of either sex were studied. All animals were maintained in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996) and the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical Center.

Antibodies

The hybridoma cell line secreting the 6A5 anti-ART2b (rat IgG1) is maintained in our laboratory. Monoclonal antibodies (mAbs) directed against αβTCR (clone R73),
CD8α (clone OX-8), CD8β (clone 341), CD4 (clone OX-35), B220 (CD45R, clone HIS24), CD25 (IL-2 receptor α-chain, clone OX-39), CD2 (LFA, clone OX-34), CD3 (clone G4.18), CD5 (clone OX-19), CD28 (clone JJ-319), CD45RC (OX-22), NKR-P1A (10/78), anti-γδ T cell receptor (clones V45 and V65), and the appropriate isotype controls for each mAb were obtained from BD PharMingen (San Diego, CA).

Flow cytometry

Single, two, and three-color flow cytometric analyses were performed as previously described (121;306). Briefly, 1 x 10⁶ viable lymph node cells or IELs were reacted with a mixture of fluorescein isothiocyanate- (FITC-), biotin-, and/or phycoerythrin- (PE-) conjugated mAbs for 20 min. at 4°C. All IEL analyses were performed after nylon wool or Percoll-density gradient purification to remove the large number of contaminating dead epithelial cells. Cells were then washed, reacted with Cy-Chrome®-conjugated streptavidin (BD PharMingen) to visualize biotinylated mAbs, washed again, and fixed with 1% paraformaldehyde. FITC-, biotin- and PE-conjugated isotype control immunoglobulins were used for all analyses. Cells were analyzed using a FACScan® instrument (Becton Dickinson, Sunnyvale, CA). Lymphoid cells were identified by their forward and side light-scatter profiles and the detection of ART2, αβTCR, or γδTCR staining. The composite phenotypes of lymphoid cell subsets for up to four antigenic markers were determined by combining the results of multiple 2 and 3-color analyses of replicate samples of lymphocytes, each incubated with a different combination of antibodies.
To characterize the subpopulation containing NKR-P1^+ cells using three color flow cytometry, I exploited the unique staining patterns of CD8α, CD4, and αβTCR on IELs. Because <<1% of IELs express the CD8α^- CD4^- αβTCR^+ phenotype, further analysis of CD8α^- CD4^- αβTCR^- IELs was possible by labeling with CD8α and CD4, selecting the CD8α^- CD4^- IEL subset, and performing three color analysis using a third fluorochrome.

To characterize the CD8^+ CD4^- αβTCR^- subpopulation, I again exploited the unique staining patterns of CD8α, CD4, and αβTCR on IELs. Because CD8α^+ CD4^- αβTCR^- IELs comprise <<1% of IELs, it was possible to stain for CD8α and αβTCR, select the CD8α^+ αβTCR^- cells, and examine this population using a third fluorochrome, e.g. anti-γδTCR using the V65 mAb. IELs expressing the CD8α^+CD4^-αβTCR^- phenotype were shown to be γδTCR^+ (see Figure 13). Subpopulations of cells with this phenotype were therefore identifiable by dual labeling for V65 and a second fluorochrome.

Strategies for analyzing the remaining three αβTCR^+ populations were as follows. Because CD8α^- CD4^+ αβTCR^- cells comprise <1% of total IELs, CD8α^- CD4^+ αβTCR^+ IELs could be analyzed by staining for CD8α and CD4, selecting for CD8α^- CD4^+ cells, and examining this population using a third fluorochrome. Because CD8α^+ CD4^+ αβTCR^- cells comprise <1% of total IELs, CD8α^+ CD4^+ αβTCR^+ IELs could be analyzed by staining for CD8α and CD4, selecting for CD8α^+ CD4^+ cells, and examining this population using a third fluorochrome. Finally, because CD8α^- CD4^- αβTCR^+ cells comprise <1% of total IELs, CD8α^+ CD4^- αβTCR^+ IELs could be analyzed by staining
for CD4 and αβTCR, selecting for CD4⁻ αβTCR⁺ cells, and examining this population using a third fluorochrome.

**Intraepithelial lymphocyte preparation**

As shown schematically in Figure 7, IELs were prepared by dissecting free of the pancreas the proximal 3-4 cm of intestine including the duodenum, and the bowel was transected at the level of the pyloric sphincter. The remainder of the small intestine was dissected free of omentum by gentle blunt dissection, transected 1 cm proximal to the ileo-cecal junction, and removed *en bloc*. Care was taken to avoid perforation of the intestine during dissection. Intestinal contents were removed by gently flushing the lumen with 50 ml of cold (4°C) RPMI-1640 over a 3 minute period using a 1 mm gavage tube inserted into the proximal lumen. Flushing to remove fecal material was performed gently, at low pressure, and without squeezing the bowel, so as not to perforate the bowel wall or disturb the epithelial mucus coating that contains large numbers of lymphoid cells. The intestine was then immersed in cold RPMI 1640 and incubated on ice for 60-120 minutes. IELs were recovered in a two step procedure.

Step 1: After cold incubation, the intestinal lumen was flushed at low pressure (over several minutes) with 10 ml of medium consisting of HEPES-buffered HBSS containing 1 mM DTE and 10% Fetalclone® I (a defined neonatal bovine serum, HyClone, Logan, UT) at 37°C. The lumenal eluate was saved.

Step 2: The weakly adherent epithelial cells and mucus coating remaining in the intestine were then squeezed out by manual compression as follows. The proximal end of
Figure 7: Schematic for the isolation of rat intraepithelial lymphocytes
Figure 7: Schematic of IEL isolation protocol. The entire small intestine is removed and gently flushed with cold RPMI-1640 to remove lumenal contents and then incubated in ice-cold RPMI-1640 for 60-120 minutes to delaminate epithelial cells and IELs from the basement membrane. Detached cells were recovered by flushing the intestine two times with a total of 50 ml of modified HEPES-buffered HBSS media at 37°C. IELs were then purified by nylon wool purification or Percoll density gradient centrifugation, and viable cells exhibiting lymphoid morphology were quantified by the method of Trypan blue using a hemocytometer. Listed are yields and purity for IELs isolated from adult WF rats.
the intestine was grasped between thumb and index finger and suspended vertically over a polystyrene container. The thumb and index finger of the other hand were then slid along the entire length of the gut, compressing it from duodenum to distal ileum to extrude mucus and cellular contents. Care was taken to avoid tearing the tissue.

The specimen was subjected to a total of 5 cycles of flushing (Step 1) and squeezing (Step 2) to extrude all intra-lumenal contents. The contents of all five 10 ml flushes and extrusions were combined in one 50 ml polystyrene test tube. The tube was then gently swirled to suspend all cells. Clumps of material present in the dispersed sediment were allowed to settle for 10 min. The supernatant containing single dispersed epithelial cells and IELs was then removed by pipetting into a second tube and centrifuged at 350 x G for 5 minutes. The resulting pellet, containing IELs, erythrocytes, and epithelial cells, was then recovered and subjected to either nylon wool or density gradient centrifugation for further purification.

The viability of IELs prepared in this way was assessed immediately after their recovery by the method of Trypan blue exclusion. I observed that >95% of the lymphoid cells were viable. In contrast, >90% of the epithelial cells in the freshly isolated population failed to exclude the dye.

**Lymph node cell preparation**

Cervical and mesenteric lymph nodes were removed and single cell suspensions were prepared by gentle extrusion through stainless steel sieves into cold medium (306). Intestine was removed prior to the recovery of mesenteric lymph nodes.
Nylon wool purification of IELs

Nylon wool purification of IEL suspensions was performed as described previously (16). Briefly, pelleted cells were suspended in 10 ml cold RPMI containing 5% Fetalclone® I, and passed through a loosely packed nylon wool column at the rate of 1 drop every 1-2 seconds. The column was washed with cold medium containing 5% Fetalclone® I. The number of viable cells with lymphoid morphology was quantified using a hemocytometer and the method of Trypan blue exclusion.

Discontinuous percoll density gradient purification of IELs

A two step discontinuous Percoll density gradient centrifugation procedure was used in some experiments (216). Briefly, pelleted cells were re-suspended in 35 ml RPMI containing 5% Fetalclone® I at room temperature; 15 ml of Percoll was then added to generate a 30% Percoll solution. The cell suspension was centrifuged at 350 x G for 15 minutes at room temperature. Non-viable epithelial cells were excluded from the 30% Percoll solution, whereas erythrocytes, lymphocytes, and viable epithelial cells localized to the pellet. The cell pellet was suspended in 15 ml of a 45% Percoll solution. A second 75% Percoll solution was layered under the 45% solution, and the discontinuous Percoll gradient was then centrifuged at 350 x G for 30 minutes. Viable epithelial cells were recovered from the supernatant at the 45% interface. Erythrocytes and debris migrated into the layer of 75% Percoll. Viable IELs were collected from the interface between the 45% and 75% Percoll layers. The number of viable cells that excluded Trypan blue was quantified using a hemocytometer and was >95% in all cases.
Histology

Samples of 16 week old WF rat intestine for histological analysis were taken at three different stages of the IEL isolation procedure. The first was obtained immediately after the animal was killed in an atmosphere of 100% CO₂. The second was recovered after incubation at 4°C, but before perfusion with medium at 37°C. The third was recovered after 5 perfusions and extrusions. Specimens were immediately fixed in 10% buffered formalin or Bouin’s fixative. Paraffin-embedded tissue sections of all samples were stained with hematoxylin and eosin and examined by light microscopy. Samples of the extruded intra-lumenal contents were embedded in paraffin, stained with hematoxylin and eosin, and examined by light microscopy. Cytospin-prepared slides of Percoll-purified IEL were prepared and stained with Giemsa for analysis of purity.

Results of Chapter II

Intestinal morphology before and after IEL isolation: Peyer’s patches and lamina propria remain intact

Samples of WF intestine were taken at different stages of the isolation procedure and examined by light microscopy. Figure 8, Panel A shows the anatomy of normal 16 week old WF rat jejunum. Panels B, C, and D show progressive changes in gut morphology during processing for the recovery of IELs. They document delamination (Panel B) and removal (Panels C and D) of the intestinal epithelium from the basement membrane. Panels C and D also document the presence of intact lamina propria and Peyer’s patches in the residual processed intestine. Panel E shows the morphology of cells present in the
Figure 8: Histology of IEL methodology
**Figure 8:** Histological analysis of gut and isolated cell populations at various stages of IEL preparation. A: Normal 16 week old WF rat jejunum showing epithelium closely apposed to lamina propria (x 10). B: Appearance of rat jejunum after incubation for 120 min at 4°C. The epithelial layer is delaminating from the basement membrane along cleavage planes (arrows) with no disruption of the lamina propria (x 10). C and D: Appearance of the lumenal surface of the jejunum after completion of the five cycles of intestinal perfusion and extrusion. Panel C shows that most epithelial cells are absent from the villi, although some remain (x 20). The arrow identifies a point of demarcation between residual epithelium and denuded, but intact, lamina propria from which the epithelium has sloughed. Panel D shows additional areas of lamina propria (arrows) and an intact Peyer’s patch (asterisk); both are denuded of gut epithelium (x 20). E: Hematoxylin and eosin stained paraffin-embedded sample of the cells present in the crude lumenal perfusate of the intestine. Small round cells consistent with lymphocytes are abundant, as are larger epithelial cells (x 20). F: Giemsa-stained cytospin sample of Percoll density gradient-purified IELs. Larger cells containing granules are indicated by arrows. (x 60).
unpurified lumenal washing after five cycles of extrusion. Numerous mononuclear cells are present together with epithelial cells.

**Yield and purity of IEL preparations**

IEL yield and purity were measured after nylon wool or Percoll purification of the lumenal washings. Nylon wool purification resulted in higher yields of IELs but slightly lower purity than did Percoll purification.

**Nylon wool purification**

Yields following nylon wool purification averaged $33.6 \times 10^6$ viable IELs per rat (range 27.2 to $48.0 \times 10^6$, N=7, Table 3). Flow cytometric analysis of the purified cells based on forward- and side-scatter demonstrated that lymphocytes comprised ~80% of total cells (Table 3 and Figure 9).

**Discontinuous percoll gradient purification**

Compared with nylon wool purification, discontinuous Percoll density gradient centrifugation resulted in lower IEL recovery but greater purity. Average yield was $21.8 \times 10^6$ viable IELs per rat (range 17.8 to $26.6 \times 10^6$, N=4, Table 3). Flow cytometric analysis revealed that >97% of cells were lymphocytes (Figure 9). Analysis of the light-scattering properties of both the nylon wool and Percoll-purified lymphoid cells revealed that they were slightly larger than peripheral lymph node cells (Figure 9). Morphological analyses of Percoll-purified cells revealed large numbers of mononuclear cells similar in
# TABLE 3

YIELD OF PURIFIED WF RAT INTRAEPITHELIAL LYMPHOCYTES

<table>
<thead>
<tr>
<th>Purification Technique</th>
<th>Percentage of Cells in Lymphoid Gate</th>
<th>Lymphoid Cells Recovered (x 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nylon Wool</td>
<td>79.9 ± 8.9 (N=7)</td>
<td>33.6 ± 8.1 (N=7)</td>
</tr>
<tr>
<td>Percoll Gradient</td>
<td>97.4</td>
<td>21.8 ± 3.6 (N=4)</td>
</tr>
</tbody>
</table>

*Legend to Table 3:* The total number of viable mononuclear cells present in the purified preparations was determined using a hemocytometer. Contaminating large epithelial cells were excluded. The percentage of lymphocytes present in the purified cell populations was measured by flow cytometry on the basis of forward- and side-scatter. The number of individual samples tested is shown in parenthesis except in the case of the percentage of lymphocytes present in Percoll-purified cells. In this instance, the percentage was measured on a single pool of IELs from 4 animals. Data are expressed as the mean ± s.d.
Figure 9: Flow cytometric profiles based on light scatter of WF rat IELs and peripheral lymph node cells
Figure 9: Flow cytometric profiles based on light scatter of WF rat IELs and peripheral lymph node cells. Forward scatter (horizontal axis) is plotted against side scatter (vertical axis). Shown are representative profiles of nylon wool-purified WF rat IELs (top panel), Percoll gradient purified IELs (middle panel), and peripheral lymph node cells (bottom panel). The gate was established on the peripheral lymph node cell population. Comparable results were obtained in 4-7 individual animals (Table 3).
appearance to unfractionated lymph node cells (Figure 8, Panel F). Some of these mononuclear cells are large and appear to contain granules.

**Phenotype of purified IELs**

The phenotype of purified intestinal lymphocyte populations was determined by flow cytometry. In preliminary studies, it was determined that the phenotypes of the intestinal lymphoid cells purified by nylon wool and by Percoll density gradient centrifugation were qualitatively similar (data not shown). The phenotypic analyses presented below were performed on nylon wool-purified cells.

**Isolated cells are predominantly ART2⁺ CD8α⁺ B220⁻**

The phenotype of cells isolated using my methodology displayed the consensus phenotype of IELs (54;216;217;294;301;302). Consistent with previous reports (213;298;302;304), the percentage of ART2⁺ cells was very high (92 ± 3%, Figure 10). More than half (62 ± 5%) of the IELs expressed CD8α, and ~60% of the CD8α⁺ cells were CD8α⁺β⁻. Less than 5% of the IEL population was B220⁺ (i.e. CD45R⁺ B cells).

Consistent with previous reports (213;298;302), the ART2⁺ cells in the IEL population (Figure 10) expressed ART2 at a surface density ~10-fold greater than lymph node cells (Figure 10). Taken together, these phenotypic and morphological results confirm that my methodology selectively isolates IELs while excluding contaminating lamina propria and Peyer’s patch lymphocytes, a large number of which express B220.
Figure 10: Flow cytometric analysis of ART2b expression on WF rat IELs and peripheral lymph node cells
Figure 10: Flow cytometric analysis of ART2b expression on WF rat IELs and peripheral lymph node cells. Fluorescence intensity (horizontal axis) is plotted against cell number (vertical axis). Shown are representative profiles of ART2b expression on lymphocyte-gated nylon wool purified WF rat IELs (left panel) and WF rat cervical lymph node cells (right panel). The insets show fluorescence profiles of the same cell populations reacted with FITC-conjugated isotype control immunoglobulin. Comparable results were obtained in 3-6 individual animals.
Differential expression of CD4, CD8, and αβTCR classifies rat IELs into five major subpopulations

Differential expression of CD8α, CD4, and αβTCR expression segregated >99% of the total population of purified IELs into one of five major non-overlapping subsets (Table 4, Figure 11). As expected on the basis of previous reports (21;54;215;217;294;301), CD8α⁺CD4⁻αβTCR⁺ cells comprised a major subpopulation (32%) of IELs (Figure 11). CD8α⁻CD4⁺αβTCR⁺ and CD8α⁺CD4⁺αβTCR⁺ cells comprised ~9% and ~6% of IELs, respectively. Surprisingly, CD8α⁻CD4⁻αβTCR⁻ and CD8α⁺CD4⁻αβTCR⁻ cells comprised ~30% and ~25% of the purified IEL population, respectively (Figure 11). The three remaining permutations of these three surface antigens (CD8α⁺CD4⁺αβTCR⁻, CD8α⁻CD4⁺αβTCR⁻, and CD8α⁻CD4⁻αβTCR⁻) together were observed on less than 1% of the total population (Figure 11, Table 4).

Natural killer cells and γδTCR⁺ T cells are abundant in the two αβTCR⁻ rat IEL populations

Unexpectedly, 23.7 ± 4.2% (N=6) of all IELs were observed to express the NKR-P1 antigen characteristic of NK cells and NK T cells (Figure 12, top panel). To determine if any of these were NK T cells, I characterized further the each of the five IEL subpopulations. I observed that 64.1 ± 9.1% (N=6) of CD8α⁻CD4⁻IELs (~99% of which are αβTCR⁻, see above) were NKR-P1⁺, accounting for most (87.1 ± 6.5%) of the NKR-P1 IEL population (Figure 12, middle panel). This phenotype identifies them as NK cells. In
TABLE 4

EXPRESSION OF CD4, CD8α, AND αβTCR DEFINES FIVE SUB-POPULATIONS OF WF RAT INTRAEPITHELIAL LYMPHOCYTES

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>CD8α⁺ CD4⁺</th>
<th>CD8α⁺ CD4⁻</th>
<th>CD8α⁺ CD4⁻</th>
<th>CD8α⁻ CD4⁺</th>
<th>CD8α⁻ CD4⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>αβTCR⁺</td>
<td>5.7 ± 2.8</td>
<td>31.8 ± 3.3</td>
<td>24.2 ± 4.1</td>
<td>8.6 ± 1.1</td>
<td>30.5 ± 5.1</td>
</tr>
<tr>
<td>αβTCR⁻</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Legend to Table 4: Nylon wool-purified WF rat IELs were analyzed by three-color flow cytometry for the expression of CD4, CD8α, and αβTCR (see Methods). Five of the eight possible combinations of these markers comprised >99% of all IELs. The percentage of CD8α⁺ CD4⁺ αβTCR⁻, CD8α⁻ CD4⁺ αβTCR⁻, and CD8α⁻ CD4⁻ αβTCR⁺ IELs was <1% of total cells. Each data point represents the mean ± s.d. of 6 individual samples.
Figure 11: Three color flow cytometric analysis of the expression of CD4, CD8α, and αβTCR by WF rat IELs
Figure 11: Three color flow cytometric analysis of the expression of CD4, CD8, and αβTCR by WF rat IELs. The expression of CD4 (vertical axis) and CD8 (horizontal axis) was determined on lymphocyte-gated, nylon wool purified αβTCR\(^+\) (left panel) and αβTCR\(^-\) IELs (right panel). Shown is a single representative analysis of the five major IEL sub-populations defined by these surface markers. Comparable results were obtained in six individual analyses (Table 4).
Figure 12: Flow cytometric analysis of NKR-P1 and ART2b expression on WF rat IELs
Figure 12: Flow cytometric analysis of NKR-P1 and ART2b expression on WF rat IELs. Fluorescence intensity (horizontal axis) is plotted against cell number (vertical axis). Shown are representative profiles of NKR-P1 expression on lymphocyte-gated nylon wool purified WF rat IELs (top panel), NKR-P1 expression on CD8^- CD4^- cells (middle panel) and ART2b expression on NKR-P1^+ IELs (bottom panel). The insets show fluorescence profiles of the same cell populations reacted with FITC-conjugated isotype control immunoglobulin. Comparable results were obtained in 6 individual animals.
addition, I observed that the majority of NKR-P1* NK IELs express high levels of cell surface ART2 (Figure 12, bottom panel). In dual label analyses, I observed that ~8% of the IELs co-expressed CD3 and NKR-P1, a phenotype that is consistent with that of NK T cells. Additional dual-label analyses using anti-CD8α and anti-CD3 antibodies revealed that <1.5% of the IEL population is CD3−CD8α+, making it unlikely that the NK IEL population is significantly contaminated with CD8+ peripheral NK cells.

A second surprising observation was that 24.4 ± 3.3% (N=6) of all IELs were labeled by the V65 anti-γδTCR mAb (Figure 13, left panel). Because αβTCR and γδTCR are not co-expressed on T cells, and because the data above indicate that the great majority of CD8−CD4−αβTCR− cells are NK cells, I next tested the hypothesis that the γδTCR+ cells would be found in the CD8+CD4−αβTCR− subpopulation. I observed that 94.9 ± 1.5% of the CD8α+αβTCR− population was γδTCR+ (Figure 13, right panel), accounting in turn for 95% of all γδTCR+ IELs.

To confirm that this high percentage of γδTCR+ IELs was not specific to the WF rat, I also measured the number of CD8+αβTCR− IELs isolated in the same way from rats of five additional strains. The number of CD8+αβTCR− IELs in these strains was 19.0 ± 6.3% (range 10.0% to 27.8%, N=9 samples). This percentage is comparable to that observed in WF rats of the same age (Table 4).

I also measured for the percentage of γδTCR+ IELs using the V45 mAb that is known to react with a subset of γδTCR+ cells (307). I observed that V45 labeled 2.0 ± 0.4% (N=6) of total IELs and 7.1 ± 1.1% (N=6) of V65+ IELs (data not shown).
Figure 13: Flow cytometric analysis of γδTCR expression on WF rat IELs
**Figure 13:** Flow cytometric analysis of γδTCR expression on WF rat IELs. Fluorescence intensity (horizontal axis) is plotted against cell number (vertical axis). Cells were reacted with the V65 mAb to identify γδTCR⁺ cells. Shown are representative profiles of γδTCR expression on lymphocyte-gated nylon wool purified total WF rat IELs (left panel) and on CD8⁺ αβTCR⁻ cells (right panel). The insets show fluorescence profiles of the same cell populations reacted with Cy-Chrome⁰-conjugated isotype control immunoglobulin. Comparable results were obtained in 6 individual animals.
Phenotypic characteristics of the five major IEL subsets

Each the five IEL populations defined on the basis of differential expression of CD8α, CD4, and αβTCR was further analyzed for expression of seven additional phenotypic markers (Table 5). To do so, I again exploited the unique staining patterns of CD8α, CD4, and αβTCR on IELs (see Methods).

The expression of CD2, CD3, CD5, CD25, CD28, CD45RCintermediate, and ART2b on each of the five major subpopulations of WF IELs is shown in Table 5. Consistent with previous reports (213;298;302), ART2 was expressed uniformly and at high density on 4 of the 5 IEL subsets. In contrast, its expression on the CD4+ CD8- IEL subset was heterogeneous and at low density, a pattern also observed on CD4+ peripheral T cells (308).

Consistent with previous reports (213), I observed that, unlike peripheral T cells, few IELs expressed CD2. Those cells that were CD2+ were found in the CD4+ CD8- subpopulation. Only one subpopulation failed to express CD3; this was the CD4- CD8- subset, which also failed to express αβTCR or γδTCR and, as documented above, was comprised predominantly of NK cells.

Most IELs, including γδTCR+ IELs, appeared to be in an activated state as evidenced by their uniform expression of CD25. Surprisingly, despite the expression of CD25, many IELs, including those in the γδTCR+ subset, did not express CD28. CD28 is a co-stimulatory molecule expressed constitutively on most peripheral T cells and at high levels on activated T cells.
<table>
<thead>
<tr>
<th></th>
<th>CD8⁺ CD4⁺</th>
<th>CD4⁻ αβTCR⁺</th>
<th>CD8⁺ αβTCR⁻</th>
<th>CD8⁻ CD4⁺</th>
<th>CD8⁻ CD4⁻</th>
<th>Total IELs</th>
<th>CD4⁺</th>
<th>LNCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>ART2b</td>
<td>96.3 ± 3.2</td>
<td>98.9 ± 0.5</td>
<td>99.3 ± 0.3</td>
<td>78.4 ± 3.3</td>
<td>77.9 ± 10.2</td>
<td>91.6 ± 2.7</td>
<td>83.9 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>CD2</td>
<td>14.8 ± 9.9</td>
<td>2.3 ± 0.5</td>
<td>2.6 ± 1.6</td>
<td>59.4 ± 6.0</td>
<td>1.1 ± 0.2</td>
<td>7.2 ± 0.9</td>
<td>99.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td>99.9 ± 0.2</td>
<td>99.9</td>
<td>99.9</td>
<td>93.4 ± 1.7</td>
<td>22.3 ± 14.0</td>
<td>73.7 ± 4.8</td>
<td>99.5 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>CD5</td>
<td>95.1 ± 3.8</td>
<td>58.0 ± 9.3</td>
<td>5.7 ± 3.0</td>
<td>94.2 ± 1.8</td>
<td>5.0 ± 4.2</td>
<td>33.7 ± 8.7</td>
<td>98.1 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>CD25</td>
<td>78.2 ± 7.9</td>
<td>90.3 ± 4.3</td>
<td>98.6 ± 0.4</td>
<td>79.3 ± 2.0</td>
<td>72.2 ± 13.6</td>
<td>84.5 ± 5.4</td>
<td>8.6 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>CD28</td>
<td>71.5 ± 11.0</td>
<td>15.6 ± 2.3</td>
<td>5.0 ± 2.2</td>
<td>86.5 ± 2.5</td>
<td>2.3 ± 0.5</td>
<td>16.5 ± 2.0</td>
<td>97.3 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>CD45RC冲洗</td>
<td>13.1 ± 10.0</td>
<td>34.4 ± 9.4</td>
<td>7.6 ± 2.5</td>
<td>8.8 ± 1.3</td>
<td>4.5 ± 2.5</td>
<td>13.8 ± 3.1</td>
<td>56.4 ± 2.1</td>
<td></td>
</tr>
</tbody>
</table>
Legend to Table 5: Subset percentages for each major IEL subset, total IELs, and peripheral lymph node cells (LNCs). Each the five nylon wool-purified WF rat IEL populations defined in Table 4 on the basis of differential expression of CD8α, CD4, and αβTCR was further analyzed for expression of the seven additional phenotypic markers listed in the leftmost column. Phenotypes shown in parentheses are inferred, not directly determined (See Methods). For comparison, the expression of these seven additional phenotypic markers was also analyzed using samples of total IELs and CD4+ peripheral T cells. Each IEL and lymph node data point represents the mean percentage ± s.d. measured in 6 and 3 individual samples, respectively. The percentages of CD4+ peripheral lymph node cells expressing each of these seven phenotypes were essentially identical to the percentages expressed by CD8α+ peripheral lymph node cells (data not shown).
Most subsets of IELs contained low percentages of CD45RC\textsuperscript{intermediate} cells. Only the CD4\textsuperscript{−}αβ\textsuperscript{+}TCR\textsuperscript{+} subset contained levels of CD45RC\textsuperscript{intermediate} cells (34%) that approached those observed in peripheral CD4\textsuperscript{+} T cell populations (56.4%, Table 5). The percentages of CD4\textsuperscript{+} peripheral lymph node cells expressing each of these seven phenotypes was essentially identical to the percentages expressed by CD8\textsuperscript{+} peripheral lymph node cells (data not shown).

The percentage of CD5\textsuperscript{+} cells varied among the different IEL subsets. CD5 was expressed by a very low percentage of γδ\textsuperscript{+}TCR\textsuperscript{+} and CD8\textsuperscript{−}CD4\textsuperscript{−} IELs, approximately half of CD4\textsuperscript{−}αβ\textsuperscript{+}TCR\textsuperscript{+} IELs, and the great majority of CD8α\textsuperscript{+}CD4\textsuperscript{+} and CD8α\textsuperscript{−}CD4\textsuperscript{+} IELs.

**Discussion of Chapter II**

The analysis of rat IEL populations recovered using my new isolation procedure has generated three principal findings. First, the method leads to the recovery of 5-10 fold more IELs than are recovered using older methods (213;216;292;294;299). The method is reproducible and yields lymphoid cell preparations that are of high purity and exhibit the consensus morphologic and phenotypic characteristics of true IELs. Second, analysis of rat IELs prepared using the new method revealed the presence of γδ T cells at levels not previously appreciated. In general, ∼25% of the cells in each preparation were γδ\textsuperscript{+}TCR\textsuperscript{+} cells. Third, three-color microfluorometric analysis classified >99% of the rat IELs into one of 5 major subpopulations based on their expression of TCR, CD4 and CD8. Surprisingly, one of these subpopulations was comprised predominantly of NK cells.
Isolation technology

The new method that I describe is rapid and reproducibly yields large numbers of viable IELs. The technique is based on the propensity of intestinal epithelial cells to slough off the gut basement membrane after exposure to cold and hypoxic culture conditions that leave the basement membrane relatively undisturbed. The sloughed epithelium carries with it the IEL population, effectively dissecting away the lamina propria and Peyer’s patches.

The procedure yields a population of cells that fulfill three criteria that define a successful IEL purification process. First, the lamina propria and Peyer’s patches remain intact throughout the isolation procedure. Second, the morphology and light-scattering properties of the recovered cells are those of intestinal epithelial lymphocytes. Finally, the phenotype of the purified cells is consistent with that of an IEL population (216;304).

The methodology is noteworthy for two additional reasons. The first is its technical simplicity. It requires no special reagents or equipment, nor does it require extensive or vigorous mechanical disturbance of the intestine. It takes only 2-3 hours, yet reproducibly generates large numbers of pure IELs.

Second, and more importantly, the methodology appears to generate cell populations that are more representative of the entire IEL compartment than are populations generated by older methods. I believe this to be the case because my method overcomes two obstacles that have previously impeded recovery of pure IELs. First, it dissects away epithelial cells from the intestine without disrupting the basement membrane. This prevents contamination of IEL preparations with lamina propria and Peyer’s patch lympho-
cytes. Second, it eliminates many purification steps that have previously been required. These include incubation in chelating agents, mincing, vigorous shaking, eversion of the intestine, and panning (16). Each of those procedures can cause substantial non-specific cell losses, leading to low cell yields. Previously published isolation protocols yield 5-15 million IELs per rat (213;216;292). The IEL population in the rat has, however, been estimated to be 10-20 fold larger (309;310). The method I have developed routinely yields 30-50 million IELs per rat.

**γδTCR⁺ rat IELs**

Consistent with previous reports (213), many of the IELs isolated with my procedure were CD8⁺ ART2⁺ αβTCR⁺ (21;216;217;304). Few were CD4⁺ CD8⁺ or CD8⁻ CD4⁺. A major new finding, however, was the identification of a population of γδTCR⁺ IELs that comprise ~25% of total IELs recovered. Previously it has been reported that V65-stained γδTCR⁺ IELs comprise either <10% (54;216;300) or 10-20% (214;215) of the rat IEL population. The percentage of γδTCR⁺ cells has been reported to be 40-50% of the total IEL population in the mouse (18), and the present data suggest that the rat IEL population may be more like that of the mouse than previously thought.

It could be argued that the high percentage of γδTCR⁺ IELs I observed reflects a selective loss of αβTCR⁺ IELs due to hypoxic isolation conditions, but I think such an explanation is unlikely. First, the absolute number of αβTCR⁺ cells that I recovered was higher than that produced by other methods. In addition, analysis of lymphoid cells immediately after the flushing step in my procedure revealed >90% viability.
I recognize, however, that a number of factors other than isolation methodology could influence γδTCR⁺ IEL percentages. For example, there could be compartmentalization of γδTCR⁺ cells in the gut, and the percentage of these cells could vary along its length. My procedure isolated IELs from the entire small bowel, and it is possible that previous reports based on the older methodology simply used different regions of the bowel. In a preliminary study, however, I found high percentages of γδTCR⁺ IELs in proximal, middle, and distal small intestine (Derrick Todd, unpublished observations).

It could also be argued that the proportion of γδTCR⁺ cells in the WF rat IEL compartment may differ from the proportion in rats of other strains or ages. My analysis of 5 phenotypically normal strains at ~10 weeks of age suggests that, although there is some strain-to-strain variability, the percentage in WF rats is not uniquely high. Whether the percentage is also high in older or younger animals analyzed by my method is not yet established, however.

**Rat IEL subpopulations**

My three-color microfluorometric analysis identified five major subpopulations of IELs based on the expression of CD4, CD8, and TCR. These five major subpopulations were further categorized using additional phenotypic criteria.

One of these five subpopulations, comprising CD8⁻ CD4⁺ αβTCR⁺ cells, has previously been interpreted to be indicative of contamination with lamina propria and/or Peyer’s patch lymphocytes (16). I believe, however, that this interpretation is not correct. B-lymphocytes are a sensitive measure of contamination of IEL populations with lamina
propria and Peyer’s patch lymphocytes (216), and I observed very low numbers of B cells in my IEL preparations (16;213).

Essentially all of the cells in each of the five major IEL subpopulations expressed the activation marker CD25, suggesting that the intestinal environment activates IELs irrespective of their intestinal or thymic origin. The presence of CD25+ IELs further suggests that IL-2 may be produced in the intestinal tissue and may in part control the functional activity of IELs. This inference is supported by the detection of abundant IL-2 mRNA in preparations of purified IELs (216).

The variable expression of CD5 I observed in the IEL subpopulations has been previously noted, and it has been suggested that the variability is age-related (213;217). My data suggest that, in addition to age, expression of CD5 may be subset-dependent. Depending on subset, the proportion of CD5+ cells varied from very low to very high (Table 5).

**Intraepithelial NK cells**

The present studies have also identified and characterized a population of αβ– and γδTCR+ IELs that express the NKR-P1 marker; such cells are NK cells (44). This population of cells was almost completely restricted to the CD4+CD8+ IEL population and are CD2+. They are unlike peripheral NK cells in the rat, which are CD2+ (311) and CD8+ (53). Cells positive for NKR-P1 staining comprised 25-30% of IELs. The majority of the NKR-P1+ NK cells were also ART2+ and CD25+. In a preliminary report, it has been suggested that peripheral NK cells express low levels of ART2 (149), and recent
work in our laboratory has confirmed this report (Chapter III). In contrast, I observed that ART2 is expressed on NK IELs at a very high density that was comparable to that observed on TCR$^+$ IELs (213;298;302).

**Conclusion to Chapter II**

In conclusion, I report a new isolation method for rat IELs that is noteworthy for its reproducible high yields. IELs isolated using this method include five major subpopulations, among them significant numbers of $\gamma\delta$TCR$^+$ and NK cells. The rapidity, efficiency, and product purity that characterize the method should facilitate functional and developmental studies of IEL subpopulations in the rat. Detailed analysis of IEL subpopulations using this new method may facilitate analyses of the mechanisms of oral tolerance.
CHAPTER III

AN ATYPICAL POPULATION OF NATURAL KILLER CELLS THAT SPONTANEOUSLY SECRETE IFN-γ AND IL-4 IS PRESENT IN THE INTRAEPITHELIAL LYMPHOID COMPARTMENT OF THE RAT

Summary of Chapter III

The intestinal lymphoid compartment of the rat is large and diverse, but the phenotype and functions of its constituent cell populations are not fully characterized. Using new methodology for the isolation and purification of rat intestinal intraepithelial lymphocytes, I previously identified a population of αβ- and γδ-TCR- NKR-P1A+ NK cells. These cells were almost completely restricted to the CD4- CD8- IEL population, and unlike peripheral NK cells in the rat, they were CD2+. I now report that rat intraepithelial NK and peripheral NK cells are similar in morphology, in their ability to lyse NK-sensitive targets, and in their ability to suppress a one-way mixed lymphocyte culture. In contrast, however, intraepithelial and splenic NK cells differ markedly in two respects. First, IENK cells express high levels of ART2 (a marker of regulatory T cells in the rat) and CD25, whereas peripheral NK cells do not. Second, unlike splenic NK cells, a substantial fraction of IENK cells appear to spontaneously secrete IL-4 and/or IFN-γ. I conclude that the rat IEL compartment harbors a large population of NKR-P1A+ CD3- cells
that function as NK cells but display an activated phenotype and unusual cytokine profile that clearly distinguish them from splenic NK cells. Their phenotypic and functional characteristics suggest that these distinctive intraepithelial NK cells may participate in the regulation of mucosal immunity.

Introduction to Chapter III

The intestine is the largest lymphoid organ in the body, challenged constantly by an enormous quantity and diversity of antigens. The intestinal lymphoid compartment is not only large but also very diverse, and the phenotype and functions of its constituent cell populations are not fully characterized (185). In addition, mucosal lymphoid tissues appear to be regulated by unique mechanisms that govern highly specialized processes like oral tolerance and controlled (or physiologic) chronic inflammation. Defects in the function of intestinal immune system regulation have been associated with celiac disease (88) and inflammatory bowel diseases (199;201;312). In addition, there are specialized lymphoid populations, in particular intraepithelial T lymphocytes (209), which respond to alternative pathways of activation. Coupled with the specialized antigen-presenting capabilities of intestinal epithelial cells (261), these lymphoid populations are thought to participate in immune responses that are under the control of distinct but as yet poorly characterized regulatory factors (185).

Using new methodology for the isolation and purification of rat intestinal IELs, I previously identified a population of αβ- and γδ-TCR− NKR-P1A+ intraepithelial NK cells (26). These cells were almost completely restricted to the CD4− CD8− IEL popula-
tion, and unlike peripheral NK cells in the rat, they were CD2⁻. I now report the phenotypic and functional characteristics of these IENK cells. The data suggest that the rat IEL compartment harbors a large population of NKR-P1A⁺ CD3⁻ cells that function as NK cells but display an activated phenotype and an unusual cytokine profile that clearly distinguish them from splenic NK cells. Their phenotypic and functional characteristics suggest that these distinctive intraepithelial NK cells may participate in the regulation of mucosal immunity.

**Materials and Methods of Chapter III**

**Animals**

WF, DA, and BN rats of both sexes were obtained from Harlan Sprague Dawley (Indianapolis, IN). Additional WF rats of both sexes were obtained from Charles River Laboratories (Wilmington, MA). All animals were certified to be serologically free of Sendai virus, pneumonia virus of mice, sialodacryoadenitis virus, rat corona virus, Kilham rat virus, H1 (Toolan's virus), mouse poliovirus (GD7), Reo-3, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, mouse adenovirus, Hantaan virus, and *Encephalitozoon cuniculi*. All animals were housed in a viral-antibody free facility until used and maintained in accordance with recommendations in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996) and the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.
Antibodies

For flow microfluorometry, fluorochrome- or biotin-conjugated mAbs directed against NKR-P1A (clone 10/78), CD2 (clone OX-34), CD3 (clone G4.18), CD5 (clone OX-19), CD8α (OX-8), CD25 (IL-2 receptor α-chain, clone OX-39), CD28 (clone JJ319), CD45RC (clone OX-22), CD54 (ICAM-1, clone 1A29), and αβ-TCR (clone R73) were obtained from BD PharMingen (San Diego, CA). The hybridoma secreting the 6A5 rat anti-rat ART2b (IgG1) monoclonal antibody (mAb) is maintained in our laboratory (26).

For measurement of IL-4-secreting cells by ELISpot, paired mAbs (purified OX-81 and biotinylated B11-3) directed against IL-4 were obtained from BD PharMingen. For measurement of IFN-γ by ELISpot, the capture mAb (clone DB-1) was obtained from BioSource International (Camarillo, CA) and the detection rabbit anti-rat IFN-γ polyclonal antibody was obtained from Torrey-Pines Biolabs (La Jolla, CA) and biotinylated as described (313). Isotype control mouse IgG1, mouse IgG2a, and mouse IgG3, secondary antibody (anti-rat IgG1), alkaline phosphatase-conjugated streptavidin, and CyChrome®-conjugated streptavidin were purchased from BD PharMingen. Horseradish peroxidase-(HRP-) conjugated avidin D was purchased from Vector Laboratories, Inc. (Burlingame, CA). Recombinant rat IL-2, IFN-γ, and IL-4 were purchased from R&D Systems (Minneapolis, MN).
Cell preparation

Intraepithelial lymphocytes were prepared from rat small intestine using previously described methods with minor modifications (26). Briefly, the entire small intestine was gently flushed with cold RPMI-1640 (Gibco, Grand Island, NY) to remove lumenal contents and then incubated in ice-cold RPMI-1640 for 60-120 minutes to delaminate epithelial cells and IELs from the basement membrane. Detached cells were recovered by flushing the intestine two times with a total of 50 ml of HEPES-buffered HBSS media containing 1 mM dithioerythritol (Sigma) and 10% Fetalclone® I (a defined neonatal bovine serum, HyClone, Logan, UT) at 37°C. IELs were then purified by Percoll density gradient centrifugation, and viable cells exhibiting lymphoid morphology were quantified by the method of Trypan blue using a hemocytometer.

Thymi, spleens, and mesenteric lymph nodes were removed from rats killed in an atmosphere of 100% CO₂. Single cell suspensions were prepared by gentle extrusion through stainless steel sieves into medium consisting of cold RPMI-1640 supplemented with 5% Fetalclone® I as described (26;121). Spleen cell preparations were washed in medium and erythrocytes were lysed in a hypotonic buffer (0.15 M NH₄Cl, 1.0 mM KHCO₃, 0.1 mM Na₂EDTA pH 7.4). For cytotoxicity assays, erythrocyte-depleted splenocytes were suspended in medium, adsorbed against polystyrene plates for one hour at 37°C, and non-adherent spleen cells were recovered by gentle rinsing of the plates with ice-cold medium.
Flow cytometry

Single, two, and three-color flow microfluorometric analyses were performed as described (26). Briefly, $1 \times 10^6$ viable splenocytes or IELs were suspended in ice-cold FACS medium (isotonic PBS supplemented with 1% Fetalclone® I) and reacted with a mixture of 6A5 anti-ART2b mAb and/or biotin-conjugated antibodies for 30 min. at 4°C. Cells were then washed with FACS medium, reacted with FITC- and PE-conjugated antibodies, and/or Cy-chrome®-conjugated streptavidin for 30 min. at 4°C, washed with FACS medium, and fixed with 1% paraformaldehyde in PBS. FITC-, biotin- and PE-conjugated isotype control immunoglobulins were used for all analyses. Cells were analyzed using a FACScan® instrument (Becton Dickinson, Sunnyvale, CA). Lymphoid cells were identified by their forward and side light-scatter profiles.

IEL and splenocyte subpopulations were purified by flow microfluorometry. Freshly isolated IELs and splenocytes were reacted either with FITC-conjugated antibodies directed against NKR-P1A or with FITC- and PE-conjugated antibodies directed against NKR-P1A and CD3, respectively, and sorted by FACScan®. As documented in Figure 14 for one representative sample, the purity of all sorted IEL populations was >95%. The purity of sorted splenic T and NK cell populations was also documented by flow microfluorometry to be >97%. The percentage of contaminating NKR-P1A⁺ CD3⁺ (NK T) cells was consistently very low (<0.1%) in the sorted cell populations in all experiments.
Figure 14: Purity of sorted intraepithelial NK cells
Figure 14: Purity of sorted intraepithelial NK cells. IELs were isolated from 4-8 week old WF rats, reacted with anti-NKR-P1A and anti-CD3 mAbs (Panel A), and sorted by flow microfluorometry using the indicated gates into NKR-P1A⁺ CD3⁻ (Panel B) and NKR-P1A⁻ CD3⁺ (Panel C) populations as described in Methods. The percentage of purified NKR-P1A⁺ CD3⁻ IENK cells and NKR-P1A⁻ CD3⁺ T cells, as indicated, was very high. Shown is the result of a single representative experiment. Comparable purity was achieved in all sorts of both IELs and splenocytes performed as part of subsequent experiments.
Morphology

Purified preparations of IEL and splenocyte subpopulations were suspended in isotonic PBS and spun onto glass slides using a Shandon Cytospin 2 (Shandon, Inc., Pittsburgh, PA). Cells were reacted with Wright-Giemsa stain and evaluated by light microscopy by a qualified pathologist (Bruce Woda, M.D.).

Cytotoxicity assay

Cytotoxic activity of lymphoid populations was measured using a previously described $^{51}$Cr-release microcytotoxicity assay with minor modifications (314). NK-sensitive YAC-1 virus-induced mouse T cell lymphoma (315) and NK-resistant C58NT(D) (316) target cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in our laboratory in AIM-V media (Gibco) supplemented with 2.2 x 10^{-5} M 2-mercaptoethanol. Target cells in growth phase were labeled with $^{51}$Cr as sodium chromate (150 μCi/million cells, New England Nuclear, Boston, MA), and 1.0 x 10^4 $^{51}$Cr-labeled cells were added to each well of a 96 well microtiter plate. Unfractionated IELs, purified subpopulations of IELs, and adherent-cell-depleted splenocytes were added at effector to target (E:T) cell ratios ranging from 75:1 to 3:1, and the plates were incubated for four hours at 37°C in a humidified atmosphere of 95% air-5% CO₂. All assays were performed in triplicate and averaged.

Total releasable radioactivity ("cpm_{maximal}") was determined by incubating an aliquot of $^{51}$Cr-labeled target cells with 1% Triton X-100. After incubation, cells were pelleted by centrifugation, and 100 μl aliquots of supernatant were transferred to a separate
microtiter plate containing 100 μl Optiphase Supermix β-scintillation fluid (Wallac, Inc. Gaithersburg, MD) and counted (“cpm_{test}”) using a 1450 Microbeta Trilux instrument (Wallac). In all assays reported here, release of $^{51}$Cr in the absence of effector cells (“cpm\textsubscript{spontaneous}”) was <12% and <22% of total $^{51}$Cr release for YAC-1 and C58NT(D) cells, respectively. Specific cytotoxicity was calculated as a percentage using the raw counts per minute (cpm) and the formula:

\[
\text{Specific Lysis (\%)} = \left( \frac{\text{cpm}_{\text{test}} - \text{cpm}_{\text{spontaneous}}}{\text{cpm}_{\text{maximal}} - \text{cpm}_{\text{spontaneous}}} \right) \times 100\%
\]

**Suppressor cell assays**

**Mixed lymphocyte culture**

Inhibition of peripheral T lymphocyte proliferation by syngeneic WF IELs was measured in a one-way mixed lymphocyte culture (MLC). Responder cells were WF rat MLN cells. Stimulator cells were either syngeneic WF (RT1\textsuperscript{a}) thymocytes or allogeneic DA (RT1\textsuperscript{b}) or BN (RT1\textsuperscript{n}) thymocytes. Stimulator thymocytes were given 2 Gy of gamma radiation (~0.1 Gy/min) using a $^{137}$Cs source (Gammacell 40, Atomic Energy of Canada, Ottawa, ON, Canada). Assays were performed in 96 well plates incubated at 37°C in a final volume of 200 μl of supplemented AIM-V media in a humidified atmosphere of 95% air-5% CO$_2$. In preliminary experiments it was determined that optimal MLN cell proliferation in the absence of IELs was obtained after 5 days of incubation using 4.0 x $10^5$ responder cells and 8.0 x $10^5$ allogeneic stimulator cells. Unfractionated IELs and purified subpopulations of IELs syngeneic to the responder cells were added to cultures
at IEL:responder cell ratios ranging between 1:400 and 2.5:1. $^3$H-thymidine (1 μCi/well, New England Nuclear) was added to each well for the final 18-24 hours of culture, and total incorporation of $^3$H-thymidine into DNA was determined as described (317). Radioactivity was measured using a 1450 Microbeta Trilux instrument (Wallac). All assays were performed in triplicate and averaged. The extent of suppression was calculated as a percentage using the raw cpm and the formula:

$$\text{% Suppression} = \left[1 - \left(\frac{\text{cpm}_{\text{test allo}} - \text{cpm}_{\text{test syn}}}{\text{cpm}_{\text{max allo}} - \text{cpm}_{\text{max syn}}}\right)\right] \times 100\%$$

where $\text{cpm}_{\text{test allo}}$ are counts from the incubation of IEL, responder, and allogeneic stimulator cells, $\text{cpm}_{\text{test syn}}$ are counts from incubation of IEL, responder, and syngeneic stimulator cells, $\text{cpm}_{\text{max allo}}$ are counts from the incubation of responder and allogeneic stimulator cells only, and $\text{cpm}_{\text{max syn}}$ are counts from the incubation of responder and syngeneic stimulator cells.

**Transwell® assay**

To determine if a soluble factor mediated IEL-induced suppression, mixed lymphocyte cultures were performed using 24-well Costar Transwell® plates (3 μM pore diameter, Fisher Scientific, Pittsburgh, PA). Responder WF MLN cells were incubated in PBS (5.0 x 10^7 cells/ml) containing 1 μm carboxyfluorescein succinimidyl ester (CFSE) for 15 minutes at 37°C as described (318). After labeling, responder cells were incubated with stimulator thymocytes from syngeneic WF or allogeneic DA or BN rats prepared as described above, with 1.0 x 10^6 WF responder cells and 3.2 x 10^6 stimulator cells placed
in the lower chamber of each test well. IELs from 5-7 week old WF rats (2.0 x 10^5 cells) were added to either the lower chamber or the upper insert chamber of test wells. Cell mixtures were cultured in supplemented AIM-V media at a final volume of 800 μl per well. Plates were incubated at 37°C for 5 days in a humidified atmosphere of 95% air-5% CO₂. After harvest, the intensity of the intracellular CFSE signal in viable lymphocytes was measured by flow microfluorometry and used as an index of cell proliferation as described (319). The absolute number of mitotic events that had occurred per 10⁴ responder cells in the analysis was calculated using the method of Wells, et al. (320).

**ELISpot analyses**

Interferon-γ and IL-4 production was first measured using a previously described ELISpot assay with minor modifications (321;322). Briefly, 96-well Nunc Maxisorp plates (Fisher Scientific) were incubated overnight at 4°C with coating antibodies (1 μg per well) diluted in sterile PBS. Plates were washed once with PBS (4°C) and blocked for 1-2 hours with PBS containing 4% bovine serum albumin (BSA, w/v). Plates were then washed once with PBS at 4°C, and cells to be assayed were suspended in supplemented AIM-V medium and 100 μl of the cell suspension was added to each well. The optimal number of IELs assayed per well was determined in preliminary experiments and varied from 1 to 9 x 10⁴. The optimal number of splenocytes assayed per well was also determined in preliminary experiments and varied from 1 to 52 x 10⁴. For all ELISpot analyses, plates were incubated at 37°C in a humidified atmosphere of 95% air-5% CO₂ for 20-24 hours in the absence of additional stimulation except as noted. Stimuli included
recombinant rat IL-2 (10 or 6000 U/ml) or the combination of phorbol myristate acetate (PMA, 5 ng/ml) and ionomycin (0.4 μg/ml). After incubation, culture media was decanted, and distilled H₂O containing 0.05% Tween 20 was added to the wells for 5-10 minutes to lyse the cells. Plates were then washed 5 times with PBS-0.05% Tween 20 using an Ultrawash Plus plate-washer (Dynatech Laboratories, Burlington, MA). Biotinylated detection antibodies (0.5 μg/well diluted with PBS containing 1% BSA) directed against IL-4 and IFN-γ or an irrelevant antigen (trinitrophenol, TNP) were then added to test wells. Plates were agitated on a plate shaker at room temperature for 60 to 120 minutes and then washed 5 times with PBS-0.05% Tween 20.

Next, 100 μl of a 1:1000 dilution of alkaline phosphatase-conjugated streptavidin in PBS containing 1% BSA was added to each well. Plates were again agitated at room temperature for 60-120 min, washed 10 times with PBS-0.05% Tween 20, and then washed once with PBS alone. A solution of 0.6% agarose (w/v, type VII low gelling temperature, Sigma) containing 0.1 M 2-amino-2-methyl-1-propanol (Sigma), 16.2 mM MgCl₂, 0.008% Triton X-405 (v/v, Sigma), and 2.3 mM 5-bromo-4-chloro-3-indolyl phosphate (Sigma) was preheated to 42°C and layered onto the plates, which were then incubated overnight at 37°C in a humidified atmosphere of 95% air-5% CO₂. The number of discrete spots visible in each well was counted using a light microscope at 2x power. Each cytokine assay was performed using 4 wells, three of which were assayed using the cytokine-specific developing antibody and one of which was assayed using the irrelevant biotinylated control antibody. For analysis, the number of spots observed in the control well for each quadruplicate assay was subtracted from the number of spots counted in
each of the three test wells. The maximum number of spots observed in any control well was three. The adjusted number of spots present in each of the test wells was then averaged. To account for the various numbers of cells added per well, data have been normalized to numbers of spots per $10^4$ cells.

**Quantification of cytokine production by cell-ELISA**

The amount of IFN-$\gamma$ and IL-4 secreted by IELs was quantified using a modification of the cell-ELISA assay (323), which is designed to avoid artifact influenced by the consumption of the cytokines that are secreted by cultured cells. For these assays, unstimulated unsorted, sorted and remixed IELs ($1\times 10^4$ per well) were processed exactly as detailed in the first paragraph of the ELISpot method described above (through the end of the culture, addition of biotinylated antibodies, and washes). For quantification, additional wells were prepared in the same way at the same time, but graded amounts of IFN-$\gamma$ or IL-4 instead of cells were added to these wells. Threefold dilutions of cytokine were added to wells in amounts that ranged from 1.2–900 pg for IFN-$\gamma$ and 0.1–33.3 pg for IL-4.

After the incubation, addition of detection antibodies directed against IL-4 and IFN-$\gamma$ or TNP, and washes, 100 μl of a 1:2500 dilution of HRP-conjugated avidin D in PBS containing 1% BSA was added to each well. Plates were agitated at room temperature for 60-120 min, washed 10 times with PBS-0.05% Tween 20, and then washed once with PBS alone. Next, 100 μl of a solution containing 0.05M Na$_2$HPO$_4$, 0.025 M NaCitrate, 1 mg/ml o-phenylenediamine (OPD, Sigma), and 1 μl/ml 30% v/v H$_2$O$_2$ was
added to each test well, and plates were incubated at room temperature for 5-10 minutes. The reaction was stopped by adding 25 µl of 3N HCl to each well, and absorbance at 790 nm was measured using a microplate reader (Model 3550, BioRad Laboratories, Hercules, CA). Each IEL population (unsorted, sorted, remixed) was assayed in 6 wells of which 3 were developed using the cytokine specific antibody and 3 with the anti-TNP antibody. Each standard was assayed in 3 wells that were developed using the cytokine specific antibody.

For wells to which the cytokine alone had been added, a standard curve was generated by linear regression using the software supplied with the plate reader (Microplate Manager PC, version 4.0, Biorad Laboratories). Curves for both IFN-γ and IL-4 were linear in the region of interest (r≥0.98 for each assay). The amount of cytokine present in each well that had been incubated with IELs was determined by the software. Because the cytokines produced in the course of the ELISpot were captured and not degraded, the data are presented as pg of cytokine produced/10⁴ cells/24 h. No cytokine was detected in wells that were developed with the TNP-specific detection antibody or in wells to which neither cells nor cytokines had been added.

**Statistics**

Parametric data are presented as arithmetic means ± 1 s.d. except in figures depicting the results of a single experiment performed in triplicate or quadruplicate. In those instances, the result is shown as the mean ± standard error of the mean. Pairs of means
were compared using two-tailed t-tests with separate variance estimates and the Bonferroni adjustment for multiple comparisons as required (324;325).

**Results of Chapter III**

The relative percentage of NKR-P1A+ cells in the intraepithelial compartment of rats decreases with age

In a preliminary experiment, IELs were isolated from WF rats of different ages, and the percentage of NKR-P1A+ cells present was measured by flow cytometry. As shown in Figure 15, the percentage of NKR-P1A+ cells present in the IEL compartment declined with age, decreasing from ~50% in weanling rats to ~17% in retired breeder rats >16 weeks old. At certain time points, the percentages of NKR-P1A+ CD3+ or NKR-P1A+ αβ-TCR+ (NK T) cells present in the IEL compartment were also measured and were consistently <2% (Figure 15). The absolute number of IENK cells was determined at two time points. I observed that the yield of IELs from 4-6 week old rats was 5.1 ± 1.9 x 10^6 per rat (N=7), of which an average of ~2.5 x 10^6 were IENK cells. The yield of IELs from much larger rats >16 week old was 27.2 ± 6.4 x 10^6 per rat (N=11), of which an average of ~5 x 10^6 were IENK cells. Except as noted, rats 4-8 weeks old were used in subsequent experiments.
Figure 15: Percentages of NKR-P1A⁺ IELs as a function of age
Figure 15: Percentage of NKR-P1A+ IELs as a function of age. IELs were isolated from WF rats of either sex at various ages and reacted with either anti-NKR-P1A alone, or the combination of anti-NKR-P1A mAb plus either anti-CD3 or anti-αβ-TCR mAb as described in Methods. The percentages of NKR-P1A+, NKR-P1A+ CD3+, and NKR-P1A+ αβ-TCR+ cells were measured by flow microfluorometry. Each data point represents the mean ± 1 s.d.; the number of independent measurements for each data point is indicated in parentheses.
Intraepithelial and splenic NKR-P1A⁺ CD3⁻ NK cells express different cell surface antigens

It is known that intraepithelial and splenic NK cells differ with respect to the expression of at least two cell surface antigens. The majority of splenic NK cells express CD2 and CD8α, whereas <10% of IENK cells express these surface antigens (26,54). To investigate whether intraepithelial and splenic NKR-P1A⁺ CD3⁻ NK cells differ phenotypically in other respects, I measured the expression of a panel of cell surface antigens on both populations. Using 7 week old WF rats, I confirmed that the percentage of cells expressing CD2 and CD8α was significantly lower in the IENK than in the splenic NK cell population (Table 6). In addition I observed several other differences of substantial magnitude. The percentage of cells expressing CD5, CD45RC, and CD54 was also significantly lower in the IENK than in the splenic NK cell population. Conversely, the percentage of cells expressing CD25 and ART2b was significantly higher in the IENK than in the splenic NK cell population. Only CD28 was expressed on similar percentages of IENK and splenic NK cells. Representative histograms documenting the expression of CD2, CD8α, ART2b, CD25, and CD45RC on IENK and splenic NK cells are shown in Figure 16.

Purified populations of intraepithelial and splenic NK cells are morphologically similar

Because IENK cells differ from splenic NK cells phenotypically, these populations were next purified by flow cytometry as described in Methods and examined for the
TABLE 6

PHENOTYPE OF INTRAEPITHELIAL AND SPLENIC NKR-P1A⁺ CD3⁻ LYMPHOCYTES

<table>
<thead>
<tr>
<th></th>
<th>% CD2⁺</th>
<th>% CD8α⁺</th>
<th>% CD5⁺</th>
<th>% CD25⁺</th>
<th>% CD28⁺</th>
<th>% CD45RC⁺</th>
<th>% CD54⁺</th>
<th>% ART2⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>IELs</td>
<td>1.4 ± 1.0ᵃ</td>
<td>9.2 ± 3.8ᵃ</td>
<td>1.4 ± 1.8ᵃ</td>
<td>95.4 ± 2.5ᵃ</td>
<td>0.3 ± 1.3ᵇ</td>
<td>0.0 ± 1.9ᵃ</td>
<td>53.4 ± 4.0ᵃ</td>
<td>96.6 ± 1.5ᵃ</td>
</tr>
<tr>
<td>SPCs</td>
<td>94.5 ± 0.3</td>
<td>73.2 ± 3.8</td>
<td>31.5 ± 2.7</td>
<td>2.0 ± 0.7</td>
<td>15.5 ± 6.0</td>
<td>95.8 ± 1.7</td>
<td>97.6 ± 0.7</td>
<td>9.9 ± 1.0</td>
</tr>
</tbody>
</table>

Legend to Table 6: IELs and splenocytes isolated from 7 week old WF rats were reacted with antibodies directed against NKR-P1A, CD3, and a third surface antigen, and analyzed by flow cytometry as described in Methods. Fluorescence associated with the isotype control antibody was subtracted. Each data point represents the mean ± 1 s.d. of three independent experiments. a: p<0.001 vs. splenocyte population. b: p=N.S. (Bonferroni adjusted) vs. splenocyte population.
Figure 16: Phenotypic analysis of intraepithelial and splenic NK cells
**Figure 16:** Phenotypic analysis of intraepithelial and splenic NK cells. Shown in the left-most upper and lower panels are representative flow cytometric profiles of NKR-P1A (horizontal axis) and CD3 (vertical axis) staining of intraepithelial lymphocytes (IELs, upper panel) and splenocytes (lower panel) prepared from 7 week old WF rats. Cells in the indicated gated regions (NKR-P1A⁺ CD3⁻) were selected and analyzed for expression of each indicated antigen. In each of the histograms, intensity of labeling (horizontal axis) is plotted against cell number (vertical axis). Solid lines indicate fluorescence associated with specific staining, and the dashed lines indicate fluorescence associated with isotype control antibody staining. The experiment was repeated on cell samples from at least 3 individual animals, all of which yielded comparable results. The overall data set for each population is presented in Table 6.
presence of the azurophilic cytoplasmic granules that are characteristic of peripheral NK cells (314). Light microscopic analysis revealed that nearly all cells in the purified IENK population had the appearance of large granular lymphocytes with azurophilic granules (Figure 17A). These granules were even more prominent than those present in purified splenic NK cells (Figure 17B). Azurophilic granules were absent in the majority of intraepithelial (Figure 17C) and splenic (Figure 17D) T cells.

**Both intraepithelial and splenic NK cells lyse NK-sensitive targets**

I next measured specific lysis of NK-sensitive YAC-1 cells (315) and NK-resistant C58NT(D) cells (316) in the presence of unfractionated IELs and monocyte-depleted spleen cells. Both IELs and monocyte-depleted spleen cells killed YAC-1 but not C58NT(D) targets (Figure 18A). Flow microfluorometric analysis of the IEL and spleen cell populations in these two experiments revealed that 44-58% and 8-9% of each population, respectively, consisted of NKR-P1A+ cells. Re-analyzing the data in Figure 18A using effector-to-target cell ratios based on these percentages, I found that the specific killing of YAC-1 cells by NKR-P1A+ IENK and splenic NK cells was similar at similar calculated NK-to-target cell ratios (Figure 18B).

To exclude the possibility that cytotoxic T cells may have contributed to YAC-1 killing by unfractionated IELs, the experiment was repeated using purified populations of NKR-P1A+ and NKR-P1A- IELs. In this experiment, 35% of IELs were NKR-P1A+. I observed that NKR-P1A+ IELs efficiently killed YAC-1 targets, whereas NKR-P1A- IELs did not (Figure 18C). In control experiments, I observed that unstained, unsorted
Intraepithelial

Splenic

Figure 17: Morphology of intraepithelial and splenic lymphocytes.
Figure 17: Morphology of purified IENK cells and T cells in IEL preparations. Intraepithelial lymphocytes (Panels A, C) and splenocytes (Panels B, D) were isolated from 4-8 week old WF rats and sorted into NKR-P1A+ CD3− (Panels A, B) and NKR-P1A− CD3+ (Panels C, D) fractions, which were then reacted with Wright-Giemsa stain. Purified NK cells exhibit the morphology of large granular lymphocytes, with abundant azophilic granules (arrows) present in the cytoplasm (Panels A, B). Few purified T cells exhibit azurophilic granules (Panels C, D). Splenic but not intraepithelial T cells also appear in general to be smaller than splenic NK cells. (x 100).
Figure 18: Cytotoxic activity of IENK cells
Figure 18: Cytotoxic activity of IENK cells. Panel A: IELs and monocyte-depleted spleenocytes (SPCs) were prepared from WF rats 5-6 weeks of age and immediately tested for their ability to kill YAC-1 and C58NT(D) cells as described in Methods. Each data point represents the average of two individual experiments, each performed in triplicate. Panel B: For both experiments in Panel A, the percentage of NKR-P1A+ cells in each effector cell population was determined by flow microfluorometry. In those two individual experiments, the percentages of NKR-P1A+ IELs were 59% and 44%, and the percentages of NKR-P1A+ spleen cells were 8% and 9%. These percentages were then used to calculate NK:target cell ratios using the raw data from Panel A. Open and filled symbols represent the data from the individual experiments, and each data point represents the mean of a triplicate determination. Panel C: IELs were isolated from 6-week-old WF rats. An aliquot of these cells was not manipulated further. Another aliquot was reacted with anti-NKR-P1A antibody and not manipulated further. A final aliquot was reacted with anti-NKR-P1A antibody and then fractionated by flow microfluorometry. A final population of effector cells was generated by remixing sorted NKR-P1A+ and NKR-P1A− cell populations at the 35:65 ratio of the pre-sort IEL preparation. Each cell population was tested for its ability to kill YAC-1 cells. Each data point represents the mean of a single experiment performed in triplicate. The experiment was repeated a second time with similar results.
IELs and stained, unsorted IELs exhibited similar YAC-1 killing activity. Finally, sorted NKR-P1A$^+$ and NKR-P1A$^-$ IEL fractions that were remixed at the pre-sort ratio of 35:65 also exhibited YAC-1 killing at IEL:Target ratios comparable to those observed using unsorted IELs (Figure 18C).

**Intraepithelial NK cells suppress mixed lymphocyte cultures**

Because splenic NK cells are known to suppress mixed lymphocyte cultures (64;68), I analyzed NK cells of intraepithelial origin for similar capability. In preliminary experiments, unfractionated IELs isolated from 7-15 week old WF rats were tested for their ability to suppress a one-way allogeneic MLC. I observed that responder cell proliferation to alloantigen was substantially reduced in the presence of IELs at IEL:responder ratios of approximately 1:10 or greater (Figure 19A). At such ratios, proliferation was reduced to levels observed in control experiments using syngeneic stimulator cells. In additional control experiments, responder cells did not proliferate in the presence of IELs alone, nor did IELs proliferate in the presence of allogeneic stimulator cells alone (data not shown).

Having demonstrated that IELs can suppress a one-way MLC, I next sought to determine which subpopulation of IELs was responsible for the effect. IELs from WF rats were fractionated into NKR-P1A$^+$ CD3$^-$ IENK and NKR-P1A$^-$ CD3$^+$ intraepithelial- (IE-) T cell subpopulations, and their ability to suppress alloreactive T cell proliferation was compared. As shown in Figure 19B, the suppressive activity of sorted IELs appeared to be restricted exclusively to the IENK subpopulation. There was no suppressive activity
Figure 19: Suppressor cell activity of IENK Cells
Figure 19: Suppressor cell activity of IELs. Panel A: IELs were isolated from WF rats 7-15 weeks of age and immediately tested for their ability to suppress a one-way MLC as described in Methods. Responder cells were WF mesenteric lymph node cells. Stimulator cells were thymocytes from either syngeneic WF rats or allogeneic DA (filled symbols) or BN rats (open symbols). Shown are the results of two individual experiments. The absolute cpm (mean ± SEM of a triplicate determination) associated with positive control allogeneic MLCs performed in the absence of IELs was 4.2 ± 0.3 x 10^4 for trial 1 and 8.5 ± 0.3 x 10^4 for trial 2. The cpm for negative control syngeneic MLCs was 0.2 ± 0.0 x 10^4 in both trials. Panel B: IELs were isolated from 5-7 week old WF rats, reacted with anti-NKR-P1A and anti-CD3 antibodies, and fractionated into NKR-P1A^+CD3^- IENK and NKR-P1A^-CD3^+ IE-T cell populations by flow microfluorometry. The purity of each fractionated subpopulation was >95%. For the assay of remixed cells, the ratio of IENK to IE-T cells was 3:2, approximately the same as that in the pre-sort IEL preparation. The data shown in Panel B represent the result of a single experiment performed using BN allogeneic stimulator cells; comparable results were obtained in a second independent trial using DA allogeneic stimulator cells. The absolute counts per minute for allogeneic and syngeneic MLCs performed in the absence of IELs was 9.2 ± 0.5 x 10^4 and 0.1 ± 0.0 x 10^4, respectively. Each data point in both panels represents the mean percent suppression of a triplicate assay ± SEM, calculated as described in Methods. There were no differences in outcome as a function of the strain of the stimulator cell donor (DA or BN).
associated with sorted IE-T cells. The suppressive activity of sorted IENK and IE-T cells that were subsequently remixed was comparable to that of both sorted IENK cells alone (Figure 19B) and unsorted IELs (Figure 19A).

Having demonstrated that NKR-P1A⁺ CD3⁻ (IENK) cells are responsible for IEL-mediated suppression of a one-way MLC, I next sought to determine whether a soluble mediator or cell-to-cell contact was more likely to be responsible for this effect. In this experiment, the MLC was performed using Transwell® methodology, and the proliferation of CFSE-labeled responder cells in contact with unfractionated IELs was compared with the proliferation of responder cells separated from IELs cells by a permeable membrane. As shown in Figure 20, retention of fluorescence, indicative of suppression, occurred when responder cells, allogeneic stimulator cells and IELs were incubated on the same side of the Transwell® membrane. Loss of fluorescence, indicative of responder cell proliferation and an increase in the absolute number of mitotic events, occurred when responder cells and allogeneic stimulator cells were separated from IELs by the membrane.

**IENK cells spontaneously produce IFN-γ and IL-4**

To investigate the possible immunoregulatory role of IENK cells, I measured their secretion of IFN-γ and IL-4 using ELISpot methodology. In a preliminary experiment, IELs were isolated from 5-11 week old WF rats and incubated overnight in the presence of PMA/ionomycin or IL-2 (10 U/ml). I observed that the frequencies of IFN-γ secreting IELs under these conditions of stimulation were 25 and 45 spots/10⁴ cells, respectively. In three separate assays, however, the frequency of cells secreting IFN-γ in the absence of
Figure 20: Suppressive activity of IELs in a one-way mixed lymphocyte culture requires cell-cell contact
Figure 20: Suppressive activity of IELs in a one-way MLC. A one-way MLC was performed using a Transwell® apparatus. IELs were isolated from 5-7 week old WF rats. WF responder cells were labeled with CFSE, and WF syngeneic and DA allogeneic stimulator cells were prepared as described in Methods. Cells were harvested after 5 days of culture and the intensity of CFSE labeling in the recovered cell population was determined by flow microfluorometry using a lymphocyte gate. The overall percentage of responder cells with reduced CFSE fluorescence, indicative of proliferation, is indicated for each experimental condition. In addition, the absolute number of mitotic events that had occurred per 10⁴ responder cells in the analysis was estimated by the method of Wells, et al. (320); these calculated numbers are shown in rectangles in each panel. In the absence of IELs, as expected, proliferation of allogeneic but not syngeneic responder cells was observed (Panels A, B). When IELs were incubated with responder and stimulator cells on the same side of the Transwell® membrane, the proliferation of allogeneic responder cells was reduced to a level comparable to that observed for syngeneic responder cells (Panels C, D). In contrast, when IELs were separated from responder and stimulator cells by the Transwell® membrane, the proliferation of allogeneic responder cells (Panels E, F) was similar to that observed in the absence of IELs (Panels A, B). The two insets present magnified views of the low intensity CFSE gate demonstrating periodic peaks of labeling, consistent with waves of proliferation as described (319). Shown are the results of one of two experiments using different IEL preparations that yielded similar results.
any *in vitro* stimulating agent was comparable: 33, 43, and 23 spots/10^4 cells. The results for IL-4 in this same experiment were similar. PMA/ionomycin and IL-2 stimulated cells generated 22 and 55 spots/10^4 cells, respectively, and cells cultured in the absence of *in vitro* stimulation yielded 35, 39, and 46 IL-4 spots/10^4 cells.

I next tested the hypothesis that IENK cells spontaneously secrete IFN-γ and IL-4 and were the source of these cytokines in my preliminary experiment. Because the secretion of IL-4 has not previously been associated with peripheral NK cells, populations of spleen cells were assayed in parallel with cells of intestinal origin. As shown in Figure 21, I confirmed by ELISpot that the frequency of un-stimulated IELs that produced either IFN-γ or IL-4 was substantial. In contrast, few un-stimulated splenocytes produced IFN-γ and none appeared to produce IL-4. Addition of up to 6000 U/ml IL-2 to these cultures resulted in only a modest increase in the frequency of cells that secreted IFN-γ and no increase in the number of cells that secreted IL-4.

Analysis of sorted populations of IENK and IE-T cells localized the production of both IFN-γ and IL-4 by IELs to the IENK subpopulation (Figure 21). Additional assays confirmed that un-stimulated splenic NK and T cells did not spontaneously secrete IL-4 and only a few secreted IFN-γ. Addition of 10 U/ml IL-2 to the splenic NK cell cultures had little effect on the number of cells that produced either cytokine. When 6000 U/ml was added to the splenic NK cell cultures, the frequency of cells that produced IFN-γ rose to levels comparable to those observed for un-stimulated IENK cells, but the number of cells that produced IL-4 remained low. The frequency of sorted intraepithelial and splenic T cells that secreted IFN-γ was small, and no cells that secreted IL-4 were detectable.
Figure 21: IENK cells spontaneously produce IFN-γ and IL-4
Figure 21: IENK cells spontaneously produce IFN-γ and IL-4. Intraepithelial lymphocytes and splenocytes were isolated from 4-8 week old WF rats as described in Methods. Pools of IELs from 8-10 donors and splenocytes from 2 donors were processed to generate five populations for assay. One aliquot of cells of each type (unstained unsorted cells) was not further manipulated. The remaining cells were reacted with anti-NKR-P1A and anti-CD3 antibodies; one aliquot of these (stained unsorted cells) was not further manipulated, and the remaining cells were fractionated by flow microfluorometry into NK cell (purified NK cells) and T cell (purified T cells) populations. A final population of cells (remixed NK and T cells) was prepared from aliquots of fractionated NK and T cells that were re-mixed at the same ratio as that determined by flow microfluorometry to have been present in the pre-sort cell preparation. IEL samples (white bars) were incubated overnight in the absence of any in vitro stimulation. Splenocyte samples were incubated in the presence of 0 (crosshatched bars), 10 (black bars), or 6000 (diagonal bars) U/ml of IL-2. Each preparation of sorted, unsorted, and re-mixed cells was then assayed in triplicate for the presence of IFN-γ (Panel A) or IL-4 (Panel B) by ELISpot as described in Methods, and data are reported as spots per $10^4$ cells added to the assay. The analysis of IELs (open bars) was repeated four times, including two assays that were also used to generate the additional data shown in Figure 22. The analysis of all other cell populations was repeated twice. Each bar represents the average of the two or four independent trials. In the case of data points with values greater than $\sim 10$ spots/$10^4$ cells, markers (○) indicate the actual values obtained in the trials.
Comparable and consistent results were obtained in control assays of stained, unsorted cells and a remixed population of sorted NK and T cells (Figure 21).

To exclude further any possibility that contaminating IE-T cells may have been responsible for the cytokine production attributed to the IENK population, an additional ELISpot assay was conducted. In this assay, graded numbers of purified IE-T cells were added to a constant number of purified IENK cells, and the number of IFN-γ and IL-4 producing cells measured as above. As shown in Figure 22, there was no detectable change in the number of cytokine producing cells as a function of the number of IE-T cells that were added to the IENK cells. In each experimental condition, the number of cytokine-positive cells, when expressed as positive cells per 10^4 IENK cells in the assay, remained constant.

**Quantification of IFN-γ and IL-4 production by IENK cells**

Having quantified the number of IENK cells that produced IFN-γ and IL-4 spontaneously, I next sought to determine the amount of cytokine produced. To circumvent the problem of reutilization of cytokines inherent in such determinations, I developed the modified “cell-ELISA” (323) described in Methods. As shown in Figure 23, I again confirmed that un-stimulated IELs and IENK cells, but not IE-T cells, produce readily detectable quantities of both IFN-γ and IL-4. IENK cells produced IFN-γ at a rate of ~150 pg/10^4 cells/24 h and IL-4 at a rate of ~1 pg/10^4 cells/24 h. Results for unsorted and remixed IELs gave consistent results based on the fraction of IENK cells present in these populations.
Figure 22: IE-T cells do not affect the production of IFN-γ and IL-4 by IENK cells.
**Figure 22:** The presence of IE-T cells does not affect the spontaneous production of IFN-\(\gamma\) and IL-4 by IENK cells. Purified populations of IENK and IE-T cells were prepared from 8-10 WF rats (4-8 weeks old) by flow microfluorometry as described in Methods. Graded numbers of purified IE-T cells were added to a constant number of purified IENK cells as indicated, and each cell mixture was assayed in triplicate by ELISpot to quantify the number of cells secreting IFN-\(\gamma\) (Panel A) or IL-4 (Panel B). Data are reported as spots per 10\(^4\) IENK cells added to the assay. The experiment was performed twice; markers (●) indicate the values obtained in each trial, and the bars indicate the average value.
Figure 23: Quantity of IFN-γ and IL-4 produced by un-stimulated IENK cells
Figure 23: Quantity of IFN-γ and IL-4 produced by un-stimulated IENK cells. Purified IENK and IE-T cells were prepared from 8-10 WF rats (4-8 weeks old) as described in Methods. Unsorted, sorted, and remixed populations were incubated overnight in the absence of any in vitro stimulation. The amount of IFN-γ (Panel A) and IL-4 (Panel B) secreted by each preparation was then quantified by "cell-ELISA" as described in Methods. Data is reported as pg of cytokine produced/10^4 cells/24 h. The experiment was performed twice; markers (●) indicate the values obtained in each trial, and the bars indicate the average value.
Discussion of Chapter III

Summary of findings

Situated at the intestinal epithelium, IELs are in direct contact with lumenal antigens and are among the first cells to encounter intestinal pathogens (15;209). Although numerous reports have identified in vitro and in vivo functions for IELs, the role of these cells in the intestinal immune system remains unclear in part because IELs have been notoriously difficult to isolate. In developing an isolation protocol that yields large numbers of highly pure IELs, I have recently overcome this impediment and described a population of NKR-P1A⁺ CD3⁻ IELs.

Lymphocytes that express the NKR-P1 surface antigen but not a T cell receptor are NK cells (44;45;52;55;326). This definition distinguishes NK cells from NKT cells, which co-express NKR-P1 and a functional TCR (46;50;51). Accordingly, I have termed NKR-P1A⁺ CD3⁻ IELs “intraepithelial natural killer” cells. My preliminary observation that IENK cells comprise a significant percentage of rat IELs, particularly in young animals, warranted their further study. Using the new IEL isolation methodology, I was able to investigate phenotypic and functional characteristics of IENK cells. Although these experiments were conducted in WF rats, significant numbers of IENK cells are present in at least one other rat strain (54).
Similarities between intraepithelial and splenic NK cells

Morphology

Azurophilic, cytoplasmic granules were present in the majority of purified NKR-P1A<sup>+</sup> CD3<sup>-</sup> IELs and splenocytes. Such granules have long been observed in peripheral NK cells (327) and harbor cytotoxic molecules like perforin (56). Granules in IENK cells were noticeably more prominent than those in splenic NK cells, but the significance of this observation is unknown. Contaminating IENK cells may have accounted for the occasional granular cell present in the purified NKR-P1A<sup>-</sup> CD3<sup>+</sup> IE-T cell fraction, but γδ T cells, which constitute a significant percentage of CD3<sup>+</sup> IELs in rats (26), also exhibit a granular morphology (328).

Cytotoxicity

The ability of freshly isolated lymphocytes to kill YAC-1 cells is a hallmark of NK cytotoxicity (314;315), and rat IELs kill these NK-sensitive targets (228). My experiments confirmed that, when normalized for NKR-P1A<sup>+</sup> cells, freshly isolated IELs and splenocytes kill YAC-1 cells at comparable efficiencies. I extended these findings by localizing YAC-1 specific lysis to purified NKR-P1A<sup>+</sup> IELs, which actually demonstrated enhanced NK activity as compared to unsorted or remixed populations. This suggests that NKR-P1A<sup>-</sup> IELs interfere with IENK cytotoxicity, but it remains to be determined whether NKR-P1A<sup>-</sup> IELs actively suppress IENK cytotoxicity or whether purified NKR-P1A<sup>+</sup> IELs, removed from NKR-P1A<sup>-</sup> cells, simply encounter YAC-1 targets more frequently. A similar observation has been made in mice splenocytes (327). It should be
noted that, under the experimental conditions described here, labeling IENK cells with anti-NKR-P1A mAb does not enhance killing activity. Moreover, the exclusion of NKT cells from analyses of purified NKR-P1A⁺ IELs was unnecessary because, in the absence of high-dose IL-2 stimulation in vitro, NKT cells exhibit poor YAC-1 killing (46). Interestingly, maximal YAC-1 specific lysis by rat IELs occurs in weanling animals (229), an observation consistent with my ontogeny data. In summary, the experiments presented here show that YAC-1 specific lysis by rat IELs is attributable to IENK cells.

**Suppression of oneway mixed lymphocyte culture**

A growing number of reports have demonstrated that NK cells possess suppressive activity in vitro and in vivo (64-71), and NK-mediated suppression of a oneway MLC is thought to occur via killing of allogeneic antigen presenting cells (64;68). In my oneway MLC, IENK cells act as potent suppressors of T cell proliferation. Equal numbers of IE-T cells do not suppress the MLC, arguing against competition for nutrients in the media as a mechanism of suppression. Furthermore, both IENK and IE-T cells express similar levels of CD25 (26), so the explanation that IENK cells act as an “IL-2 sink” is equally unlikely. The blockade of IEL suppression by a Transwell® membrane suggests that cell-to-cell contact is necessary for suppressive activity, although a highly localized release of inhibitory cytokines by IENK cells cannot be ruled out. The exact mechanism whereby cell-to-cell contact would result in IENK-mediated suppression of T cell proliferation remains unanswered, but it may involve killing of allogeneic APCs or direct suppression of allo-reactive T cells. My results do not necessarily contradict those
of previous studies, which have shown that rat IELs exert suppressive activity through soluble mediators (214;292;322;329). IEL preparations from such studies may have been contaminated by significant numbers of intestinal epithelial cells that may interfere with *in vitro* IEL assays (296;330).

**Differences between intraepithelial and splenic NK cells**

**Phenotypic differences**

Phenotypic differences between intraepithelial and circulating T cells have contributed to the theory that IELs represent a distinct T cell compartment with unique origins and functions (209). My flow microfluorometric analyses of CD2, CD8α, CD5, CD25, CD45RC, CD54, and ART2 suggest that the same is true for IENK cells as compared to splenic NK cells. With respect to expression of ART2, CD45RC, and CD25, IENK cells more closely resemble IE-T cells (26) than splenic NK cells, implying that the intraepithelial environment may influence the expression pattern of these surface antigens. Nearly all IENK cells display the ART2⁺ CD45RC⁻ phenotype associated with peripheral CD4⁺ regulatory T cells in the rat (133;331). Intraepithelial but not splenic NK cells also express CD25, the IL-2Rα molecule expressed by activated T and NK cells (332). Interestingly, a population of CD25⁺ cells has recently been associated with regulatory activity mediated by cell-to-cell contact (333-335). In fact, ~85% and ~30% of overall IELs in rats and humans, respectively, express CD25 (26;336).
Spontaneous secretion of IFN-γ and IL-4

Upon activation, peripheral NK cells secrete IFN-γ (56), and activated NKT cells can produce both IFN-γ and IL-4 (46). At the time of this article, however, no manuscript known to the authors has reported IL-4 production by NKR-P1+ CD3- NK cells. Therefore, I was surprised to find that 1-2% of freshly isolated IENK cells tested positive in both IFN-γ and IL-4 ELISpots. Convincing evidence for the presence of dual-secretors was not attainable by this assay. The frequencies observed for spontaneous IFN-γ and IL-4 secretion are similar to those for mouse IE-T cells, which spontaneously secrete IFN-γ (and IL-5) (209;253;337). Clearly, however, mouse and rat IELs are different, as very few rat NKR-P1A- CD3+ IE-T cells spontaneously produce IFN-γ or IL-4. Spontaneous cytokine production provides additional evidence that rat IENK cells are activated. Interestingly, freshly isolated IENK cells appear maximally activated because, despite their expression of CD25, addition of IL-2 to the ELISpot assay fails to up-regulate production of either cytokine. The observation that splenic NK cells, stimulated in vitro with 6000 U/ml IL-2, produce IFN-γ at frequencies similar to those of unstimulated IENK cells challenges the hypothesis that these are truly distinct cell populations. Perhaps the phenotypic and functional differences between intraepithelial and splenic NK cells are simply a consequence of IENK cells being activated in vivo. Such reasoning would not have predicted, however, the marked difference in IL-4 secretion between unstimulated IENK cells and stimulated splenic NK cells. Thus, despite similarities in morphology, cytotoxicity, and MLC suppression, the different phenotypic and functional characteristics of
intraepithelial and splenic NK cells provide strong evidence that these are indeed two distinct cell populations.

**IENK cells and active immune responses in the intestine**

Because of their location at the intestinal epithelium, IELs are believed to function as a "first line of defense" against intestinal pathogens (8;19), and numerous *in vitro* studies have suggested a role for IELs as effector cells in intestinal immune responses. IELs display several cytotoxic activities in addition to NK cytotoxicity, including antigen-specific cytotoxicity, antibody-dependent cell-mediated cytotoxicity, and activity in redirected cytotoxicity assays (209). Upon stimulation, IELs express inflammatory Th1-associated cytokines like IFN-γ (15;209). Ligands for intraepithelial γδ T cells include mycobacterial and synthetic non-peptide phosphoantigens, mycobacterial and autologous heat shock proteins, and the epithelial stress antigens MICA and MICB (8;19;247;338).

Several *in vivo* studies support the argument that IELs play a role in intestinal immune responses to pathogens. Viral infection induces expansion of IFN-γ secreting IELs (272;273). Adoptive transfer of IELs from *Toxoplasma*-infected mice protects recipients from disease presumably via mechanisms involving IFN-γ (268-270). In rats, the CD4+ IEL population expands with age in conventionally housed but not in germ free animals (217). Taken as a whole, both *in vitro* and *in vivo* experiments support the argument that IELs play a protective role against pathogens in intestinal immune responses.
Peripheral NK cells are a critical component of the innate immune system, and mice inoculated with a murine cytomegalovirus exhibit enhanced viral replication if depleted of NK cells prior to infection (339-341). In this manuscript, I have demonstrated that IENK cells share phenotypic and functional similarities with peripheral NK cells, suggesting that IENK cells play a role in mucosal defense. To this end, natural cytotoxicity may delete infected or transformed epithelial cells, and IFN-γ has been shown to increase epithelial cell permeability and up-regulate expression of MHC class II (209). The expression of CD25 and spontaneous secretion of IFN-γ by IENK cells suggest that these cells are constitutively activated in vivo, perhaps scouring the epithelium for stressed/damaged epithelial cells.

**IENK cells and “Physiologic Inflammation” in the intestine**

Despite their vast numbers and continuous exposure to intestinal antigens, T lymphocytes of the intestinal epithelium and lamina propria appear to be restricted to a state of “physiologic inflammation” by as of yet undefined regulatory mechanisms (185). A systemic manifestation of this mucosal tolerance is the phenomenon of oral tolerance, whereby orally administered{antigens induce antigen-specific nonresponsiveness in systemic lymphocytes (285). Disruption of mucosal tolerance has been postulated to contribute to inflammatory and allergic disorders that are not necessarily limited to the intestine (185). Autoreactive T cells also exist in the IEL, presumably because a portion of IELs are non-thymically derived and therefore evade traditional selection processes
(230;231;243;342;343). Consistent with mucosal tolerance, autoreactive IELs do not normally cause disease (244;344;345).

Several factors appear to contribute to mucosal tolerance. Intestinal epithelial cells present antigen via non-classical CD1d molecules and induce CD8+ suppressor cells that may help protect patients from inflammatory bowel diseases (185;201;261). Furthermore, regulatory cytokines like IL-4, IL-10, and TGF-β protect animals from inflammatory bowel disease and are critical to the induction and maintenance of oral tolerance diseases (199;280;285;291;346). In addition, altered mechanisms of TCR signaling make IELs relatively nonresponsive to mitogen stimuli when compared to peripheral lymphocytes (257-259;347;348).

Consistent with mucosal regulatory activity, IENK cells suppress T cell proliferation and secrete the immunoregulatory cytokines IFN-γ and IL-4. Whether IENK cells communicate with other mucosal lymphocytes remains to be determined, however. It is conceivable that IENK cells inhibit aberrant mucosal immune responses by directly suppressing T cell proliferation or by killing inappropriately activated APCs. With respect to the secretion of IFN-γ and IL-4, IENK cells resemble peripheral NKT cells which have recently been shown to possess regulatory activity in vivo (46;47;349;350). The mechanism whereby NKT cells regulate immune responses may involve IL-4 driven deviation from cell-mediated immunity (Th1) to humoral mediated immunity (Th2) (46). Co-secretion of IL-4 and IFN-γ is also a function of Th0-type T cells, a T cell population that is postulated to be the precursor to Th1-type and Th2-type T cells. Because IENK cells lack surface expression of a T cell receptor complex and because I am unable to show
IFN-γ and IL-4 dual secreting cells, I cannot claim that IENK cells are NK T cells or Th0 cells. It remains an attractive hypothesis that IENK cells regulate immune responses at the intestinal epithelium through the differential expression of IFN-γ or IL-4. It should be noted that IENK-mediated secretion of IL-4 is independent of a TCR signal, further supporting the argument that the IEL compartment is unique.

Conclusion to Chapter III

In conclusion, I have demonstrated that the rat IEL compartment harbors a large population of NKR-P1A<sup>+</sup> CD3<sup>-</sup> cells that function as NK cells but display an activated phenotype and highly unusual cytokine profile that clearly distinguish them from splenic NK cells. Their phenotypic and functional characteristics suggest that these distinctive intraepithelial NK cells may participate in the regulation of mucosal immunity.
CHAPTER IV

DEFICIENCIES IN GUT NATURAL KILLER CELL NUMBER AND FUNCTION PRECEDE DIABETES ONSET IN BB RATS

Summary of Chapter IV

BBDP rats spontaneously develop autoimmune diabetes mellitus and are severely deficient in peripheral T cells. BBDR rats do not spontaneously develop autoimmune diabetes, have normal numbers of peripheral T cells, and can be induced to become diabetic by injection of a cytotoxic anti-ART2a mAb and low doses of poly I:C. Using a new, quantitative method of isolating large numbers of highly purified intraepithelial lymphocytes, I have documented previously the presence of a major IEL subpopulation that exhibits phenotypic and functional properties of activated natural killer cells. Intraepithelial NK cells also secrete spontaneously the immunoregulatory cytokines IFN-γ and IL-4. I now demonstrate that, prior to diabetes, both BBDP and ART2a-depleted BBDR rats have a reduced total number of IELs and exhibit a selective deficiency of IENK cell number and function as compared to control BBDR rats. The deficiency of BBDP rat IELs can be corrected by engraftment of bone marrow from histocompatible WF donors. My results suggest 1) that the peripheral lymphopenia in BBDP rats extends to the IEL compartment, particularly to IENK cells, 2) that in BBDR rats the diabetes-inducing treatment depletes
IELs, particularly IENK cells, and 3) that the defect in BBDP rat IELs is intrinsic to hematopoietic cells, not intestinal stromal cells.

**Introduction to Chapter IV**

The cause of autoimmune diabetes in BB rats, NOD mice, and humans is still unknown, but both genetic and environmental factors appear to participate. One important class of environmental factors—diet and enteromicrobial agents—participates in this pathogenic process through the mediation of the gut immune system. Gut immune tissue is regulated by unique mechanisms that control the physiological inflammation induced by huge antigen loads. It harbors unique lymphoid populations that respond to alternative activation pathways and subserves oral tolerance (185). I hypothesize that defects in gut immunity predispose to systemic autoimmunity and diabetes, and I have tested that hypothesis in the BB rat.

BBDP rats, which are severely T cell lymphopenic, spontaneously develop autoimmune diabetes mellitus (4). BBDR rats, which were derived from BBDP forebears, are non-lymphopenic and never become spontaneously diabetic when housed in a VAF environment (4). BBDR rats do, however, become diabetic in response to infection with KRV, depletion of ART2a+ regulatory T cells, and/or immune system activation with poly I:C (4). Both BBDP and BBDR rats appear to share a common diabetes susceptibility locus, iddm4, but only the former is homozygous for the lyp locus responsible for lymphopenia (351;352).
In recent studies, I have documented the existence of a new population of intraepithelial gut natural killer cells in the rat that spontaneously secrete IL-4 and IFN-γ. Here I report that cells with these characteristics appear to be dramatically deficient in BBDP rats and in BBDR rats that are treated to induce autoimmune diabetes.

**Materials and Methods of Chapter IV**

**Animals**

BBDR and BBDP rats were obtained from BMR, Inc (Worcester, MA). WF rats were obtained from Harlan Sprague Dawley (Indianapolis, IN). Animals of either sex were used in all experiments. All animals were certified to be serologically free of Sendai virus, pneumonia virus of mice, sialodacryoadenitis virus, rat corona virus, Kilham rat virus, H1 (Toolan's virus), mouse poliovirus (GD7), Reo-3, Mycoplasma pulmonis, lymphocytic choriomeningitis virus, mouse adenovirus, Hantaan virus, and Encephalitozoon cuniculi. All animals were housed in a VAF facility until used and maintained in accordance with recommendations in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996) and the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

**Antibodies**

Fluorochrome-conjugated mAbs directed against CD3 (clone G4.18), NKR-P1A (clone 10/78), and RT7.2 (clone HIS41) were obtained from BD PharMingen (San Diego,
CA). Tissue culture supernatant containing the NDS58 rat anti-rat RT7.1 (IgG2b) was obtained from Serotec, Ltd. (Oxford, UK). Hybridoma cells secreting the DS4.23 rat anti-rat ART2a (IgG2b) are maintained in our laboratory. For measurement of IL-4-secreting cells by ELISpot, paired mAbs (purified OX-81 and biotinylated B11-3) directed against IL-4 were obtained from BD PharMingen. For measurement of IFN-γ by ELISpot, the capture mAb (clone DB-1) was obtained from BioSource International (Camarillo, CA) and the detection rabbit anti-rat IFN-γ polyclonal antibody was obtained from Torrey-Pines Biolabs (La Jolla, CA) and biotinylated as described (313). Isotype control mouse IgG1 and mouse IgG3, secondary antibody (anti-rat IgG2b), and alkaline phosphatase-conjugated streptavidin were purchased from BD PharMingen.

**Diabetes induction protocol**

To induce diabetes, BBDR rats 25-28 days of age were treated with intraperitoneal injections of DS4.23 mAb (50 µg 5 times weekly) and poly I:C (5 µg/100 g body weight 3 times weekly) as described (125). Rats were treated for one week, approximately half the time required for diabetes onset (125).

**Cell preparation and bone marrow transplantation**

Intraepithelial lymphocytes were prepared from rat small intestine by Percoll® density gradient centrifugation using minor modifications of a method developed in our laboratory (26). Viable cell numbers were quantified by the method of Trypan blue using a hemocytometer. Cells for bone marrow transplantation were prepared from 6 week old donor rats as described (353). Bone marrow recipients 6 weeks of age were treated with
750 rads of gamma radiation (~0.1 Gy/min) using a $^{137}$Cs source (Gammacell 40, Atomic Energy of Canada, Ottawa, ON, Canada) and immediately injected intravenously with 150-200 x $10^6$ bone marrow cells.

**Flow cytometry**

Single and two-color flow cytometric analyses were performed as described (26). Briefly, 1 x $10^6$ viable lymphocytes were suspended in ice-cold FACS medium (isotonic PBS supplemented with 1% Fetalclone® I) and reacted as indicated with purified NDS58 anti-RT7.1 mAb for 30 min. at 4°C. Cells were then washed with FACS medium and reacted with FITC- and PE-conjugated antibodies for 30 min. at 4°C. Cells reacted with NDS58 mAb in the primary incubation were reacted with FITC-conjugated anti-rat IgG2b mAb in the secondary incubation to visualize RT7.1 staining. All samples were then washed with FACS medium and fixed with 1% paraformaldehyde in PBS. FITC- and PE-conjugated isotype control immunoglobulins were used for all analyses. Cells were analyzed using a FACScan® instrument (Becton Dickinson, Sunnyvale, CA). Analyses were gated on lymphoid cells, which were identified by forward and side light-scatter profiles.

**Cytokine analyses**

Interferon-γ and IL-4 production was measured by ELISpot assay as described (354). The optimal number of IELs assayed per well varied from 2.5 to 20 x $10^4$. The number of discrete spots visible in each well was counted using a light microscope at 2x power. Each cytokine assay was performed using 4-6 wells, with all wells but one as-
sayed using the cytokine-specific developing antibody and the remaining well assayed using the irrelevant biotinylated control antibody. For analysis, the number of spots observed in the control well for each multiplicate assay was subtracted from the number of spots counted in each of the test wells. The maximum number of spots observed in any control well was three. The adjusted number of spots present in each of the test wells was then averaged. To account for the various numbers of cells added per well, data are normalized to number of spots per 10^4 cells.

**Statistics**

Parametric data are presented as arithmetic means ± 1 s.d. except in figures depicting the results of a single experiment performed in triplicate or quadruplicate. In those instances, the result is shown as the mean ± standard error of the mean. Pairs of means were compared using two-tailed t-tests with separate variance estimates and the Bonferroni adjustment for multiple comparisons as required (324).

**Results of Chapter IV**

**Decreased intraepithelial lymphocytes in pre-diabetic BBDP and BBDR rats**

The number of IELs recovered from normal, untreated BBDR rats was comparable to that recovered from normal WF rats and tended to increase somewhat with age (Table 7). In contrast, the number of IELs recoverable from the BBDP rat was substantially less, with the magnitude of the difference increasing with age. Treatment of BBDR rats with poly I:C and an anti-ART2a mAb for one week, according to a protocol known
### TABLE 7

**IEL NUMBER IN WF AND BB RATS**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>4-6 weeks old</th>
<th>7-9 weeks old</th>
</tr>
</thead>
<tbody>
<tr>
<td>WF</td>
<td>None</td>
<td>6.8 ± 1.5 (6)⁴</td>
<td>12.8 ± 1.1 (5)⁵</td>
</tr>
<tr>
<td>BBDR</td>
<td>None</td>
<td>8.7 ± 2.3 (8)</td>
<td>14.1 ± 2.4 (8)⁶</td>
</tr>
<tr>
<td>BBDR</td>
<td>anti-ART2a mAb plus poly I:C</td>
<td>3.3 ± 0.4 (4)⁷</td>
<td>N.D.</td>
</tr>
<tr>
<td>BBDP</td>
<td>None</td>
<td>2.2 ± 0.8 (4)</td>
<td>1.6 ± 0.7 (8)⁸</td>
</tr>
</tbody>
</table>

**Legend to Table 7:** IELs from WF and BB rats were isolated and counted as described in Methods. BBDR rats were either untreated or treated for one week with anti-ART2a mAb plus poly I:C to induce diabetes as described in Methods. Each data point represents the mean ± 1 s.d.; the number of rats tested is indicated in parentheses. The BBDP rats at these time points were not yet diabetic. ⁴: Statistically similar to untreated BBDR of the same age and p<0.001 vs. treated BBDR and BBDP of the same age and vs. 7-9 week old WF. ⁵: Statistically similar to BBDP of the same age. ⁶: Statistically similar to untreated BBDR and p<0.001 BBDP of the same age. ⁷: p<0.001 vs. BBDP of the same age and vs. 4-6 week old untreated BBDR. ⁸: Statistically similar to 4-6 week old BBDP.
to induce diabetes in these animals, was associated with a reduction in the number of IELs to levels similar to those observed in the spontaneously diabetic BBDP rat.

**Intraepithelial natural killer cell number and function are reduced in pre-diabetic BBDP and BBDR rats**

Intraepithelial NKR-P1A⁺ CD3⁻ NK cells may play an important immunoregulatory function in the gut (Chapter III). I therefore tested the hypothesis that the reduction in IEL number associated with the prediabetic state in BB rats is associated with defects in IE NK cell number or function. Consistent with previous observations (354), the percentage of IELs expressing the NKR-P1A⁺ CD3⁻ NK phenotype was ~50% (Table 8). Also consistent with previous observations (354), I observed that a substantial number of unfractionated WF IELs spontaneously secreted both IFN-γ and IL-4 (Table 8). These cytokine-producing IELs are known to be IENK cells (354). In contrast, both the percentage of IENK cells and the number of IFN-γ and IL-4 producing cells in both BBDP and untreated BBDR rats was dramatically reduced, and treatment of BBDR rats to induce diabetes was associated with an even greater deficit in both parameters.

**The IENK cell deficiency in BBDP rats results from a hematopoietic defect**

I next sought to determine if the deficiencies in IENK cell number and function in BB rats were the result of a defect in hematopoietic progenitors or the developmental microenvironment of the host. To do so, I performed a reciprocal bone marrow transplantation analysis using BBDP and normal WF rats. Using the RT7 allotypic marker to distinguish cell origin, I observed that WF bone marrow appeared to generate an IEL com-
TABLE 8
INTRAEPITHELIAL NK CELLS IN WF AND BB RATS

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>IENK Cells (%)</th>
<th>IFN-γ (spots/10⁴ cells)</th>
<th>IL-4 (spots/10⁴ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WF (4)</td>
<td>None</td>
<td>50.6 ± 5.0</td>
<td>44 ± 12ᵇ</td>
<td>47 ± 10ᶜ</td>
</tr>
<tr>
<td>BBDR (8)</td>
<td>None</td>
<td>17.4 ± 2.1</td>
<td>7 ± 2</td>
<td>10 ± 4ᵈ</td>
</tr>
<tr>
<td>BBDR (4)</td>
<td>anti-ART2a mAb</td>
<td>1.2 ± 0.8</td>
<td>1 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td></td>
<td>plus poly I:C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BBDP (4)</td>
<td>None</td>
<td>11.8 ± 1.7</td>
<td>3 ± 1</td>
<td>1 ± 0</td>
</tr>
</tbody>
</table>

Legend to Table 8: IELs from WF and BB rats 4-6 weeks old were isolated as described in Methods. BBDR rats were either untreated or treated for one week with anti-ART2a mAb plus poly I:C to induce diabetes as described in Methods. The percentage of NKRP1A⁺ CD3⁻ (IENK) cells was determined by flow microfluorometry. The number of unfractionated, unstimulated IELs secreting IFN-γ or IL-4 was determined by ELISpot after overnight culture as described in Methods. Each data point represents the mean ± 1 s.d.; the number of independent determinations is indicated in parentheses. a: Each of the 4 values differs from all others at the p<0.005 level. b: p<0.001 vs. all other groups; no other paired comparisons are statistically significant. c: p<0.001 vs. all other groups. d: p<0.025 vs. treated DR and BBDP.
partment in BBDP rats that was normal with respect to both the number of IELs recoverable and the percentage of IENK cells (Table 9). Both parameters were comparable to those observed in both unmanipulated WF rats and radiated WF rats of the same age transplanted with syngeneic WF bone marrow (Table 9). In contrast, transplantation of BBDP bone marrow into radiated WF recipients failed to generate a normal IEL compartment of BB rat origin (Table 9). Although the number of IELs recovered was high, most of these cells were of WF origin, and the percentage of IENK cells was intermediate between that of unmanipulated WF and BBDP rats (Table 9).

**Discussion of Chapter IV**

**Summary of findings**

The potential regulatory properties of IENK cells led me to investigate these cells in pre-diabetic BB rats, which are known to have defects in peripheral ART2+ regulatory cells (118). I first analyzed overall IEL numbers and found that IEL recovery was reduced in pre-diabetic BB rats. Upon further inspection, I observed reduced relative numbers of IENK cells in untreated BBDR rats as compared to normal WF rats. This deficiency was more pronounced in BBDR rats following one week of diabetes-inducing treatment and also in pre-diabetic BBDP rats. Spontaneous IFN-γ and IL-4 secretion by unfractionated IELs was likewise decreased in untreated BBDR rats and further decreased in treated BBDR and in BBDP rats. Using reciprocal bone marrow chimeras, I was able to show that the IENK cell deficiency in BBDP rats results from a hematopoie-
### TABLE 9
**IENK CELLS IN BONE MARROW TRANSPLANTED RATS**

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Yield (x $10^6$)</th>
<th>% IENK</th>
<th>% RT7.1⁺</th>
<th>% RT7.2⁺</th>
<th>% RT7.1⁺</th>
<th>% RT7.2⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>WF</td>
<td>7.6 ± 2.8</td>
<td>10.6 ± 4.6</td>
<td>0.0 ± 0.0</td>
<td>98.9 ± 1.0</td>
<td>0.1 ± 0.1</td>
<td>44.0 ± 11.1</td>
</tr>
<tr>
<td>WF</td>
<td>WF⁺</td>
<td>8.0 ± 1.4</td>
<td>9.0 ± 1.3</td>
<td>0.0 ± 0.0</td>
<td>99.6 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>60.6 ± 8.0</td>
</tr>
<tr>
<td>None</td>
<td>BBDP</td>
<td>1.4 ± 0.7</td>
<td>1.0 ± 0.6</td>
<td>92.2 ± 2.3</td>
<td>2.5 ± 1.8</td>
<td>82.8 ± 6.7</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>BBDP</td>
<td>BBDP⁺</td>
<td>1.4 ± 0.7</td>
<td>1.4 ± 0.5</td>
<td>95.8 ± 2.7</td>
<td>0.1 ± 0.1</td>
<td>85.7 ± 4.3</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>WF</td>
<td>BBDP</td>
<td>5.0 ± 1.4</td>
<td>8.2 ± 1.3[^b]</td>
<td>2.7 ± 1.6</td>
<td>95.9 ± 2.1</td>
<td>1.4 ± 0.9[^b]</td>
<td>67.1 ± 4.7[^b]</td>
</tr>
<tr>
<td>BBDP</td>
<td>WF</td>
<td>5.9 ± 1.9</td>
<td>2.1 ± 0.5</td>
<td>0.9 ± 0.1</td>
<td>98.5 ± 0.2</td>
<td>37.5 ± 13.1</td>
<td>11.0 ± 5.4</td>
</tr>
</tbody>
</table>
Legend to Table 9: WF (RT7.2+) and BBDP (RT7.1+) rats 6 weeks of age were randomized and either not treated further or irradiated and transplanted with either WF or BBDP bone marrow as indicated. When 12 weeks of age, all rats were killed, and IELs and bone marrow lymphocytes were isolated and analyzed by flow microfluorometry. Each data point represents the mean ± 1 s.d. for four individual animals. a: No paired comparisons between the transplanted hosts and non-transplanted controls are statistically significant. b: p<0.05 vs. BBDP → WF chimeras. No other paired comparisons between these two groups are statistically significant.
tic defect. Details of these findings are discussed in this section, and broader implications of these results are also addressed.

**Overall IEL deficiency in pre-diabetic BB rats**

Untreated BBDR rats resembled normal WF rats with regards to overall IEL numbers recovered using my new IEL isolation methodology. This relationship was consistent regardless of rat age. Treatment of BBDR rats with anti-ART2 mAb plus poly I:C for 1 week, approximately half the time required to induce diabetes (125), dramatically reduced the number of IELs recovered from these rats. The number of IELs recovered from BBDP rats was also reduced and comparable to that of treated BBDR rats. BBDP rat lymphocytes are more prone to apoptosis (121), so it could be argued that mechanical manipulation of IELs during the isolation protocol resulted in lower IEL recovery in BBDP rats. Histologic analysis, however, has also suggested previously that IEL numbers are decreased in BBDP rats (302). My quantification of IEL yields therefore establishes that pre-diabetic BB rats are deficient in overall IEL numbers, and it is plausible to hypothesize that such a deficiency impacts normal mechanisms of mucosal tolerance.

**IENK cell deficiency in BBDR rats**

Treatment of BBDR rats with anti-ART2 mAb plus poly I:C depletes peripheral ART2+ regulatory T cells and induces diabetes (125). I therefore analyzed IELs from untreated and treated BBDR rats for the presence and function of IENK cells, which may represent a regulatory lymphocyte population in the intestinal mucosa. Despite normal overall numbers of IELs, reduced relative numbers of IENK cells were observed in un-
treated BBDR rats. I have already established that IENK cells in WF rats are entirely responsible for spontaneous IFN-γ and IL-4 secretion, so unfractionated BBDR rat IELs were measured for this activity as a reflection of IENK cell function. Consistent with the argument that IENK cells are responsible for spontaneous IFN-γ and IL-4 secretion, this activity was reduced in unfractionated IELs from untreated BBDR rat.

Treatment of BBDR rats with anti-ART2 mAb plus poly I:C for 1 week further reduced the relative number of IENK cells and spontaneous cytokine secretion by unfractionated IELs to nearly undetectable levels. The relative number of intraepithelial T cells was preserved in treated BBDR rats, suggesting that the diabetes-inducing treatment preferentially depleted IENK cells. This relatively selective depletion of IENK cells by my diabetes-inducing treatment was a surprising result given that nearly all IELs express high levels of ART2 by flow cytometry analysis (26). Diabetes-inducing treatment does not show such selectivity peripherally, as >95% of peripheral ART2+ cells are depleted in treated BBDR rats (164). Although the anti-ART2 mAb definitely reaches the IEL compartment following intraperitoneal injection (unpublished observation), it remains unclear whether complement proteins are able to access and lyse IELs in situ.

Several mechanisms may explain the relatively selective IENK cell depletion in treated BBDR rats. If antibody-labeled IELs are not depleted in situ, then perhaps only ART2+ precursor cells en route to the IEL compartment are depleted by the diabetes-inducing treatment. A more rapid turnover by IENK cells as compared to intraepithelial T cells would therefore explain the relatively selective depletion of IENK cells in treated BBDR rats. Indeed, peripheral NK cells are exquisitely sensitive to radiation treatment
(355), supporting the hypothesis that turnover of peripheral NK cells is more rapid than that of peripheral T cells. It could also be argued that, if IEL depletion does occur \textit{in situ}, IENK cells may be more sensitive to complement-mediated lysis, thus accounting for their relatively selective depletion in treated BBDR rats. Heightened sensitivity to complement is often observed with cells that have down-regulated expression of decay accelerans factor (DAF, CD59), another GPI-linked protein (356), but there is currently no evidence for a decreased expression of DAF by IENK cells. Possible implications of selectively depleting a potential regulatory population from the IEL compartment of BBDR rats are discussed below.

\textbf{IENK cell deficiency in BBDP rats}

Given the peripheral lymphopenia of BBDP rats (124), it is not overly surprising to find a similar lymphopenia in the IEL compartment of BBDP rats, especially since the majority of IELs in normal rats are \textit{CD8\alpha}+ and ART2+ (26). Interestingly, however, there was a relatively selective decrease in the percentage IENK cells in BBDP rats that was even more pronounced than in untreated BBDR rats. This observation contrasts that of splenic NK cells, which are relatively increased in BBDP rats and decreased in BBDR rats (53). Accordingly, spontaneous IFN-γ and IL-4 secretion by unfractionated BBDP rat IELs was likewise dramatically reduced to undetectable levels.

One possible explanation for the relatively selective deficiency in IENK cells observed for BBDP rats is that there is a defect in one or more stroma-derived IEL growth factors. Such is the case in several other animal models that exhibit selective IEL defi-
ciencies. In Sl/Sld mice, which are naturally deficient in SCF, there is a selective and nearly complete lack of γδ TCR+ IELs (241). In addition, experimental mice genetically deficient in IL-7 also exhibit a relatively selective deficiency in γδ TCR+ IELs (238).

It is unlikely, however, that a stroma-derived defect accounts for the selective IENK deficiency in BBDP rats because normal WF bone marrow completely restores the IEL compartment in BBDP rats. Thus, the overall IEL deficiency and the relatively selective IENK deficiency in BBDP rats were attributable to a hematopoietic-derived defect. Similar results have been demonstrated for peripheral lymphocyte populations of BBDP rats reconstituted with bone marrow from BBDR rats (353). The exact nature of the hematopoietic-derived defect that leads to peripheral lymphopenia in BBDP rats is unknown, but it may involve the enhanced apoptosis of deficient lymphocyte subpopulations (121).

Several animal models besides the BBDP rat exhibit relatively selective deficiencies in IEL subpopulations secondary to one or more hematopoietic-derived defects. In W/Wv mice, which are naturally deficient in c-kit (the receptor for stem cell factor), and in genetically engineered IL-7R knockout mice there are predictable deficiencies in γδ TCR+ IELs similar to that of Sl/Sld and IL-7−/− mice, respectively (238;241). Mice that are genetically deficient in the Linker of Activated T cells (LAT) lack CD4+ and CD8β+ IEL subpopulations (357). In addition, genetically engineered mice lacking the IL-2 receptor β (IL-2Rβ) chain exhibit a selective deficiency of CD8αα+ αβ TCR+ and γδ TCR+ IELs; interestingly, these mice also lack NK cells peripherally (358).
Implications of IENK cell deficiency in BB rats

Regardless of the exact mechanism of selective IENK cell deficiencies in treated BBDR rats or in BBDP rats, it should be emphasized that the decreased relative number of IENK cells precedes diabetes in both cases. Furthermore, there is also a loss of IENK-associated spontaneous IFN-γ and IL-4 secretion by unfractionated IELs in pre-diabetic BB rats. Peripheral NK T cells, which have repeatedly been implicated as regulatory cells in mice, also express IFN-γ and IL-4 independent of exogenous cytokine stimulation (46;47;349). A loss of the potentially regulatory IENK cells in the intestinal epithelium of pre-diabetic BB rats may contribute to the disruption of mucosal tolerance and predispose these animals to activation of autoreactive T cells peripherally.

Conclusion to Chapter IV

With respect to diabetes, I have documented that spontaneously diabetic BBDP rats are severely deficient in IENK cells. Similarly, BBDR rats are also deficient in IENK cells as compared to the normal WF strain rat. Interestingly, however, when BBDR rats are induced to become diabetic using a standard protocol consisting of anti-ART2 mAb plus poly I:C (4), levels of IENK cells and associated spontaneous IFN-γ and IL-4 activity become nearly undetectable. The NK-like phenotype and function, together with an unusual cytokine profile—constitutive expression of IL-4 and IFN-γ in the local microenvironment of the gut—suggests that IENK cells are specialized cells that may be serving gut-specific functions. The present data suggest that one such function may relate to the expression of autoimmune diabetes in genetically susceptible individuals.
CHAPTER V

PRELIMINARY DATA: A DISRUPTION OF MUCOSAL TOLERANCE IN BBDP RATS TRIGGERS AUTOREACTIVE T CELLS AND LEADS TO AUTOIMMUNITY

Summary of Chapter V

BBDP rats spontaneously develop autoimmune diabetes mellitus and are severely deficient in peripheral ART2+ T cells. Dietary manipulation influences expression of diabetes in BBDP rats, suggesting a role for the intestinal immune system in disease etiopathogenesis. BBDR rats do not spontaneously develop autoimmune diabetes, have normal numbers of peripheral ART2+ T cells, and can be induced to become diabetic by injection of a cytotoxic anti-ART2a mAb plus low doses of poly I:C. Using a new, quantitative method of isolating large numbers of highly purified intraepithelial lymphocytes, I have documented previously the presence of a unique IEL subpopulation in non-autoimmune susceptible WF rats that consists of intraepithelial natural killer cells and is selectively deficient in pre-diabetic BB rats. In this chapter, I establish that, unlike BBDR and WF rats, BBDP rats are also deficient in γδTCR+ IELs, a population of T cells that may play a role in normal mucosal tolerance mechanisms. I also report preliminary data showing that systemic autoreactivity may be initiated in the intestine; peripheral autoreactive lymphocyte populations appear to emanate first from mesenteric lymph nodes that drain the intestine, and such cells may initiate a type 2 autoimmune phenomenon driven
by IL-4. These findings support the argument that a failure in mucosal tolerance in BBDP rats, perhaps secondary to deficiencies in one or more IEL subpopulations, triggers activation of autoreactive T cells peripherally and contributes to the immunopathogenesis of spontaneous diabetes in BB rats. They also call into question the general applicability of the Th1-model of autoimmune diabetes that has been the benchmark of disease pathogenesis in the NOD mouse.

**Introduction to Chapter V**

The majority of BBDP rats spontaneously develop autoimmune diabetes by 120 days of age (4). BBDP rats have a severe peripheral lymphopenia with a reduction of CD4+ T cells (124) and an almost complete lack of CD8+ (359;360) and ART2a+ T cells (118). The basis for the severe peripheral T cell deficiency, which results from reduced thymic emigration (120) and an enhanced rate of intrahepatic T cell apoptosis (121), has been traced to the hematopoietic stem cell (361). Transfusion of lymphocytes from normal histocompatible rats prevents the development of diabetes provided that ART2a+ cells become engrafted (122;123). Coisogenic BBDR rats, which have normal numbers of peripheral T cells, never spontaneously develop autoimmune diabetes when maintained in a VAF environment, but can be induced to become diabetic by injection of a cytotoxic anti-ART2a mAb and low doses of poly I:C, an immune system activator (125).

The phenotype and functions of intestinal lymphocytes are not fully characterized, but these cells appear to govern mucosal processes like oral tolerance and controlled (or physiologic) chronic inflammation (185). Defects in regulation of the intestinal immune
system are associated with celiac disease (88), inflammatory bowel disease (199;201;312), and possibly autoimmune diabetes (362). Gluten-rich diets and perhaps oral insulin – which protects NOD mice from diabetes (288) – may accelerate autoimmune diabetes in BBDP rats (170;177;290). In contrast, hydrolyzed casein-containing diets reduce the incidence of spontaneous diabetes in BBDP rats (170;177). Lack of tolerance to dietary gluten has been implicated in the immuno-pathogenesis of celiac disease (87;88), and several studies have documented a correlation between type 1 DM and celiac disease in humans (86;363-365).

I have recently described a new method for the isolation and purification of rat intestinal IELs that routinely yields 30-50 million IELs per adult rat (26). Analysis of IELs isolated by this new method has demonstrated the presence of five IEL subpopulations based on the differential expression of αβTCR, CD8α and CD4, with a major subpopulation consisting of intraepithelial NK cells (26). IENK cells are unique in that they display an activated phenotype and spontaneously secrete the immunomodulatory cytokines IFN-γ and IL-4. I have shown previously that BB rats, particularly pre-diabetic BBDR and BBDP rats, are deficient in the relative number and function of IENK cells.

A second subpopulation of IELs expresses the γδ TCR and was shown by me to comprise a second major IEL compartment in normal WF rats. I now establish that, prior to diabetes, BBDP rats but not BBDR rats exhibit a selective deficiency of γδTCR⁺ IEL cells as compared to WF rats. I extend these findings to peripheral lymphocyte compartments and demonstrate that mesenteric lymph nodes draining the intestine of pre-diabetic BB rats preferentially harbor autoreactive lymphocytes as compared to cervical lymph
node lymphocytes. By analyzing the cytokine profile of mesenteric lymph nodes, serum IgE levels, and tissue sections of intestine and pancreas, I also present preliminary data suggesting that the autoimmune phenomenon in BB rats results from a disruption of normal mucosal tolerance mechanisms and is a type 2-driven autoimmune process, similar to allergic hypersensitivity.

**Materials and Methods of Chapter V**

**Animals**

BBDR and BBDP rats were obtained from BMR, Inc (Worcester, MA). WF rats were obtained from Harlan Sprague Dawley (Indianapolis, IN). Athymic WAG-\textit{rnu/rnu} rats were obtained from the colony at the University of Massachusetts (Worcester, MA) and used at 12 weeks of age. Animals of either sex were used in all experiments. All animals were certified to be serologically free of Sendai virus, pneumonia virus of mice, sialodacryoadenitis virus, rat corona virus, Kilham rat virus, H1 (Toolan's virus), mouse poliovirus (GD7), Reo-3, \textit{Mycoplasma pulmonis}, lymphocytic choriomeningitis virus, mouse adenovirus, Hantaan virus, and \textit{Encephalitozoon cuniculi}. All animals were housed in a VAF facility until used and maintained in accordance with recommendations in the \textit{Guide for the Care and Use of Laboratory Animals} (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996) and the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.
Antibodies

Fluorochrome-conjugated mAbs directed against CD3 (clone G4.18) and γδTCR (clone V65) were obtained from BD PharMingen (San Diego, CA). For measurement of IL-4-secreting cells by ELISpot, paired mAbs (purified OX-81 and biotinylated B11-3) directed against IL-4 were obtained from BD PharMingen. For measurement of IFN-γ by ELISpot, the capture mAb (clone DB-1) was obtained from BioSource International (Camarillo, CA) and the detection rabbit anti-rat IFN-γ polyclonal antibody was obtained from Torrey-Pines Biolabs (La Jolla, CA) and biotinylated as described (313). Isotype control mouse IgG1 and mouse IgG3 and alkaline phosphatase-conjugated streptavidin were purchased from BD PharMingen.

Cell preparation

Intraepithelial lymphocytes were prepared from rat small intestine by Percoll® density gradient centrifugation using minor modifications of a method developed in our laboratory (26). Viable cell numbers were quantified by the method of Trypan blue using a hemocytometer. Single cell suspensions of splenocytes and lymph node lymphocytes and were prepared by gentle extrusion through stainless steel sieves into medium consisting of cold RPMI-1640 supplemented with 5% Fetalclone® I as described (121). For preparation of lymph node lymphocytes, cervical and mesenteric cells were processed separately.
Manipulation of BBDR and BBDP rats

Treatment of BBDR Rats with a diabetes-induction protocol

BBDR rats 25-28 days of age were treated with intraperitoneal injections of anti-ART2a mAb (50 μg 5 times weekly) and poly I:C (5 μg/100 g body weight 3 times weekly) as described (125). Rats were treated for 7-12 days, less than the time required for diabetes onset (4).

Prevention of diabetes in BBDP rats by BBDR splenocytes

BBDP rats 25-28 days of age were injected intraperitoneally with splenocytes from age-matched, untreated BBDR donors. Splenocytes were not depleted of erythrocytes, and each recipient animal received the equivalent of one entire donor spleen.

Adoptive transfer of lymph node lymphocytes and diagnosis of diabetes

For adoptive transfer experiments, 20 x 10⁶ mesenteric or cervical lymph node lymphocytes from pre-diabetic BBDR rats that had received 7 days of the anti-ART2a plus poly I:C diabetes-induction protocol (~5 weeks old) were isolated and infused intravenously into athymic WAG-rnu/rnu recipients at 12 weeks of age. BBDP rats and adoptive transfer recipients were monitored for diabetes by testing for glycosuria three times weekly (Tes-Tape, Eli Lilly, Indianapolis, IN). The diagnosis of diabetes in glycosuric animals was confirmed by a plasma glucose concentration of >200 mg/dl.
**Flow cytometry**

Single and two-color flow cytometric analyses were performed as described (26). Briefly, $1 \times 10^6$ viable intraepithelial and splenic lymphocytes were suspended in ice-cold FACS medium (isotonic PBS supplemented with 1% Fetalclone® I) and reacted with FITC- and PE-conjugated antibodies for 30 min. at 4°C. All samples were then washed with FACS medium and fixed with 1% paraformaldehyde in PBS. FITC- and PE-conjugated isotype control immunoglobulins were used for all analyses. Cells were analyzed using a FACScan® instrument (Becton Dickinson, Sunnyvale, CA). Analyses were gated on lymphoid cells, which were identified by forward and side light-scatter profiles.

**Cytokine analyses**

Interferon-γ and IL-4 production was measured by ELISpot assay of MLN and cervical lymph node (CLN) lymphocytes from BB and WF rats as described (354) except that cells were incubated for the overnight culture in the presence of PMA and ionomycin. Included in this study were untreated WF rats, untreated BBDR rats, untreated BBDP rats, BBDR rats that had been treated for 12 days with the diabetes-induction protocol, and BBDP rats that had received an infusion of splenic lymphocytes from untreated BBDR rats to prevent diabetes. All rats were 5-6 weeks old, and none were diabetic. The optimal number of IELs assayed was determined in preliminary experiments to be $1 \times 10^5$ cells per well.

The number of discrete spots visible in each well was counted using a light microscope at 2x power. Each cytokine assay was performed using 4-6 wells, with all wells but...
one assayed using the cytokine-specific developing antibody and the remaining well assayed using the irrelevant biotinylated control antibody. For analysis, the number of spots observed in the control well for each multiplicate assay was subtracted from the number of spots counted in each of the test wells. The maximum number of spots observed in any control well was three. The adjusted number of spots present in each of the test wells was then averaged. To account for the various numbers of cells added per well, data are normalized to number of spots per 10^4 cells.

**Histology**

Samples of intestine and pancreas were obtained from 16 week old untreated BBDR and diabetic BBDP rats. Specimens were immediately fixed in 10% buffered formalin. Paraffin-embedded tissue sections of all samples were stained with hematoxylin and eosin and examined by light microscopy.

**Preliminary Results of Chapter V**

**Deficiency in γδTCR⁺ IELs in pre-diabetic BBDP rats**

Using flow cytometry, I measured directly the relative numbers of γδ T cells in IELs and splenocytes isolated from 4-6 week old BB rats, none of which were diabetic. The percentage of CD3⁺ IELs expressing the γδTCR in untreated and treated BBDR rats was similar to that of WF rats and greater than that of BBDP rats (Figure 24). In addition, the percentage of overall IELs exhibiting a CD3⁺ γδTCR⁺ phenotype in untreated and treated BBDR rats (32.5 ± 2.7% and 50.5 ± 4.1%, respectively) was greater than that ob-
Figure 24: Flow cytometric analysis of γδTCR expression on intraepithelial T cells in WF and BB rat IELs
Figure 24: Flow cytometric analysis of γδTCR expression on intraepithelial T cells in WF and BB rat IELs. IELs were isolated from 4-6 week old rats, reacted with mAb specific for CD3 and γδTCR (V65 clone), and analyzed by flow cytometry. Fluorescence intensity (horizontal axis) is plotted against cell number (vertical axis). Shown are percentages and representative profiles of γδTCR expression on gated CD3+ lymphocytes as described in Methods (solid line). Fluorescence associated with isotype staining is also indicated (dashed line). Included in this study were WF rats, untreated BBDR rats, BBDR rats that had received one week of diabetes-inducing anti-ART2a plus poly I:C (treated BBDR), and BBDP rats. Comparable results were obtained in at least 4 individual animals.
served in BBDP rats (5.3 ± 2.0%). The percentage of CD3+ splenocytes expressing the γδTCR was actually increased in BBDP rats (12.1 ± 2.9%) versus WF and untreated BBDR rats (6.9 ± 0.9% and 7.7 ± 1.6%, respectively). In a separate analysis of IELs and splenocytes isolated from 7-9 week old BB rats, BBDP rats exhibited comparable differences in IEL but not splenic γδ T cells (data not shown).

Mesenteric lymph node cells in BBDR rats harbor autoreactive cells prior to diabetes expression

I next sought to ascertain whether any potential defects in intestinal mucosal tolerance might have implications on peripheral autoreactive lymphocyte populations. Accordingly, MLN cells, which drain the intestine (205), and CLN cells were isolated from BBDR rats that had received 7 days of the diabetes induction protocol and then tested independently for their ability to adoptively transfer autoimmune diabetes to athymic recipients. Gut-draining MLN from treated BBDR rats transferred diabetes to athymic rats earlier than did CLN (Figure 25).

Mesenteric lymph node cells in pre-diabetic BB rats exhibit a type 2 cytokine profile

To better understand the mechanism that permits the early appearance of autoreactive cells in the MLN of BB rats, IFN-γ and IL-4 production was analyzed by ELISpot as described in Methods. Low levels of each cytokine were detected in the MLN and CLN populations of control WF and untreated BBDR rats (Table 10). Treatment for 12 days with anti-ART2 mAb plus poly I:C to induce diabetes induced a surprisingly high
Figure 25: Diabetes expression in athymic rats that received mesenteric or cervical lymph node lymphocytes from pre-diabetic BBDR rats
Figure 25: Diabetes expression in athymic rats that received mesenteric or cervical lymph node lymphocytes from pre-diabetic BBDR rats. Athymic WAG rnu/rnu rats were intravenously administered 20 x 10⁶ mesenteric (broken line) or cervical (solid line) lymph node lymphocytes from BBDR rats that had been treated for 1 week with the anti-ART2a plus poly I:C diabetes-induction protocol. Recipient animals were monitored for diabetes as described in Methods (n = 5 for each group). Shown is a Kaplan-Meyer graph of the cumulative percentage of diabetes-free recipients (y-axis) plotted versus time (x-axis).
TABLE 10

CYTOKINE PRODUCTION BY CLN AND MLN FROM WF AND BB RATS

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>IL-4 (spots/10⁵ cells ± SEM)</th>
<th>IFN-γ (spots/10⁵ cells ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CLN</td>
<td>MLN</td>
</tr>
<tr>
<td>WF</td>
<td>None</td>
<td>0 ± 0</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>BBDR</td>
<td>None</td>
<td>0 ± 0</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>BBDR</td>
<td>anti-ART2a plus poly I:C</td>
<td>1 ± 1</td>
<td>38 ± 5</td>
</tr>
<tr>
<td>BBDP</td>
<td>None</td>
<td>2 ± 1</td>
<td>19 ± 3</td>
</tr>
<tr>
<td>BBDP</td>
<td>Splenocyte Transfusion</td>
<td>2 ± 0</td>
<td>7 ± 2</td>
</tr>
</tbody>
</table>

Legend to Table 10: MLN and CLN lymphocytes from WF and BB rats 4-6 weeks old were isolated as described in Methods. BBDR rats were either untreated or treated for one week with anti-ART2a mAb plus poly I:C to induce diabetes as described in Methods. BBDP rats were either untreated or transfused intraperitoneally at 4 weeks of age with lymphocytes from one BBDR spleen to prevent diabetes as described in Methods. The number of PMA plus ionomycin stimulated lymphocytes secreting IFN-γ or IL-4 was determined by ELISpot after overnight culture as described in Methods. Each data point represents the mean ± 1 SEM for one individual animal.
production of IL-4 in MLN but not CLN of pre-diabetic BBDR rats, whereas just a modest rise in IFN-γ production was detected only in the CLN of treated BBDR rats (Table 10). A similar situation occurred in BBDP rats; IL-4 but not IFN-γ was markedly increased in the MLN but not CLN of pre-diabetic BBDP rats, whereas infusion of splenic lymphocytes from BBDR rats to prevent diabetes abrogated IL-4 production by BBDP rat MLNs (Table 10). Using Light Cycler® technology to quantify IL-4 mRNA, transcript levels from CLN and MLN samples closely correlated to ELISpot results (data not shown). Levels of serum IgE were also measured (by ELISA) and found to be elevated in pre-diabetic BB rats as compared to non-diabetic controls (data not shown).

**Eosinophils in the intestine and islets of diabetic BBDP rats**

Interleukin-4 and IgE are typically associated with type 2 (Th2) immune responses like allergic hypersensitivity (181). I next analyzed samples of intestine and pancreas from 16 week old untreated BBDR rats and from age-matched BBDP rats that had diabetes for ~1 month. Gross analysis revealed that the intestine of BBDP but not BBDR rats hypercontracted upon evisceration (not shown). Histologic analysis demonstrated that the intestine of BBDR rats appeared normal, whereas the muscularis layer of BBDP rat intestine was thickened and hyperplastic (Figure 26). In addition, eosinophils were present between the circular and longitudinal muscularis layers in BBDP but not BBDR rats (Figure 26). Additional histologic analysis of pancreas tissue revealed normal islet architecture in BBDR rats, whereas eosinophils were present in the insulitic lesion of diabetic BBDP rats (Figure 26).
Figure 26: Histology of BB rat intestine and pancreas
Figure 26: Histological analysis of small intestine and pancreas from BBDR and BBDP rats. Jejunum and pancreas were isolated from 16 week old BBDR rats and from age-matched BBDP rats that had diabetes for ~1 month. Samples were paraffin-embedded, stained with hematoxylin and eosin, and analyzed via light microscopy. Samples of jejunum and pancreas from BBDR rats (Panels A and B, respectively) show no evidence of pathology. Samples of jejunum from BBDP rats (Panel C) exhibit a thickened and hyperplastic muscularis with eosinophils present between circular and longitudinal muscle layers (arrows). Samples of pancreas from BBDP rats (Panel D) demonstrate insulitis with eosinophils present in the infiltrate (arrows). Shown is a representative field for each sample. Magnification is indicated in parentheses.
Discussion of Chapter V

Environmental factors, including dietary antigens, influence the expression of spontaneous autoimmune diabetes in BBDP rats (4;170;177). In addition, oral insulin, which protects NOD mice from diabetes (278), does not prevent and may actually exacerbate diabetes in BBDP rats (289;290). What is it about the intestinal microenvironment in BBDP rats that contributes to such findings? The intestine is one site where lymphocytes encounter a major load of environmental antigens (181). Accordingly, tolerance mechanisms unique to the intestinal mucosal are necessary, including physiologic chronic inflammation and oral tolerance (185). Such processes are postulated to be governed by specialized intestinal regulatory cell populations, but the nature and function of these cells is still poorly characterized. Failure of mucosal tolerance has been implicated in the pathogenesis of inflammatory bowel disease (199;201;312), celiac disease (88) and perhaps autoimmune diabetes (362).

It has been shown previously that pre-diabetic BB rats exhibit severe deficiencies in peripheral and intestinal lymphocytes (4;366). Deficient cell populations include potential regulatory cells, a lack of which may predispose these animals to autoimmune diabetes (4;25;164;366). Preliminary data presented in this chapter suggest that, in addition to IEL deficiencies, several systemic defects are also observed in pre-diabetic BB rats that may be indicative of a failure of mucosal tolerance.
Summary of preliminary data

Prior to diabetes, intraepithelial γδ T cells are deficient in BBDP but not BBDR rats. It is likely that the γδ T cell deficiency of BBDP rats is due to a hematopoietic defect because engraftment of WF bone marrow into irradiated BBDP rats reconstitutes γδTCR⁺ IELs (unpublished observation). Several antigen-independent functions have been ascribed to intraepithelial γδ T cells, most likely reflecting the restriction in γδTCR repertoire (19). These functions include: maintenance of epithelial integrity (264;265), cytotoxicity of stressed intestinal epithelial cells (8;19), and regulation of mucosal immune responses (15;367). Interestingly, increased intestinal permeability and inflammation occurs in humans with type 1 diabetes mellitus (362;368;369) and in BBDP but not BBDR rats prior to diabetes and independent of dietary antigen (370). It is conceivable that such a finding is a direct consequence of the γδTCR⁺ IEL deficiency in BBDP rats.

These data may also provide a link between lymphocytes of the intestine and periphery. Lymphocytes of the MLN, which drain the intestine, are capable of adoptively transferring diabetes to athymic rats earlier in disease pathogenesis (prior to diabetes in donor rats) as compared to CLN lymphocytes, suggesting that activated autoreactive peripheral lymphocytes appear in the MLNs prior to systemic circulation. A similar finding has been reported in NOD mice (371;372).

I also provide surprising yet preliminary evidence that autoimmune diabetes in BB rats may be the result of an IL-4 driven type 2 immune response. Mesenteric but not cervical lymph nodes from pre-diabetic rats produced high levels of IL-4 that were associated with elevated serum IgE. By histology, the intestine of BBDP rats resembled the
allergic hypersensitivity typically seen in food allergy. These data directly challenge the
dogma established in NOD mice that autoimmune diabetes is a Th1-mediated disease
(32-34). Of note, there exists at least one report that Th2-polarized T cells induce insulitis
and diabetes when administered to NOD.scid mice (373).

Although my data argue that Th2-type MLN lymphocytes contribute to the ex-
pression of autoimmune diabetes in BB rats, how do I reconcile these preliminary data
with the observation that Th1 cytokines are elevated in acutely inflamed islets of BB rats?
Several explanations may account for these apparently contradictory data. First, there
may be an issue of location; Th2-type autoreactive T cells may preferentially home to the
intestine or MLN, whereas Th1-type autoreactive T cells may home to the pancreas. Al-
ternatively, there may be an issue of timing; with the earliest autoreactive cells acquiring
a Th2-cytokine profile in the gut, and later autoreactive cells acquiring a Th1-type cyto-
kine profile in the pancreas. Finally, it is not unreasonable to hypothesize that the Th1
model of autoimmune diabetes established in the NOD mouse (33) is not directly appli-
cable to the BB rat. Supporting this argument is the observation that both recombinant
IFN-γ (159;160) and anti-IFN-γ mAb (158) prevent diabetes in BBDP rats.

The correlation between IL-4 protein, IL-4 mRNA, serum IgE, and histology
strongly argues that the phenomenon observed in preliminary experiments is real, but
there is still room to question whether or not the Th2 profile actually contributes to dia-
betes expression. Typically, Th2 immune responses occur in response to allergy or para-
sitic infection. It is possible that, concurrent with this preliminary experiment, there was
an undetected environmental allergen or parasitic infection in our rat colony. Independent
of diabetes, immunodeficient animals (untreated BBDP and treated BBDR rats) may have therefore been more susceptible to systemic immune responses against the unknown agent. Although the possibility of parasites is raised whenever eosinophils are observed in the islet or intestine of BBDP rats (374;375), our animals are routinely screened for parasites and consistently test negative (Barbara Whalen, personal communication). Further experimentation in this subject is clearly necessary.

**Systemic implications of mucosal lymphocyte deficiencies in BBDP rats**

How might deficiencies in intraepithelial NK and γδ T cell populations contribute to systemic immune defects observed in pre-diabetic BB rats? Failure of mucosal tolerance is associated with local and systemic autoimmune disease (88;199;201;362), and both intraepithelial NK and γδ T cells may act locally as regulatory cells. Increased intestinal permeability is associated with deficiencies in intraepithelial γδ T cells (264;265) and allergic responses to dietary antigen (181;203;204). The loss of epithelial integrity in food allergy is in part attributable to “leaky” tight junctions that allow for the paracellular passage of luminal antigens (181). Therefore, LP DCs, which typically receive antigen that has been processed and transported in a transcellular fashion by IECs (181), may be directly exposed to intestinal antigens. The migration of activated dendritic cells to regional mesenteric lymph nodes (205) sets the stage for systemic immune responses (206) and may be responsible for defects observed in the MLN of pre-diabetic BB rats. Alternatively, autoreactive lymphocytes may circulate through the intestine and become activated *in situ* due to a lack of properly controlled physiologic inflammation (199).
Failure of mucosal tolerance and autoimmune diabetes

Besides being responsible for intestinal disorders like inflammatory bowel disease (IBD) and celiac disease, failure of mucosal tolerance may also influence the expression of autoimmune diabetes in humans and rodents (362). The role of cow’s milk protein in the pathogenesis of human type 1 DM is still debated (108-112). In addition, a link between celiac disease, an allergic reaction to dietary gluten, and autoimmune diabetes has been increasingly recognized in humans (86;363-365). Data presented here suggest that a similar dietary allergy may occur in BB rats. Whether or not dietary gluten induces diabetes in BB rats remains to be proven, but several studies have shown that dietary gluten abrogates the diabetes-protective effect of a hydrolyzed casein diet (170;177). In contrast, dietary supplementation with BSA or whole milk protein does not abrogate the diabetes-protective effect of a hydrolyzed casein diet (172).

Conclusion to Chapter V

Although still poorly understood, there is clearly a role for environmental factors in the pathogenesis of autoimmune diabetes in humans and rodents. The intestine is potentially a major source of such environmental perturbants. I have provided preliminary data suggesting that, prior to diabetes, several immunologic defects can be detected in the mesenteric but not cervical lymph nodes of BB rats, thus implicating defects in intestinal mucosal tolerance in the pathogenesis of systemic autoimmunity. These results need to be investigated further. If applicable to human type 1 diabetes mellitus, then perhaps diabetogenic intestinal antigens can be identified and eliminated from at-risk individuals.
CHAPTER VI

COMPREHENSIVE DISCUSSION

Chapter VI, Section 1 - General Background to Discussion

Review of clinical rationale

Type 1 diabetes mellitus continues to cause significant morbidity and mortality in children and young adults despite modern advances in patient care. No modifiable risk factors for type 1 DM have currently been identified, so disease incidence has yet to decrease and may even be on the rise (376). These concerns, together with the rising economic cost of health care, necessitate the development of alternative modalities designed to decrease the incidence of type 1 DM. Before we can hope to prevent this disease in humans, however, a greater understanding of the immunopathogenesis of type 1 DM is necessary. For any disease, ethical and moral issues surrounding experimentation in humans hinder the study of disease pathogenesis in humans. Accordingly, animal models of human disease prove to be an invaluable tool, and our laboratory has made extensive use of the BB rat, a rodent model of type 1 diabetes mellitus.

Overview of Chapter VI

The objective of this chapter is to discuss my findings as they relate to autoimmune diabetes and mucosal tolerance. Section 2 of this chapter states my working hy-
pothesis regarding the immunopathogenesis of autoimmune diabetes in BB rats. Section 3 explains my rationale for studying intestinal intraepithelial lymphocytes. Section 4 reviews in a broader context the data presented in Chapters II-V, with particular attention given to Chapters III. Section 5 postulates a mechanism whereby defects in mucosal tolerance may contribute to autoimmune diabetes in BB rats. Finally, Section 6 addresses the applicability of this work to human type 1 diabetes mellitus and suggests future directions for continuation of this work.

Chapter VI, Section 2 - The Immunopathogenesis of Autoimmune Diabetes in BB Rats: A Working Hypothesis

Based on experiments conducted prior to this dissertation, I have expanded the “Balance Hypothesis” (Chapter I, Section 4 – Figure 3) into a working hypothesis that at least three factors contribute to the immunopathogenesis of autoimmune diabetes in BB rats (summarized in Table 11). First, autoreactive T cells are generated in the thymus. Second, the relative number of peripheral ART2+ regulatory T cells is insufficient to prevent autoimmunity by autoreactive ART2- T cells (the “Balance Hypothesis”). Third, there occurs an aberrant activation of autoreactive T cells that subsequently initiate autoimmunity in the absence of sufficient regulation by ART2+ T cells.

Generation of autoreactive T lymphocytes

The development of spontaneous autoimmune diabetes in BBDP rats that can be adoptively transferred to non-diabetic rats via lymphocytes strongly argues for the pres-
TABLE 11
THREE FACTORS CONTRIBUTE TO AUTOIMMUNE DIABETES IN BB RATS

<table>
<thead>
<tr>
<th>Factor</th>
<th>Spontaneous BBDP Rat</th>
<th>Inducible BBDR Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Autoreactive cells</td>
<td>Generated in thymus (programmed by <em>iddm4</em>?)</td>
<td>Generated in thymus (programmed by <em>iddm4</em>?)</td>
</tr>
<tr>
<td>2. Insufficient ART2⁺ regulatory cells</td>
<td>Genetically programmed by <em>lyp</em>⁻⁻ mutation</td>
<td>Induced experimentally by cytotoxic anti-ART2a mAb</td>
</tr>
<tr>
<td>3. Activation of autoreactive cells</td>
<td>Environmental source: Failure of mucosal tolerance to harmless gut antigens (e.g. dietary protein)</td>
<td>Induced experimentally by immune system activator (e.g. poly I:C, KRV)</td>
</tr>
</tbody>
</table>

Legend to Table 11: I postulate that at least three factors contribute to the immunopathogenesis of autoimmune diabetes in BB rats. First, autoreactive T cells are generated in the thymus. Second, the relative number of peripheral ART2⁺ regulatory T cells is insufficient to prevent autoimmunity by autoreactive ART2⁻ T cells (the “Balance Hypothesis”). Third, there occurs an aberrant activation of autoreactive T cells that subsequently initiate autoimmunity in the absence of sufficient regulation by ART2⁺ T cells. Listed for both BBDP and BBDR rats are potential mechanisms for these three factors.
ence of autoreactive cells in BBDP rats (4;146). Thymectomy prevents diabetes in BBDP rats, suggesting further that autoimmunity is mediated in part by autoreactive T cells (377). By histologic analysis, CD4+ and CD8+ T cells are among the cells present in the insulitic lesion (4).

That diabetes can be induced in BBDR rats following experimental manipulation argues for the presence of autoreactive cells in these animals (125;164). Evidence that autoreactive cells in BBDR rats are T cells is provided by experiments in which splenocytes from diabetes-induced BBDR rats adoptively transfer disease to non-diabetic rats (147). In addition, thymocytes from BBDR rats that are adoptively transferred to athymic rats induce diabetes provided that recipient animals are depleted of ART2+ T cells (151).

In both BBDP and BBDR rats, the presence of autoreactive cells may be attributable in part to an autosomal dominant diabetes-susceptibility locus, termed iddm4, that has recently been mapped in (WF x DP) x WF rats to chromosomes 4 (4;351;352). This gene, which has yet to be identified, appears to be involved with the genetic predisposition of BB rats to autoimmunity (4).

The generation of autoreactive T cells is not unique to BB rats. Autoreactive T cells appear to be responsible for experimentally induced autoimmune syndromes in PVG and LEW rats, among others. On the other hand, some rat strains like the WF are remarkably resistant to autoimmune-inducing protocols (130). It is possible that such strains lack autoreactive T cell populations entirely.
Insufficient numbers of ART2+ regulatory T cells

A major breakthrough in the BB rat field has been the observation that the number of peripheral ART2+ T cells correlates inversely with expression of autoimmune diabetes in both BBDP (118) and BBDR rats (164). Accordingly, these cells are thought to represent a population of regulatory T cells (25). The mechanism whereby these cells may regulate immune responses is not yet known, but potential immunoregulatory functions of ART2 were discussed in Section 4 of Chapter I.

In BBDP rats, there is a natural deficiency in peripheral ART2+ T cells attributable to a homozygous mutation at the lyp gene locus (4). The lyp+/− defect in BBDP rats results in decreased thymic export of T cells (120) and increased intrahepatic apoptosis of recent thymic emigrants (121). Infusion of splenocytes from non-lymphopenic histocompatible donors (BBDR or WF rats) prevents diabetes in BBDP rats provided that ART2+ cells engraft (122;123).

BBDR rats have normal numbers of peripheral ART2+ T cells and do not spontaneously develop diabetes (4). Autoimmune diabetes can be induced in >90% of BBDR rats, though, by administration of a cytotoxic anti-ART2 mAb plus an immune system activator such as poly I:C or KRV (125;126;164). Decreased numbers of ART2+ T cells appear permissive but not required for diabetes expression in BBDR rats, however, as KRV infection alone induces diabetes in ~30% of BBDR rats (126;378). Thus, it seems that ART2+ regulatory T cells raise the threshold of activation required for autoimmunity. Activation of autoreactive ART2− T cells is elaborated upon below.
Regulatory activity has been shown for ART2+ T cells in other rat strains besides the BB rat. Diabetes is induced in PVG-RT1u rats by irradiation and neonatal thymectomy and is associated with deficiencies in CD4+ ART2+ T cells (133). Diabetes is prevented in these rats by infusion of CD4+ ART2+ T cells from unmanipulated PVG-RT1u rats. Administration of poly I:C or KRV also induces diabetes in PVG-RT1u rats, but depletion of ART2+ T cells is required for disease expression in this instance (130;131).

**Activation of autoreactive T cells**

Variations of the standard diabetes inducting protocol suggest that an activation of autoreactive T cells is required for the expression of autoimmune diabetes in BBDR rats. Administration of high-dose poly I:C or KRV alone induces diabetes in ~30% of BBDR rats (126;378). In contrast, depletion of ART2+ T cells without poly I:C or KRV fails to induce autoimmunity in VAF BBDR rats (156). Taken together, these observations argue that depletion of peripheral ART2+ T cells is not necessary for disease expression, but activation of ART2- autoreactive T cells is required.

Unlike diabetic BBDR rats, in which immune stimulation by poly I:C or KRV may allow for the activation of autoreactive cells, BBDP rats spontaneously develop diabetes between 60-120 days of age (4). In addition, the incidence of diabetes in BBDP rats is ~90% (<100% concordance), and diabetes onset in BBDP rats is influenced by environmental factors, including dietary manipulation (4;170). These data argue for the presence of one or more environmental factors that activate autoreactive cells in BBDP rats and trigger autoimmunity.
Chapter VI, Section 3 - Rationale for Studying Intestinal Lymphocytes in BB Rats: A Failure of Mucosal Tolerance Can Have Systemic Consequences

The intestinal mucosa: Environment and the Immune System

I sought to study intestinal lymphocytes in BB rats because the proposed activation of peripheral autoreactive cells by environmental agents may occur in the intestinal mucosa, a major site of interaction between the immune system and the environment (185). The intestinal mucosa sees a huge antigenic load; it is estimated that $10^{13}$ organisms inhabit the intestine at any given time, and an individual consumes at least 2,500 kg of food antigens during a normal lifetime (181). Also in the gastrointestinal tract are tremendous numbers of lymphocytes that must respond to infectious agents yet remain tolerant of antigens from typically non-pathogenic sources (185). Intestinal lymphocytes appear to regulate the balance between mucosal immunity and mucosal tolerance to gut antigens.

When mucosal tolerance fails: Local consequences

In animal models and possibly in humans, the loss of normal mucosal tolerance mechanisms is associated with several intestinal disorders, including inflammatory bowel disease (IBD) (199;201) and celiac disease (88). In these cases, absence or failure of mucosal regulatory cells has been implicated in disease pathogenesis (199). Germ-free animals do not develop IBD (276;379-381), suggesting that an overactive immune response
to commensal intestinal bacteria triggers IBD (185;201). In celiac disease, however, the offending antigen is known to be gluten, a dietary protein of wheat germ, and removal of gluten from the diets ameliorates the disease (87;88).

**When mucosal tolerance fails: Systemic consequences**

Mucosal tolerance is not limited to intestinal lymphocytes. Inflammatory bowel diseases are associated systemically with ocular, joint, and skin inflammations (382). A failure of mucosal tolerance has also been implicated in the pathogenesis of autoimmune diabetes in NOD mice (362). Mesenteric lymph nodes from NOD mice adoptively transfer disease even earlier than pancreatic lymph nodes (371;372), and the vascular endothelium of acutely inflamed islets in NOD mice expresses the gut-associated adhesion molecule MAdCAM-1, a mucosal vascular addressin (383;384). Additionally, islet-infiltrating T cells in NOD mice display the α4β7 integrin typically expressed by lamina propria lymphocytes (385), and administration of an anti-β7 integrin prevents diabetes in NOD mice (386).

**Environment and spontaneous autoimmune diabetes in BBDP rats**

Any failure of mucosal tolerance in spontaneously diabetic BBDP rats more closely resembles celiac disease than IBD because removal of known pathogens does not protect BBDP rats from diabetes; gnotobiotic animals spontaneously develop diabetes at rates similar to conventionally housed animals (157). Other environmental activation sources cannot be excluded by this study, however, because even these gnotobiotic ani-
mals received food; they suckled until weaning and were then maintained on germ-free rat chow (Aldo Rossini, personal communication) (157).

Many common food antigens stimulate intestinal lymphocytes (170;177;181), and diets with low antigenicity – with protein sources from hydrolyzed casein (172;173) or elemental amino acids (171) – decrease the incidence of spontaneous diabetes in BBDP rats. On the other hand, gluten rich diets are diabetogenic (170;175). It is therefore entirely conceivable that BBDP rats, which are deficient in peripheral regulatory cells, also lack intestinal regulatory cells and therefore lack normal mechanisms of mucosal tolerance to dietary antigens.

**Why study intraepithelial lymphocytes in BB rats?**

Intraepithelial lymphocytes are among the first cells of the immune system to encounter enteric antigens (8), but their role in mucosal tolerance is unknown. Effector mechanisms of mucosal tolerance were introduced in Chapter I and include secretory IgA, generation of Th3 and Tr1 cells, and generation of CD8+ suppressor cells. IELs may contribute to each of these processes either directly through suppression/regulation of aberrant mucosal immune responses (214;354;366) and/or indirectly through the maintenance of epithelial permeability (247;264;265). I therefore sought to address the nature of IELs in normal and BB rats with the hypothesis that defects in IEL number and/or function may contribute to a failure of mucosal tolerance and spontaneous autoimmune diabetes in BBDP rats.
Chapter VI, Section 4 - Review of Findings

In Chapter I, I stated that the overall objective of this dissertation was to investigate whether defects in IEL number and/or function may contribute to the expression of autoimmunity in the BB rat model of type 1 diabetes mellitus. To address this issue, I have established a new IEL isolation protocol that has enabled me to investigate the phenotype and function of IELs in normal WF rats. Through these studies, I identified a population of potentially regulatory intraepithelial natural killer cells. Importantly, the relative number and function of these cells are deficient in BB rats, particularly in prediabetic BBDR and BBDP rats. These data are reviewed here and discussed in the broader context of mucosal tolerance and autoimmune diabetes.

An improved methodology for the isolation of intraepithelial lymphocytes

The role of IELs in mucosal tolerance has been difficult to study in part because current methods for the isolation of IELs require extensive manipulation of the gut (16;216;295;297), and IEL preparations are often contaminated with intestinal epithelial cell that may interfere with in vitro IEL assays (296;330). I have therefore established a new method for the isolation of rat IELs that is based on the selective removal of intestinal epithelial cells under conditions that leave the basement membrane undisturbed. The method is rapid and requires neither enzymatic digestion, nor surgical removal of Peyer’s patches, nor vigorous mechanical manipulation of the intestine. The yield of rat IELs using this method is 5-10 fold greater and of higher purity than that reported for other methods (216;295). Morphological and phenotypic analyses demonstrated that the puri-
fied cell population is comprised of IELs and is not contaminated with lamina propria or Peyer's patch lymphocytes. This methodology allows me to investigate phenotypic and functional characteristics of IELs on a scale that has heretofore been unattainable.

**Identification of a potentially regulatory natural killer cell population in the intestinal epithelium of normal rats**

Using new methodology, I have identified a novel population of intraepithelial NK cells that appear constitutively to secrete IL-4 and IFN-γ. These unusual IENK cells are similar to peripheral NK cells in morphology, in their ability to lyse NK-specific targets, and in their ability to suppress a one-way mixed lymphocyte culture. They are, however, markedly different from peripheral NK cells in two respects. First, IENK cells express high levels of CD25 and ART2, whereas peripheral NK cells do not. Second, unlike splenic NK cells that secrete little or no IL-4 and secrete IFN-γ only in response to stimulation (56), a substantial fraction of rat IENK cells appear to secrete IFN-γ or IL-4 spontaneously.

**Novel function and phenotype of intraepithelial natural killer cells**

I (26) and others (54) have previously reported that cells with an NKR-P1A<sup>+</sup>CD3<sup>-</sup> phenotype are present in the IE compartment of the rat intestine. My data now extend these observations by demonstrating that IENK cells appear to represent a new, previously unrecognized subpopulation of rat NK cells. It could, of course, be argued that the IL-4 secretion I observed in rat IENK cell populations resulted from contamination of the IENK cell preparation with either NK T cells or contaminating IE-T cells, both of
which are known to secrete IL-4 (15;46;209). This is unlikely however, because my assays revealed that NK T and IE-T cells comprised <0.1% of the purified IENK cell preparations used in my ELISpot assays. This frequency of contaminating cells is insufficient to account for the 1-2% frequency of IL-4 secreting cells I observed in the purified IENK populations. In addition, I observed that the purposeful addition of IE-T cells to the IENK ELISpot had no effect on the number of cytokine-secreting cells.

It could also be argued that the apparently "spontaneous" secretion of IL-4 was simply the result of the IENK cells being activated, as are nearly all IELs (8;15;209). This is also unlikely, however, because my data show clearly that peripheral NK cells, even when activated with high levels of IL-2, secrete little IL-4. The ability of freshly isolated IENK cells to secrete IL-4 suggests that these cells may represent a new subpopulation of NK cells that, in the IEL microenvironment, are programmed to secrete IL-4 constitutively. It should be pointed out, however, that my data do not formally exclude the possibility that factors other than IL-2 or IL-4 could be present in vivo in the gut, activate IENK cells, and induce them to secrete IL-4. Whether any individual IENK cells secrete both IFN-γ and IL-4 spontaneously also cannot be determined from my data.

Consistent with the view that IENK cells comprise a distinct NK subpopulation is my documentation that they differ phenotypically from splenic NK cells. Expression of CD2, CD8α, CD5, CD45RC, and CD54 is lower in IE than in splenic NK cell populations. In contrast, expression of CD25 and ART2 is higher. Although the expression of CD25 could simply reflect the constitutively activated state of IENK cells, ART2 expression on peripheral T cells is known to be downregulated in the setting of activation (150).
Functions of IENK cells that resemble peripheral NK cells

Although the cytokine profile of rat IENK cells appears to be unique among cells with the NKR-P1A⁺ CD3⁻ phenotype, it is important to note that these cells do share important morphological and functional characteristics with peripheral NK cells. Both are large and exhibit distinctive azurophilic granules (209;327). I recognize that γδ-TCR⁺ IELs have been reported to have azurophilic granules (209;328), but this cell subset was excluded from the IENK cell specimens that I analyzed. My morphological studies also suggest that intraepithelial NK cells may be slightly larger than those in the splenic population, consistent with their activated state (56;209). This conclusion must be regarded as tentative, however, as I cannot exclude the possibility of artifact induced by the cytopsin method used for specimen preparation.

I also observed that, like peripheral NK cells (64;68;315;316), intraepithelial NK cells exhibit cytotoxic activity and suppress a one-way mixed lymphocyte culture. The cytotoxicity data, which showed that purified rat NKR-P1A⁺ IELs lyse YAC-1 cells more efficiently than do unsorted or remixed populations, are consistent with studies performed using mouse spleen cells (327). I recognize that NK T cells were present in my cytotoxicity assays, but it is unlikely that they affected the outcome because, unless they are pre-incubated in the presence of high-dose IL-2, NK T cells are ineffective in lysing YAC-1 cells (46). With respect to the suppression of MLCs by rat IENK cells, it should be pointed out that comparable numbers of intraepithelial T cells did not result in suppression, arguing against competition for nutrients in the media as a mechanism of suppression. NK-mediated suppression of a one-way MLC is thought to occur via killing of allo-
geneic antigen presenting cells (64;68), a conclusion supported by my Transwell® studies. These studies suggest that cell-cell contact between IENK and stimulator/responder cells may be required for suppression, but it must be recognized that they do not formally exclude the possibility that soluble mediators that are either short lived or present at low concentrations could be involved.

IENK cells and regulation of intestinal immune responses

The constellation of NK-like phenotype and function, together with an unusual cytokine profile—constitutive expression of IL-4 and IFN-γ in the local microenvironment of the gut—suggests that these are specialized cells that may be subserving a gut-specific function. If the IENK cell population is indeed a new subpopulation of intraepithelial cells, how might they influence gut immunity? I propose that IENK cells have previously unrecognized regulatory properties. The gut is known to exhibit unique immune responses that differ from those in the systemic circulation and can influence the response of the peripheral immune system (185).

Two unique immune responses mediated by the gut are regulation of controlled or "physiologic" inflammation (185), and mediation of oral tolerance upon encounter with exogenous antigens (8;185;362). The ability of IENK cells to spontaneously (in essence, constitutively) secrete IL-4 implies an important role vis-à-vis these two unique functions of the gut immune system. IL-4 is known to be a potent regulator of inflammatory processes and biases inflammation towards a "non-destructive" rather than a "pro-inflammatory destructive" process (31;387;388). IL-4 has also been demonstrated to
protect animals from inflammatory bowel disease (199), and it is required for oral tolerance (346). Interestingly, NKR-P1+ cell populations have been implicated in the regulation of a Th1-type immune response in a mouse model of chronic colitis, but it is not known specifically if NK cells of gut origin subserved that function (65).

With regard to possible immunoregulatory function, it is intriguing to note that ART2, an ADP-ribosyltransferase (150), and CD25 are expressed at high levels on rat IENK cells. Expression of ART2 on peripheral rat T cells is a marker of regulatory capability (133;150), as is expression of CD25 on a population of peripheral mouse T cells that can prevent autoimmune diabetes in NOD mice (389). It is plausible to speculate that ART2+ CD25+ rat IENK cells could play a role in the processes that regulate the “physiologic inflammation” that is characteristic of the gut (185).

The data suggest further that defects in IENK cell number or function might lead to un-regulated inflammatory processes in the gut that result in disease. Abnormalities in gut immunity may participate in the pathogenesis of autoimmune disorders including celiac disease (88), inflammatory bowel diseases (199;201;202;312), and autoimmune type 1 diabetes (362). With respect to diabetes, I have documented that the spontaneously diabetic BBDP strain of rats is severely deficient in IENK cells (Chapter IV). The diabetes resistant BBDR rat strain is also deficient in IENK cells, as compared to the normal WF strain rat used in the present studies. Interestingly, however, when BBDR rats are induced to become diabetic using a standard protocol consisting of anti-ART2 mAb plus polyinosinic:polycytidylic acid (4), levels of IENK cells become nearly undetectable (Chapter IV).
There is as yet little information on the abundance and function of IENK cells in species other than the rat. Some IELs in both human and mouse express NK cell surface markers (225;390;391), and mouse IELs exhibit NK-like cytotoxic activity (225;390). Most IELs in mice (15;209) and humans (260), however, are CD3+ and spontaneous secretion of IFN-γ (and IL-5) in the mouse has been associated with intraepithelial CD3+ T cell populations (253).

**Intraepithelial natural killer cells and BB rats**

With respect to diabetes, I have documented that the spontaneously diabetic BBDP strain of rats is severely deficient in IENK cells. The diabetes resistant BBDR rat strain is also deficient in IENK cells as compared to the normal WF strain rat. Interestingly, however, when BBDR rats are induced to become diabetic using a standard protocol consisting of anti-ART2 mAb plus poly I:C (4), levels of IENK cells and associated spontaneous IFN-γ and IL-4 activity become nearly undetectable.

The NK-like phenotype and function, together with an unusual cytokine profile—constitutive expression of IL-4 and IFN-γ in the local microenvironment of the gut—suggests that IENK cells are specialized cells that may be subserving gut-specific functions. The present data suggest that one such function may relate to the expression of autoimmune diabetes in genetically susceptible individuals. Based on these data, I hypothesize that BB rat diabetes is a multi-step process that starts with a deficiency of regulatory gut NK cells. Step 1 is an abnormally regulated immune response to adventitious gut antigens. During step 2, dysregulated gut immune cells traffic to the periphery, home to
pancreas, and cause non-destructive insulitis. Step 3 involves peripheral T cells that home to the inflamed tissue and, due to genetically determined lack of regulation, cause destructive insulitis. I believe that this hypothesis is deserving of additional testing.

**Evidence for systemic consequences resulting from a failure of mucosal tolerance in pre-diabetic BB rats**

In preliminary experiments I have established that, unlike BBDR and WF rats, BBDP rats are also deficient in γδTCR⁺ IELs that may play a role in normal mechanisms of mucosal tolerance. Is systemic autoreactivity in BB rats initiated in the intestine? Peripheral autoreactive lymphocyte populations appear to emanate first from mesenteric lymph nodes that drain the intestine, and such cells may initiate a type 2 autoimmune phenomenon driven by IL-4.

These findings support the argument that a failure in mucosal tolerance in BBDP rats, perhaps secondary to deficiencies in one or more IEL subpopulations, triggers activation of autoreactive T cells peripherally and contributes to the immunopathogenesis of spontaneous diabetes in BB rats. I also challenge the general applicability of the Th1-model of autoimmune diabetes that has been the benchmark of disease pathogenesis in the NOD mouse and question whether type 1 diabetes in humans is truly a Th1-mediated autoimmune disease.
Chapter VI, Section 5 - Failure of Mucosal Tolerance in BB Rats

Triggers Autoreactivity: A Model

In Section 2 of this chapter, I proposed that three factors contribute to the expression of autoimmune diabetes in BB rats: the presence of autoreactive T cells, insufficient numbers of ART2+ regulatory cells peripherally, and activation of autoreactive T cells by environmental factors. For diabetes-induced BBDR rats, fulfillment of the third stipulation may arise from experimental stimulation of the immune system by poly I:C or KRV. Less clear is the mechanism whereby environmental agents stimulate autoreactive cells in the spontaneously diabetic BBDP rat. To address this point, I have investigated in BB rats one critical site where the immune system is under tremendous environmental stimulation, the intestinal mucosa. My working hypothesis has been that a failure of mucosal tolerance in BBDP rats, and perhaps BBDR rats, contributes to the expression of autoimmune diabetes by allowing for the activation of autoreactive T cells peripherally. This section of the discussion reviews and interprets data relevant to the subject of mucosal tolerance in BB rats. I then present a model by which an activation of peripheral autoreactive cells may occur in BBDP rats.

Review and interpretation of data relevant to mucosal tolerance in BB rats

Relevant studies conducted prior to this dissertation

Prior to this dissertation, it was well established that environmental factors, particularly diet, influence the expression of autoimmune diabetes in BBDP rats (4;170). Diets comprised of hydrolyzed casein (172;173) or elemental amino acids (171) decrease
the incidence of spontaneous diabetes in BBDP rats, suggesting that diets with low mucosal antigenicity are diabetes-protective (170). Furthermore dietary gluten proteins abrogate the protective effects of a hydrolyzed-casein diet (175), implying that some dietary proteins have a greater mucosal antigenicity and contribute to diabetes expression in BB rats (170). Interestingly, oral insulin, which protects NOD mice from diabetes (288), fails to prevent (289) and may even exacerbate (290) spontaneous diabetes in BBDP rats. Such findings suggest that a failure of mucosal tolerance is a causative factor in the expression of autoimmune diabetes in BBDP rats.

How might oral antigens trigger autoimmunity in BBDP rats? One possible mechanism is through the direct activation of autoreactive T cells by dietary antigens. Alternatively, spontaneous autoimmune diabetes in BBDP rats may result from “by-stander activation” of autoreactive T cells secondary to an aberrant immune response to one or more dietary antigens. The latter interpretation implies that a given dietary antigen is not necessarily an autoantigen, yet it allows for the activation of autoreactive cells that then trigger disease. At the outset of this project, I determined that a greater understanding of the mechanism underlying the activation of autoreactive T cells was necessary. I therefore investigated the role of regulatory cells in mucosal tolerance in BB rats.

Relevant data from this dissertation

I characterized in normal WF rats a population of potentially regulatory natural killer cells in the intestinal epithelium, an important site of interaction between environmental antigens and the immune system. I extended these findings to BB rats and found
that pre-diabetic animals exhibit drastic deficiencies in IENK cell number and function. In additional studies, I showed compelling yet preliminary data that BBDP rats are deficient in intraepithelial γδ T cells and also that pre-diabetic BB rats may have a failure of intestinal mucosal tolerance. Given my findings in the context of what is already known about BB rats and mucosal tolerance, I conclude that an aberrant stimulation of the immune system secondary to a failure of mucosal tolerance contributes to the activation of autoreactive T cells and spontaneous autoimmune diabetes in BBDP rats.

**A model whereby failure of mucosal tolerance activates autoreactive lymphocytes in BB rats**

I propose a model whereby a failure of mucosal tolerance exists in pre-diabetic BB rats and contributes to disease expression in these animals though the direct or indirect activation of circulating autoreactive T lymphocytes (Figures 27-29). Normal mucosal tolerance forms the foundation of my model (Figure 6), and the first part of my model (Figure 27) describes how IELs may contribute to mechanisms of mucosal tolerance. The second part of my model (Figure 28) postulates on mechanisms whereby failure of mucosal tolerance in BB rats is disturbed secondary to deficiencies in IEL subpopulations. The third and final part of my model (Figure 29) proffers a mechanism whereby a failure of mucosal tolerance in BB rats activates circulating autoreactive T cells and triggers systemic autoimmunity.
Figure 27: Role of IELs in mucosal tolerance

Soluble Lumenal Antigen

1. Suppression
2. Maintenance

Barrier Maintained
**Figure 27: Proposed roles of IELs in mucosal tolerance.** IELs (*blue*) may participate in mucosal tolerance through two principle mechanisms: 1) the direct suppression/regulation of aberrant immune responses by potentially autoreactive lamina propria lymphocytes (*red*), and 2) the maintenance of intestinal epithelial cells (*violet*) and epithelial barrier function. Both allow for normal processing of gut antigens (Figure 6).
Figure 28: Local failure of mucosal tolerance in BBDP rats

Soluble Lumenal Antigen

1

Failure of:
1. Suppression
2. Maintenance

Barrier Permeable
Figure 28: Proposed mucosal consequences of IEL deficiency in BBDP rats. BBDP rats exhibit marked deficiencies in multiple IEL subpopulations (e.g. IENK cells, $\gamma\delta$TCR$^+$ IELs) that may interfere with mucosal tolerance at two levels: 1) the failure to suppress aberrant immune responses by potentially autoreactive lamina propria lymphocytes (red), and 2) the failure to maintain intestinal epithelial cells (violet) and epithelial barrier function.
Figure 29: Systemic failure of mucosal tolerance in BBDP rats
Figure 29: Proposed systemic consequences of a failure of mucosal tolerance in BBDP rats. Failure of mucosal tolerance to dietary antigens secondary to Peyer’s patch deficiencies (step 1), poor epithelial barrier function (step 2), and decreased mucosal regulation (step 2), and may allow for the activation of dendritic cells and autoreactive T cells in the lamina propria of BBDP rats. Activated cells then migrate to draining mesenteric lymph nodes (step 3) and, in the absence of sufficient regulation by ART2+ T cells, propagate a systemic autoimmune response that initiates β cell destruction in the islets of the pancreas (step 4).
The role of intraepithelial lymphocytes in normal mucosal tolerance

As already shown in Chapter I (Figure 6), myriad factors contribute to normal mucosal tolerance including processing of antigen by Peyer’s patches and intestinal epithelial cells (185;187;280), mucosal IgA production by lamina propria plasma cells (188), and down-regulation of aberrant immune responses locally through the activity of specialized suppressor/regulatory lymphocyte populations (199;261). I hypothesize that critically important to these processes is the integrity of the intestinal epithelium mediated by tight junctions between intestinal epithelial cells that prevent the paracellular transit of luminal antigens (181;185;203;392). In normal circumstances, therefore, Peyer’s patches and intestinal epithelial cells sample luminal antigens thus allowing for the local production of regulatory/suppressive lymphocyte populations that may contribute to oral tolerance systemically (199;278;280;285;291).

Intraepithelial lymphocytes appear to be in part responsible for maintenance of epithelial integrity (8;247;264;265) and therefore mucosal tolerance (Figure 27) (181). Cytotoxic activity, which I observed for IENK cells and has been shown for other IEL populations as well (19;328), may delete transformed or infected intestinal epithelial cells (IECs) thus preserving the integrity of the epithelium (8;19). Intraepithelial γδ T cells, which are present in large numbers in normal rats (26), appear to support epithelial integrity through the recognition of stressed IECs (247) and secretion of keratinocyte growth factor (264;265). At least one study has also shown that γδ T cells down-regulate aberrant Th2 mucosal immune responses (367).
My data also suggests that IELs (particularly IENK cells) may also contribute to mucosal tolerance through the secretion of the immunoregulatory cytokines IL-4 and IFN-γ (354). IL-4 is known to be a potent regulator of inflammatory processes and biases inflammation towards a "non-destructive" rather than a "pro-inflammatory destructive" process (31;387;388). IL-4 has also been demonstrated to protect animals from inflammatory bowel disease (199), and it is required for oral tolerance (346).

Failure of mucosal tolerance in BBDP rats

How might deficiencies in IEL number or function disrupt normal mechanisms of mucosal tolerance and contribute to systemic autoimmunity? IELs appear to play a critical role in maintaining the barrier function of the intestinal epithelium (264;265), and increased intestinal permeability is associated with 1) appropriate immune responses to active infection (181;185;203;392) and 2) inappropriate immune responses to intestinal antigens from typically non-pathogenic sources. Indeed, several intestinal disorders such as inflammatory bowel disease (201), celiac disease (87;88), and food allergy (181;203;392) are associated with an increased intestinal epithelial permeability. Based on data presented here and elsewhere, a similar process that may partly explain the diabetogenic effects of highly antigenic diets may occur in pre-diabetic BBDP rats (Figure 28).

I have shown here that BBDP rats are severely deficient in intraepithelial γδ T cells and IENK cells, which may account for their increased intestinal permeability (370). Thus, there may be increased paracellular and decreased transcellular trafficking of luminal antigens through the intestinal epithelium in BBDP rats. Transcellular processing
of lumenal antigens is associated with presentation by CD1 and generation of CD8+ regulatory/suppressor populations (185;261), whereas paracellular antigen delivery is seen in food allergy (181;203;392). By histology, the BBDP intestine resembles allergic hypersensitivity (204) and also appear deficient in Peyer’s patch lymphocytes (unpublished observation), suggesting that normal mechanisms of mucosal tolerance may be defective at multiple levels in these rats. Antigen-rich diets and even oral insulin itself may therefore trigger a cascade of events that 1) fails to generate regulatory mucosal lymphocytes and 2) activates autoreactive circulating T cells, leading to autoimmune diabetes in BBDP rats.

Deficiencies in potentially immunoregulatory IENK cells in BBDP rats may also predispose these animals to overactive (non-physiologic) mucosal inflammation due to an absence of immunoregulatory cytokines locally. Aberrant mucosal immune responses that might be aborted in immunocompetent animals are sustained in BBDP rats. Lamina propria lymphocytes and dendritic cells in these animals would therefore exist in a pro-inflammatory environment, setting the stage for systemic spreading of mucosal inflammation.

Failure of mucosal tolerance activates autoreactive lymphocytes in BB rats

Even though BBDP rats may exhibit a failure of mucosal tolerance, it is still necessary to link that shortcoming to diabetes expression. Preliminary data presented in Chapter V suggest that just such an association exists and beg the question of how defective mucosal tolerance contributes to diabetes. I propose a mechanism whereby the fail-
ure of mucosal tolerance in BB rats recruits lamina propria dendritic cells that then activate autoreactive T cells either directly or indirectly (Figure 29).

In this model, increased epithelial permeability and deficiencies in mucosal regulatory cells – defects seen in inflammatory bowel disease (201), celiac disease (87;88), and pre-diabetic BBDP rats (370) – allow for paracellular trafficking of lumenal antigens such that lamina propria dendritic cells are directly exposed to unprocessed antigen in the absence of sufficient regulation. An activation of autoreactive T cells then occurs locally and/or in draining mesenteric lymph nodes. In the absence of sufficient regulation by ART2+ T cells peripherally, activated autoreactive cells subsequently home to the pancreas and induce diabetes. It should be noted that the hyperexpression of class I antigens that occurs in diabetic BB rats (139;393) is decreased in BB rats maintained on a diabetes-protective hydrolyzed casein diet (394).

Surprisingly, the preliminary data also suggest that autoreactive lymphocytes act via a Th2 humoral-mediated immune response. Thus, the elevated MLN IL-4, elevated serum IgE, intestinal and islet eosinophils may be down-stream consequences of defects initiated at the intestinal epithelium. It remains to be established whether the Th2-type immune response in the gut contributes directly to beta cell destruction in the pancreas of BB rats. The proposed Th2 model of autoimmune diabetes in BB rats is similar to those of asthma (395;396) and food allergy (181;204). In addition, these data challenge the applicability of the Th1 model of autoimmune diabetes (established mostly in mice) in BB rats. Such limitations must therefore be considered when one attempts to apply animal studies to human disease.
Chapter VI, Section 6 - Impact, Directions, and Final Comments

Applicability of these findings to human disease

The intestinal immune system is hypothesized to participate in the pathogenesis of autoimmune diabetes in genetically susceptible humans, BB rats, and NOD mice (362). The mechanisms by which gut immune responses may lead to diabetes are still unclear in part because evidence remains largely circumstantial. In this dissertation, I have identified potential cellular mechanisms whereby abnormal gut immune responses lead to autoimmune diabetes in the BB rat. These data have already been discussed in detail, but what is their applicability to type 1 DM in humans?

Several lines of study in humans suggest that failures in mucosal regulation may contribute to expression of type 1 DM in genetically susceptible individuals (362). Environmental factors such as diet and enteromicrobial agents may participate in the initiation of disease through mediation of the intestinal immune system (4). In particular, enteroviral infections and dietary cow’s milk may be associated with type 1 DM (1). More recently, an association has been identified between type 1 DM and the intestinal disorder celiac disease (86). These observations are particularly relevant to BB rats in which viral infections and dietary gluten unmask disease expression (4;170).

With regards to diabetes and the intestine itself, increased levels of gut inflammation – beyond what might be deemed “physiologic” – have been observed in the intestine of patients with type 1 DM (369). Furthermore, an increased epithelial permeability, reminiscent of that in BBDP rats, has been documented in the intestine of humans with
type 1 DM (368). Studies of IELs in patients with type 1 DM are lacking, but humans with celiac disease have decreased numbers of potentially regulatory NKR-P1\(^+\) CD3\(^-\) IELs (391;397;398). In the context of these human studies, it is apparent that many similarities exist between autoimmune diabetes in humans and BB rats at the level of the intestine. Such similarities warrant continued study.

**Future directions**

Data presented in this dissertation broach three major avenues for future study in the BB rat. First, molecular mechanisms of immunoregulation should be identified for IELs. Second, genetic and environmental factors should be evaluated in BB rats for their influence on IELs and IENK cell number and function. Third, preliminary data presented in Chapter V should be investigated further to determine if there is truly a cause-effect relationship between the perceived lack of mucosal tolerance in BB rats and the potentially Th2-mediated autoimmune diabetes. Together, these lines of study should contribute to our knowledge about the immunopathogenesis of autoimmune diabetes in BB rats and may shed additional light on human disease.

**Molecular mechanisms of immunoregulation by IELs**

That peripheral regulatory T cells are ART2\(^+\) suggests a role for this protein in the immunoregulatory function of these cells (4;25). Interestingly, nearly all IENK cells express uniformly high levels of ART2 (26) suggesting that the potential immunoregulatory function of these cells may be related to ART2 expression. For these reasons, the role of ART2 in immunoregulation requires further investigation, particularly in IELs. Prelimi-
nary studies in rats have already shown that IELs appear to lack crucial cell-signaling molecules like LAT (Derrick Todd, unpublished observation). Furthermore, the traditional signaling pathways associated with ART2 in peripheral T cells are not the same in IELs (Hans-Peter Raue, unpublished observation). Understanding these mechanisms may unveil immunoregulatory properties of IELs and are of a high priority.

The effect of genetics and environment on IELs and epithelial permeability

I have proposed that one of the earliest defects contributing to the expression of spontaneous autoimmune diabetes in BBDP rats is their dramatic deficiency in IEL (and IENK cell) number and function. To better understand the role of genetic factors in this process, IENK cell number and function should be tested in WF $iddm4^d$ congeneric rats that carry the defective BB-origin $iddm4$ allele. In addition, the role of environmental factors in the dis regulation of IENK cell-regulated mucosal immunity can be tested in other rat models of inducible autoimmune diabetes (e.g. high-dose poly I:C).

According to my model, the IEL defect in BBDP rats results in an increased intestinal permeability that triggers a cascade of events leading to autoimmunity. Experimental modalities that increase intestinal permeability (dextran sodium sulfate) should therefore be evaluated for their ability to induce diabetes in BBDR rats when co-administered with anti-ART2 mAb. Furthermore, epithelial permeability in diabetes-induced BBDR rats, which may be increased, should be measured. In addition, restoration of the IEL compartment of BBDP rats via BMT should theoretically restore intestinal epithelial barrier function. Finally, the consequences of depleting potentially immu-
noregulatory IEL populations should be addressed. The recent commercial availability of an anti-CD103 mAb (BD Pharmingen) may make this feasible.

Expansion of preliminary data in Chapter V

Data presented in Chapter V provide compelling data that autoimmune diabetes in BBDP rats may result from a failure of mucosal tolerance that gives rise to Th2-mediated autoimmunity. Given that these data directly contradict the dogma of Th1-mediated diabetes in NOD mice, additional experiments should be performed to confirm my preliminary observations in BB rats. Furthermore, preliminary observations can be expanded along a number of avenues that include trafficking studies, adoptive transfer experiments, and analysis of genetically-susceptible iddm4d congenic rats. Intriguing is the possibility that if IgE is involved in the pathogenesis of diabetes in BB rats, then perhaps identifying the IgE-specific antigen would uncover putative autoantigens in BB rats.

Final Comments

In this chapter, I have presented a model whereby IEL defects may contribute to the expression of autoimmune diabetes in the BB rat. It is my sincere hope that, by defining the immunopathogenesis of autoimmune diabetes in BB rats, these data may provide insight into autoimmune processes involved in human type 1 diabetes. Current measures to prevent type 1 diabetes in humans are lacking; studies using oral insulin or nicotinamide have been largely unsuccessful in human subjects (399-401). A greater understanding of the immunopathogenesis of type 1 diabetes mellitus is necessary before researchers can ever hope to prevent this devastating disease in humans.
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