MAJOR HISTOCOMPATIBILITY COMPLEX ASSOCIATION OF INSULIN-DEPENDENT DIABETES IN THE BB RAT

by

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ABTRACT

BB rats spontaneously develop an insulin-dependent diabetes mellitus strikingly similar to the syndrome observed in man. The disorder requires the presence of multiple susceptibility genes and unknown environmental factors. At least one susceptibility gene resides within the u haplotype of the rat major histocompatibility complex (RT1). Restriction fragment length polymorphism analysis of genomic DNA from rats generated from a series of intercrosses between diabetic BB rats and Buffalo rats ($RT1^b$) demonstrated that animals heterozygous throughout the RT1 developed IDDM. A single dose of the high risk allele was thus shown to be sufficient for the development of IDDM if other susceptibility factors are present. RFLP analysis of DNA from rats generated in three other breeding studies involving the r8 and r4 recombinant haplotypes mapped the IDDM susceptibility genes between the RT1.A and RT1.C loci, the immune response region. As the u regions of the various haplotypes used in these studies were not derived from the BB rat, the development of IDDM in the progeny strongly suggested that the MHC requirement for IDDM is only for a "u" allele and not a particular or "diabetogenic" u allele.

Analysis of the expression of MHC genes in isolated islets of age-matched BB and Wistar-Furth rats demonstrated

enhanced class I MHC gene expression in the islets of prediabetic BB rats. Immunohistochemical analysis confirmed that enhanced class I expression was an early event during the pathogenesis of IDDM, and did not detect aberrant expression of class II antigen on beta cells. Investigation of the inducibility of class I and II MHC genes on the rat insulinoma cell line RIN5F by crude lymphokine preparations or recombinant gamma-interferon indicated that although both classes of genes were inducible, their kinetics of induction are quite different. In vitro nuclear transcriptions demonstrated that induction of the genes had a transcriptional basis. Although class II genes were induced by gamma-interferon, class II antigen was not detected by flow cytometric analysis.

RESUMÉ

Les rats BB développent spontanément un diabète insulino-dépendant ressemblant fortement le syndrome developpé chez l'humain. La maladie requiert la présence de genes de susceptibilité multiples et des facteurs environnementaux inconnus. Au moins un des gènes de susceptibilité requis se trouve dans le haplotype u du complexe d'histocompatibilité majeur du rat (RT1).

L'analyse du polymorphisme des fragments de longeur de restriction du DNA génomique provenant de rats d'une série de croisements entre les rats BB diabétiques et les rats Buffalo ($RT1^b$) ont permis d'établir que les animaux hétérozygotes sur toute la longueur du RT1 développent le diabète

La présence d'un seul gène à haut risque est donc suffisant pour permettre l'apparition du diabete quand les autres facteurs de susceptibilité sont également présents. L'analyze des RFLP du DNA produit dans trois autres études de croisement impliquant les haplotypes recombinant ont permis de localiser les gènes de susceptibilité du IDDM entre les loci RT1 A et RT1 C, la région de la réponse immunitaire.

Comme les régions u des divers haplotypes utilisés dans ces études n'étaient pas originaires du rat BB, le développement du diabète dans la progéniture suggère fortement que seule l'allèle u du MHC est requise et non pas un allèle u

précis ou "diabetogénique."

L'analyse de l expression génétique du MHC sur les îlots provenant de rats BB et WF pairés pour l âge a permis d'établir une expression accrue des gènes de classe I du MHC dans les ilots de rat BB prédiabetiques. L analyse immunohistochimique a confirmé que l augmentation de l'expression de la classe I est un évènement précoce dans la pathogénèse du diabète et n'a pas révelé d'expression aberrante des antigènes de classe II sur les cellules bêta

L'étude de l'inductibilite des gènes de classe I et II de la lignée cellulaire d'insulinoma de rat RIN5F à partir de préparations de lymphokines ou d'interféron gamma de recombinaison, indique que malgré que les deux classes soient inductibles, leurs mécanismes d'induction sont probablement différents.

Les transcriptions nucléaires in vitro ont démontré que l'induction des gènes a une base transcriptionelle Quoique les gènes de classe II sont induits par l'interféron gamma, les antigènes de classe II n'ont pas été démontré par l'analyse de débit cytométrique

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PREFACE

The work described in this thesis was carried out in the laboratory of Dr. Abraham Fuks (McGill Cancer Centre). The aim of this work was to contribute to our understanding of the molecular immunogenetics of insulin-dependent diabetes mellitus (IDDM). The spontaneously diabetic BB rat was our model system. We focused our attention first on the association of the syndrome to the u haplotype of the major histocompatibility complex (MHC). We proceeded to map the IDDM susceptibility genes within the BB rat MHC in a series of breeding studies. These studies demonstrated that the susceptibility genes were located between RT1.A and RT1.C and that their contribution to IDDM was not functionally recessive. These studies also demonstrated that IDDM was not associated with mutant genes within the MHC and thus suggested that the associated haplotype predisposed the animal for a diabetic syndrome introduced by external mutations.

At a second level of analysis, we investigated the expression of MHC genes in isolated islets from prediabetic BB rats and found that the first discernible modulation in MHC gene expression was an enhanced level of class I gene expression throughout the islets. Subsequent studies on the inducibility of MHC genes in the rat insulinoma cell line RIN5F using either crude lymphokine preparations or recombinant gamma-interferon showed that class I antigen

expression could be enhanced but class II antigen expression could not be induced on this cell line. Due to these findings, we have hypothesized that the levels of class I antigen on a given cell might be a critical determinant in the immunogenicity of that cell and that exceedingly high levels of class I antigen on that cell might trigger or amplify the autoimmune recognition of that cell. Alternatively, high levels of class I expression might be directly cytocidal or inhibit the function of beta cells.

The immunohistochemical analysis of pancreatic tissue was performed by Dr. Badia Issa-Chergui (a postdoctoral fellow in the laboratory of Dr. Thomas Seemayer) and the phenotypic analysis (RT1.A typing, total WBC counting and assay of ConA responsiveness) was performed in the laboratories of Drs. Ronald D. Guttmann and Eleanor Colle. Otherwise, the work described in this thesis was performed by the candidate. Drs. Issa-Chergui, Colle, Seemayer, Guttmann and Fuks are co-authors on the published papers that form the results section of this thesis. In accordance with the McGill University guidelines for the preparation of a thesis, I have included, "as part of the thesis the text of an original paper, or papers, suitable for submission to a learned journal for publication." I include the University policy on this form of thesis:

"The candidate has the option, subject to the approval of the Department, of including as part of the thesis the text of

an original paper, or papers, suitable for submission to learned journals for publication. In this case the thesis must conform to all other requirements explained in Guidelines Concerning Thesis Preparation (available in the Thesis Office) and additional material (e.g. experimental data, details of equipment and experimental design) must be provided in sufficient detail to allow a clear and precise judgement of the importance and originality of the research reported. In any case, abstract, full introduction and conclusion must be included, and where more than one manuscript appears, connecting texts and common abstracts, introduction and conclusions are required. A mere collection of manuscripts is not acceptable, nor can reprints of published papers be accepted.

While the inclusion of manuscripts co-authored by the candidate and others is not prohibited by McGill, the Candidate is warned to make an explicit statement on who contributed to such work and to what extent, and Supervisors and others will have to bear witness to the accuracy of such claims before the Oral Committee. It should be noted that the task of the External Examiner's is much more difficult in such cases."

ACKNOWLEDGEMENTS

I wish to express my thanks to Dr. Abraham Fuks in whose laboratory this research was carried out. His advice, encouragement and patience are greatly appreciated. I also thank Drs. Eleanor Colle, Ronald D. Guttmann and Gerald Prud'homme, other members of our research team, with whom I was fortumate to interact. I am also grateful to the many members of the Fuks lab and the McGill Cancer Centre for their support. Special thanks are due to Dr. Harry Goldsmith, Director of The Division of Experimental Medicine, for his encouragement. I thank the J.W. McConnell Foundation and Fonds de la Recherche en Sante du Quebec for financial support. Finally, I thank my family and my wife for their constant support, understanding and encouragement over the past several years.

CHAPTER ONE.

GENERAL INTRODUCTION

A. THE MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)

1. General Definition. The identification of the major histocompatibility complex originated with the work of Landsteiner that established that the successful transfusion of blood between individuals depended upon matching of "blood group antigens" (Strominger et al., 1986; Paul, 1989). Gorer provided additional evidence for this concept, identifying a group of genetically linked "histocompatibility" antigens that play a central role in determining the success or failure of tissue grafts (Gorer, 1936). Subsequently, it has become clear that these antigens have a more fundamental role in the integrity of the multicellular organism than the determination of histocompatibility. These antigens form the basis of the distinction between self and non-self via their participation in the interaction of the cells of the immune system amongst themselves and with the other cells of the organism (Strominger et al., 1986). Immunogeneticists and molecular biologists have defined a region of the genome, conserved among all mammalian species, that encodes these histocompatibility antigens. This major histocompatibility complex or MHC is referred to as H-2 in the mouse (located on chromosome 17), HLA in man (located on chromosome 6) and RT1 in the rat. The MHC occupies 1.5 centimorgans or roughly 3000

kilobases of DNA. The MHC is estimated to encompass several hundred genes of varied function in addition to the genes that encode the histocompatibility or "transplantation" antigens (for a general review on the MHC see Klein, 1975).

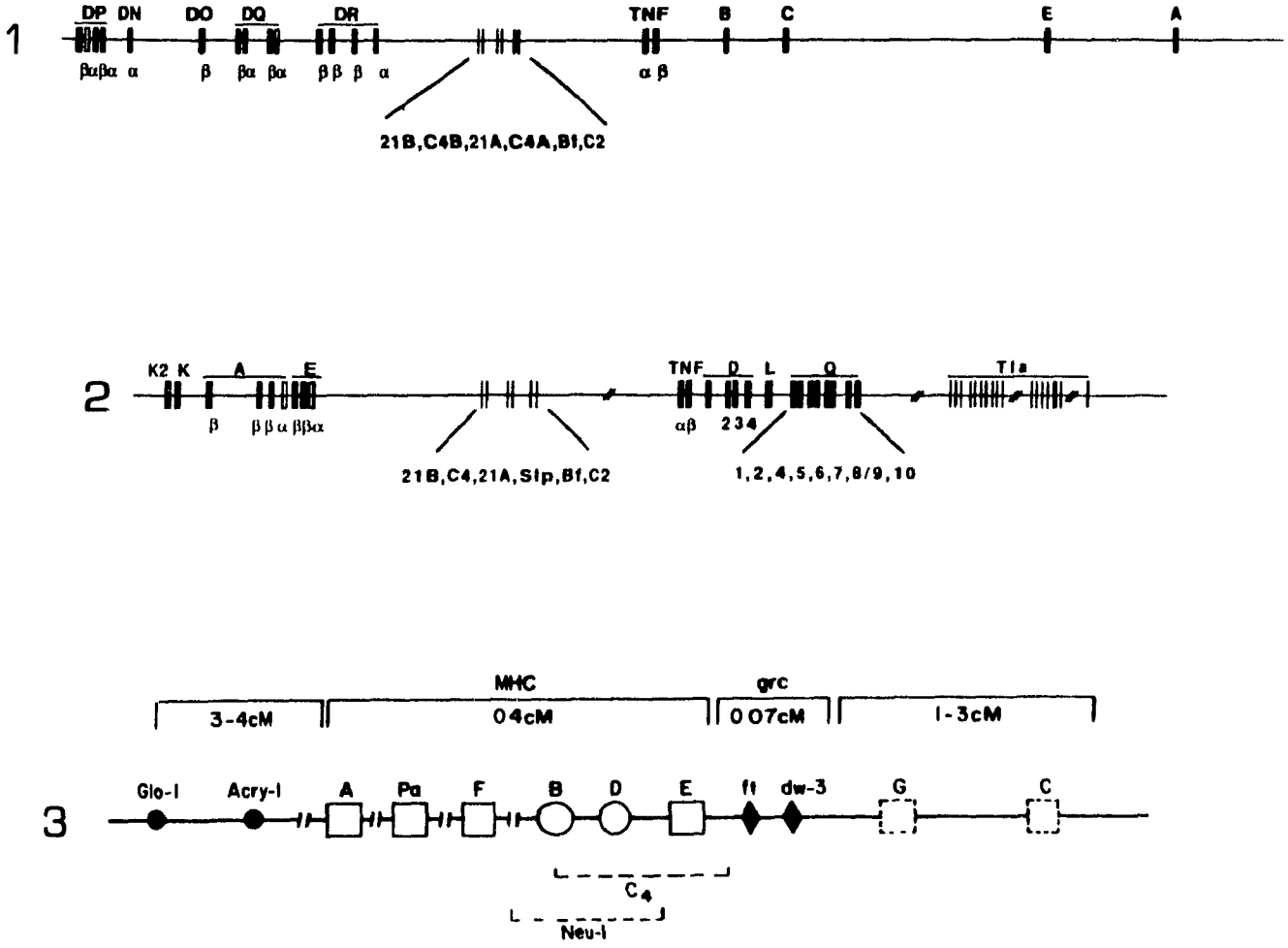

FIGURE 1. The organization of the major histocompatibility complexes of man[1], mouse[2] and rat[3] (from Todd et al., 1988)

2. Functions of the MHC antigens. Gorer and Snell first defined the MHC by grafting skin or tumors between inbred (genetically indistinguishable) and congenic (differing at a single chromosomal region) mice (Gorer, 1936). It has since become evident that the complex plays various diverse roles in the homeostasis of the multicellular organism. In this thesis, I will restrict the focus of discussion to the immunologic functions of the MHC.

The histocompatibility antigens are essential for the immune response to foreign antigens which are recognized immunologically in the context of the MHC antigens. The different classes of MHC antigens (to be discussed in depth later) restrict the recognition of antigen by different classes of T lymphocytes (Katz et al., 1983). Cytotoxic T lymphocytes, that recognize and kill (albeit with the aid of T helper cells) virally infected cells and allogeneic transplants are restricted by class I MHC antigens, while helper T cells recognize antigen in the context of class II MHC or the Ia antigens. Cytotoxic T cells recognize viral antigens that are expressed on the surface of the infected cell in the context of a class I antigen (Zinkernagel and Doherty, 1974). A given cytotoxic T cell generated in response to a viral infection will not, however, kill an allogeneic cell expressing the same viral epitope (a

phenomenon known as MHC restricted killing). Helper T cells are similarly restricted by the class II antigens A given T helper cell will recognize processed antigen on an antigen presenting cell (e.g. a macrophage) only in the context of syngeneic class II antigens. Moreover, a T helper cell can interact with B lymphocytes to stimulate the production of specific immunoglobulin or can enhance the immune response in situ by releasing lymphokines at a site of inflammation These lymphokines can (among other effects) amplify the recognition of foreign antigen by T lymphocytes by upregulating the expression of MHC antigens, enhance the microbicidal activity of macrophages, and can be directly cytocidal. In the case of both types of T lymphocyte, the (antigen/MHC antigen) complex is recognized via the T cell receptor for antigen. A third class of T lymphocyte, the T suppressor cell, counteracts the positive effects of the macrophage, T helper and B cells on the specific immune response to antigen. In this case, as well, the action of a given "primed" T suppressor cell is restricted by the class II region of the initially challenged animal. The developing T lymphocyte acquires the phenomenon of self-MHC restriction while in the thymus, where it presumably interacts functionally for the first time with MHC antigen. The expression of the T cell receptor for antigen is initiated in the thymus, and it is hypothesized that the phenomenon of restriction results from the clonal selection within the

thymus (thymic education) of those developing thymocytes that interact with a molecular complex involving self-MHC.

3. Polymorphism of the MHC. A particularly striking feature of many of the MHC antigens is their extreme polymorphism (Klein, 1975). It is thought that at least 50 alleles of the murine H-2 K and D antigens exist, and the beta chain genes of the immune response (class II) region are also highly polymorphic (Trowsdale et al., 1985). The allelic forms are also highly divergent, differing in many cases by several dozen amino acids. Interestingly, the degree of polymorphism of the genes of the MHC appear to correlate with the extent and nature of each gene's use in the functioning of the immune system. Thus, the H-2 K and D genes, that encode the MHC antigens that function most frequently in the presentation of foreign antigen, are far more polymorphic than the numerous Qa/Tla class I like genes. The mechanism by which polymorphism is generated appears to involve the intergenic exchange of sequence information with non-allelic class I or II genes (Weiss et al., 1983). There are several arguments that favor gene conversion over saturation mutagenesis as the source of polymorphism in MHC genes. First, sequence analysis of <u>de novo</u> class I mutants reveal identity to non-allelic genes (Nathenson, 1986). Second, many nucleotide substitutions are silent mutations, arguing against a selective force as an explanation for the sequence similarity

of the new gene. Finally, very similar results are observed in the analysis of class II gene polymorphism (Korman et al., 1985).

The extreme polymorphism of the genes of the MHC in face of the high degree of conservation of the complex in the animal kingdom hints at a role for polymorphism in the survival of the multicellular organism from the species point of view. In short, genetic variability at the MHC protects against the possibility of a "perfect" pathogen. Each allele at a given MHC locus is capable of presenting (and thus conferring resistance) to multiple epitopes, but is unable to present others. Polymorphism thus provides the basis for the survival of the species in the absence of complete protection for the individual (van Rood et al., 1981).

4. Classes of antigens encoded by the MHC. Alloantisera have defined two classes of MHC antigen that are involved in the immune response. Sequence analysis of the genes encoding these antigens has shown that these antigens are part of the immunoglobulin gene superfamily (Strominger et al., 1986; Williams and Barclay, 1989). The Class I antigen is composed of a polymorphic heavy chain and a non-polymorphic light chain (beta$_2$-microglobulin) encoded on a distinct chromosome. The class I heavy chain is 45,000 daltons and beta$_2$ microglobulin is 12,000 daltons (Krangel et al., 1979). Class I antigens are further subdivided into the classical "transplantation"

antigens, that mediate graft rejection (or H-2 antigens) and the multiple Qa/Tla antigens. The two sets of class I antigen are related in amino acid sequence, but differ in their tissue distribution and function (Stanton and Boyse, 1979). The transplantation antigens are expressed ubiquitously (albeit in varying degrees) while the Qa/Tla antigens have a more restricted distribution and are less polymorphic (Flaherty, 1985). Interestingly, the antigen encoded by the Q10 gene appears to encode a secreted class I antigen that may function in the promotion of tolerance (Maloy et al., 1984). The class I transplantation antigens are denoted K, D and L in the mouse, A, B and C in man, and A, C and E in the rat (Gill et al., 1987). The class I molecule consists of three globular alpha domains 90 amino acid residues in length with alpha 3 forming the association with beta$_2$-microglobulin. A short hydrophilic portion of the C-terminus of the heavy chain lies within the cytoplasm (30 residues) and a hydrophobic segment (40 residues) anchors the chain to the cell membrane. Alloantigenic sites are found on the first and second alpha domains (Ozato et al., 1985), and the molecule has a twofold axis of symmetry (Bjorkman et al., 1987a+b).

Class II antigens are composed of two distinct polypeptides (alpha and beta) that are both encoded within the MHC. The alpha chain is approximately 33,000 daltons and the beta chain approximately 29,000 daltons in size. The difference in molecular weight is mainly due to different

degrees of glycosylation. A third invariant chain is found in non-covalent association with the class II antigens. The alpha and beta chains are held together in non-covalent linkage, with the C-terminus of each chain lying within the cytoplasm. Each chain forms two globular extracellular domains stabilized by intrachain disulphide bonds (save the alpha-1 domain). Both chains are glycosylated, although the beta chain contains most of the alloantigenic sites (Jones et al., 1978). All of the chains that make up the class II antigen (except for the invariant chain) are encoded in the immune response region. The class II antigens are denoted A and E in the mouse, DP, DQ and DR in man, and B and D in the rat. The class II antigens are expressed on the cells of the immune system (e.g. macrophages, B lymphocytes, activated T lymphocytes and dendritic cells) and function in the restriction of immune recognition of antigen and the interaction of the cells of the immune system (Rosenthal and Shevach, 1973).

As stated earlier, several other genes are encoded within the MHC including those denoted class III genes. These genes encode several proteins that function in the complement cascade (Schneider et al., 1986).

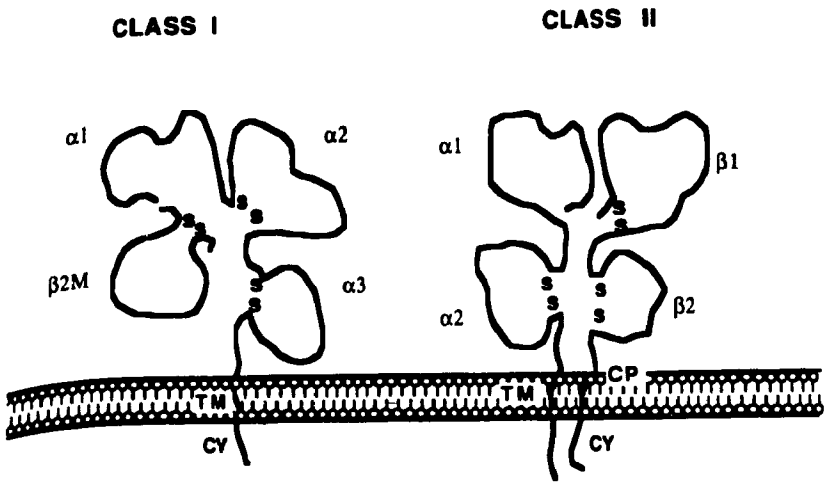

FIGURE 2. The class I and II MHC molecules (from Kaufman et al., 1984; Paul, 1989).

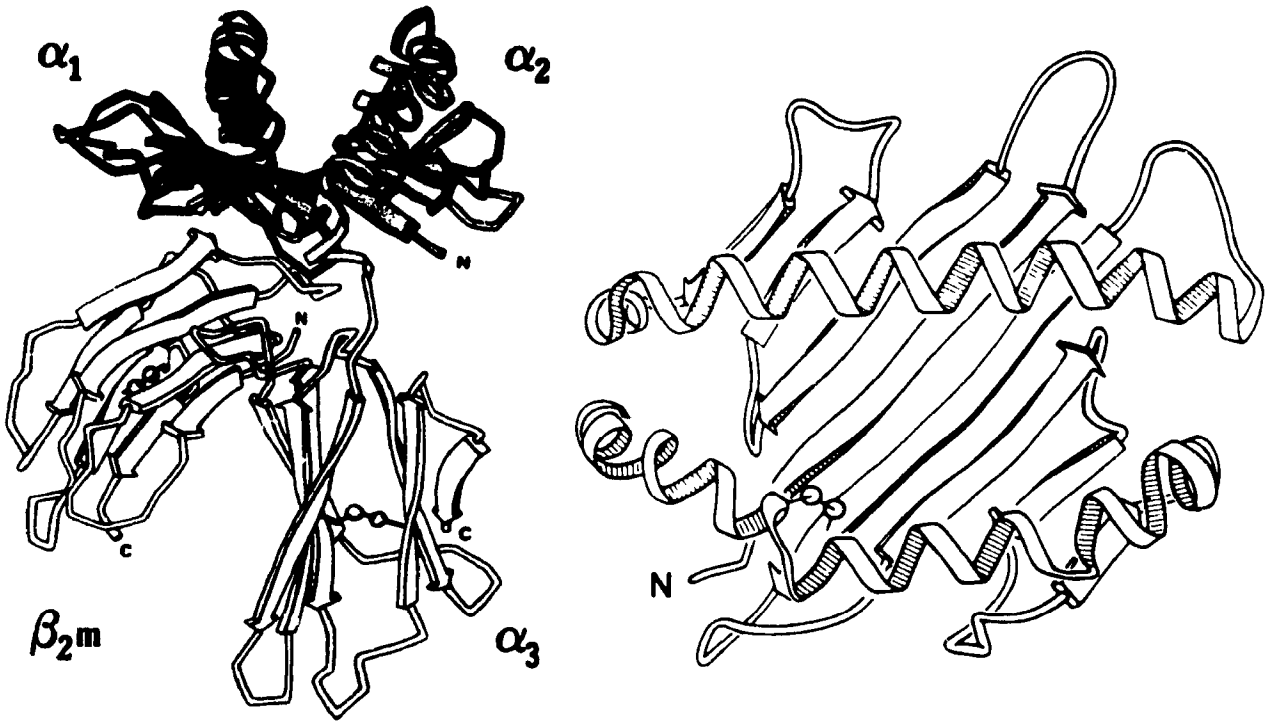

FIGURE 3. Three dimensional structure of the human class I MHC molecule HLA-A2 (from Bjorkman et al., 1987a).

5. MHC genes. The structures and organization of class I and II MHC genes have been elucidated using cDNA clones isolated from libraries using oligonucleotide probes synthesized based on amino acid sequence information (Ploegh et al., 1980; Kvist et al., 1981; Korman et al., 1982; Mengle-Gaw and McDevitt, 1985). The exon-intron organization of isolated murine class I genes is conserved. Moreover, as is the case with the immunoglobulin genes, the exon-intron organization correlates with the domains of the class I glycoproteins and contains the consensus splicing sequences GT/AG (Steinmetz et al., 1980). This exon-intron organization is also very similar in the human genomic clones (Auffray and Strominger, 1986). The transcriptional regulatory elements of the class I genes are typical of non-"house keeping" genes containing both CAAT and TATA boxes, the Kozak sequence and polyadenylation sites. An interferon responsive transcriptional enhancer has been identified in the 5′ sequence information (Shirayoshi et al., 1989). Analysis of K and D genomic clones of various haplotypes has resulted in the identification of allele-specific nucleotides in exons 4 and 8, and locus-specific nucleotides have been located in the 3′ untranslated regions of class I cDNAs (Maloy, 1987; Kress et al., 1983). Interestingly, different loci from two haplotypes can, in some instances, exhibit more homology than two alleles of a locus. This is further evidence that gene conversion mechanisms might

operate to generate polymorphism in class I genes (Klein and Figueroa, 1986; Paul, 1990). The nucleotide sequences of the Qa/Tla genes show strong homology to the genes encoding the transplantation antigens (approximately 80%) suggesting that these class I-like antigens might play a role an unknown role in immunity (Nathenson et al., 1981; Nathenson, 1986; Maloy, 1987). Finally, class I genes appear to undergo alternative splicing at their 3' ends (reminiscent of immunoglobulin) resulting in class I products differing in size and sequence, and may result in the generation of related class I antigens with distinct functions (Lew et al., 1987).

There are estimated to be 12 to 40 class I genes in mammals. Some of these genes are pseudogenes, and different class I genes may be expressed in different tissues. Although most tissues express class I products, the comparison of the genetic structure of genomic DNA (by restriction fragment length polymorphism analysis and DNA sequencing) isolated from expressing and non-expressing (sperm) tissue from syngeneic sources has indicated that rearrangement of the class I genes, unlike immunoglobulin genes, does not control gene expression (Steinmetz et al., 1982). Some progress has been made in the identification and cloning of nuclear DNA binding factors that might contribute to the activation of class I gene expression (Shirayoshi et al., 1988).

Single copy class I probes derived from cosmid libraries have been used in restriction fragment length mapping

experiments to define the order of the class I genes (Weiss et al., 1984; Carroll et al., 1987; Paul, 1990). Gene transfer experiments of murine cosmid clones into L cells followed by screening for the expression of known class I antigens using monospecific antibodies has identified the specific genomic clones encoding the expressed class I antigens (Goodenow et al., 1982; Auffray and Strominger, 1986; Paul, 1989). Similar experiments using human genomic clones provided the surprising finding that human class I heavy chains can be efficiently expressed in the context of murine beta$_2$-microglobulin. These studies also resulted in the identification of four human class I genomic clones (Germain and Malissen, 1986). These experiments have shown that all identified class I genes (including pseudogenes) cluster within the MHC. Furthermore, most of the class I genes in the rat and mouse cluster within the RT1.C or Qa/Tla regions respectively (Winoto et al., 1983; Cortese Hassett et al., 1986; Gill et al., 1987). In keeping with the different degrees of polymorphism of the two subsets of class I antigens, the genes encoding the transplantation antigens are far more polymorphic in RFLP analyses than the non-classical class I genes (Winoto et al., 1983). Similar studies using MHC chromosome loss mutants and pulsed field gel electrophoretic techniques have provided a detailed molecular map of the human class I genes (Lawrence et al., 1987; Paul, 1989).

Several class II genes have been isolated from rat, man

and mouse (Wallis et al., 1984; Holawachuk, 1985; Robertson and McMaster, 1985; Eccles and McMaster, 1985; Okada et al., 1985a+b; Spies et al., 1985; Auffray and Strominger, 1986; Paul, 1989). As is the case with many of the other members of the immunoglobulin gene superfamily, the class II gene exon-intron organization correlates well with the domain structure of the class II antigens (with some exceptions). The immune response region of the mouse appears to reside in roughly 250 kilobases of DNA isolated from a cosmid library (Flavell et al., 1985). Further localization of the specific class II genes has been accomplished by Southern blot hybridization using either synthetic oligonucleotide or cDNA probes (Paul, 1989). Mengle-Gaw and McDevitt have published a detailed review on the diversity and organization of the class II genes (Mengle-Gaw and McDevitt, 1985). In brief, the class II genes appear to have a more limited degree of polymorphism than the class I set, suggesting that a significant level of polymorphism need be present in the genes encoding the T cell receptor for antigen to account for the exquisitely specific recognition of the multitude of antigens.

Analysis of the 5′ regulatory elements of class II genes has identified two consensus "boxes" (X and Y) that are found 20 to 60 base pairs upstream of the TATA motif (Auffray and Strominger, 1986; Benoist and Mathis; 1990). These conserved motifs are required for the tissue-specific expression of class II genes as well as their induction by lymphokines such

as gamma-interferon and interleukin-4. Indeed, E_{alpha} transgenic mice bearing deletions in the Y box are unable to transcribe the E_{alpha} gene (Gerber et al., 1986), and mutagenesis of the conserved nucleotides of the X box results in markedly decreased transcription of all class II MHC genes studied to date (Boss and Strominger, 1986; Sakurai and Strominger, 1988; Sherman et al., 1989; Koch et al., 1989; Benoist and Mathis; 1990). Some of the class II genes also contain a CAAT box, but it is dispensible in many cases since the Y-box motif is an inverted CAAT box. Several genes encoding sequence-specific DNA binding proteins that interact with the conserved X- box (RF-X, hXBP-1 and 2) and Y-box (YB-1, NF-Ya and b) have been cloned, [Benoist and Mathis, 1990]. Gene transfer experiments involving class II genes altered by site-directed mutagenesis or bearing "shuffled" exons have been used to define regions of the class II molecule involved in heterodimer interaction and antigen presentation (Germain et al., 1985; Ezquerra et al., 1986; Cowan et al., 1987; Murray et al., 1988; Paul, 1989).

6. Inducibility of MHC Genes with gamma-interferon. Gamma-interferon or immune interferon can enhance the expression of class I heavy and light chains and has the unique ability to induce the de novo expression of class II genes (King and Jones, 1983; Collins et al., 1986; Rosa and Fellous, 1988; Benoist and Mathis, 1990). This lymphokine most likely

functions normally to enhance the expression of the MHC antigens with the "end" of facilitating recognition of foreign antigen (Unanue, 1984). On the other hand, the lymphokine has also been implicated in exacerbating or triggering abnormal antigen recognition processes such as allograft rejection, graft-versus-host disease and autoimmune disorders (Bottazzo et al., 1983b; Pujol-Borrell et al., 1987; Fuks et al, 1988). For these reasons, considerable effort has been invested in the elucidation of the molecular mechanisms underlying the ability of gamma-interferon to enhance MHC expression (King and Jones, 1983; Boss and Strominger, 1986; Tsang et al., 1988; Shirayoshi et al, 1989; Sherman et al., 1989b; Benoist and Mathis, 1990). These studies established that enhancement of MHC antigen expression involved increases in the levels of MHC transcripts. Mutagenesis of the proximal promotors of class I and II MHC genes and sequence comparisons with the 5′ termini of interferon-inducible genes has resulted in the localization of an interferon response element in the case of class I MHC genes (Shirayoshi et al., 1989) and a core inducible enhancer (composed of the X and Y boxes) for class II MHC genes (Boss and Strominger, 1986; Sakurai and Strominger, 1988; Benoist and Mathis; 1990)

7. **The rat MHC.** The study of the rat MHC or RT1 began in the late 1950s with the study of blood group antigens (Frenzl et al., 1960; Brdicka et al., 1962). The transplantation

antigens were thus identified by serologic methods (Stark et al., 1967; Hausman and Palm,1973; Ohhashi et al., 1981). The class I and II loci were distinguished using naturally occurring rats bearing recombinant MHCs (Kunz and Gill, 1974; Kunz et al., 1977). Subsequently, many other recombinant haplotypes have been derived through breeding that have further localized the gene order of the RT1. The current map of the RT1 is shown in Figure 1 (see Gill et al., 1987 for a general review of the RT1).

The class I antigens of the rat are heterodimers composed of a 45 kilodalton heavy chain and a 12 kilodalton light chain (beta$_2$-microglobulin). The anatomy of the heavy chain is, as expected) very similar to that of mouse and man, containing 3 extracellular domains, a transmembrane domain and an intracellular tail (Blankenhorn et al., 1978; Sporer er al., 1979). Alloantigenic sites are clustered in the first two extracellular domains (alpha 1 and alpha 2). Alpha 3 is associated non-covalently with beta$_2$-microglobulin. Sequential immunoprecipitation using monoclonal antibodies has indicated that at least four class I antigens are present in the rat MHC (Misra et al., 1982). A striking feature of the rat class I antigens is a relatively limited level of polymorphism when compared to those of man or mouse (Cramer et al., 1978).

The existence of rat class II antigens was first demonstrated using alloantisera that immunoprecipitated 35 and

28 kilodalton molecules (Shinohara et al., 1977). Subsequent studies involving sequential immunoprecipitation and tryptic peptide mapping demonstrated that two distinct class II antigens (one similar to I-A and the other to I-E) existed in the rat MHC (Shinohara et al., 1978; Shinohara et al., 1981). Monoclonal antibodies directed against rat class II antigens have confirmed in two-dimensional gel analyses that there are multiple class II alpha and beta chain products encoded by the rat MHC (McMaster and Williams, 1979; Fukumoto et al., 1982; Bayer and Reske, 1983; Natori et al., 1983; Goldner-Sauve et al., 1983; Natori et al., 1985). Cross-reactivity with monoclonal antibodies against mouse I-A and I-E antigens demonstrated significant antigenic homology between the rat and mouse and helped split the rat I region into the RT1.B and RT1.D loci (Blankenhorn et al., 1983). Subsequent amino acid and cDNA sequence information has confirmed a high degree of homology between the class II antigens of various species (Cecka et al., 1980; Robertson et al., 1985; Wallis et al., 1984; Holowachuk, 1985). An invariant chain has also been identified in the rat (Wettstein et al., 1981).

As stated earlier, there is a high degree of homology between the MHC antigens of various species and it is therefore not surprising that the MHC genes of man, mouse and rat cross-hybridize. Three groups have analyzed the genetic structure of the class I multigene family in the rat (Palmer et al., 1983; Gunther et al., 1985; Cortese Hassett et al.,

1986). They find that approximately 20% of the class I genes map to the RT1.A locus while 80% map to the RT1.C region. Cortese Hassett and colleagues have also mapped specific restriction fragments that map to RT1.B/D, E, grc and the G/C regions. As is the case in both mouse and man, there are many more class I genes (as detected by Southern blotting) than serologically defined class I antigens. This is most likely due to 1) the existence of several pseudogenes and 2) the existence of class I genes with restricted expression. Indeed, the identification of secreted class I antigens (Maloy et al., 1984; Bjork et al., 1986) and the identification of several new functional class I genes upon transfection (Shimizu et al., 1987) argues that many of the class I genes identified by Southern blotting or walking on an MHC cosmid library encode yet to be identified class I antigens.

Rat class II genes (both alpha and beta) exhibit a significant level of restriction fragment length polymorphism when probed with either mouse or human locus-specifc probes (Sawicki et al., 1984; Palmer et al., 1985). The organization of these genes appears to be RT1.A/RT1.B(beta,alpha) /RT1.D(alpha, beta)/RT1.E/C (Blankenhorn and Cramer, 1985). All four class II genes have been cloned and show extensive homology to their counterpart genes in mouse and man (Wallis and McMaster, 1984; McMaster and Wallis, 1985; Holowachuk, 1985; Eccles and McMaster, 1985; Robertson and McMaster, 1985; Scholler et al., 1985).

8. Disease association with alleles of the MHC. It has been noted since the early 1970s that many diseases are associated with particular alleles of the MHC (see McDevitt and Bodmer, 1974 for review). The majority of these diseases are autoimmune in nature but the significance of these associations remain a mystery. Some hypotheses that have been advanced to explain these associations are: 1)inability of the antigen(s) of the associated allele to present an infectious agent, 2)aberrant or hyperexpression of the MHC antigen(s) resulting in presentation of autoantigen, 3)faulty selection of the T cell repertoire that develops due to the associated immune response genes, 4)antigenic similarity between an infectious agent and an associated MHC antigen, and 5)an associated MHC antigen functioning directly as the receptor for an infectious agent (Doherty and Zinkernagel, 1975; Benacerraf and McDevitt, 1972; Kagnoff et al., 1984; Kvist et al., 1978; Bottazzo et al., 1983b. In the cases where disease is associated with the MHC, the mode of disease inheritance is usually polygenic. Often, the MHC is the only defined gene system that contributes to the expression of the disorder. For these reasons, the association of diseases to the MHC is of intense interest as it may elucidate pathogenetic mechanisms.

B. INSULIN-DEPENDENT DIABETES MELLITUS.

1. General description. Diabetes mellitus is a heterogeneous syndrome characterized by hyperglycemia, polydipsia, polyuria, polyphagia and mellituria that has afflicted mankind since 1500 B.C. 5% of the Western world suffers from the syndrome, and severe long term complications affecting multiple systems in the body make it the third leading cause of death (Notkins, 1979). It is now clear that the majority of cases of diabetes mellitus can be classified as either insulin-dependent diabetes mellitus (IDDM, juvenile onset or type 1 diabetes) or noninsulin-dependent diabetes mellitus (NIDDM, maturity onset of the young "MODY" or type 2 diabetes). Insulin-dependent diabetes usually presents during or shortly after puberty and affects upwards to 1% of North Americans. While immunologic markers of imminent IDDM (such as islet-specific autoantibodies) can precede clinical onset, the progression from early signs to full-blown disease is usually rapid. Several lines of evidence, that will be touched upon later, indicate that IDDM results at least in part from an autoimmune pathogenesis. The pancreas of the newly diabetic or prediabetic patient is characteristically infiltrated by inflammatory cells, and diabetes mellitus results from the destruction of the insulin-producing beta cells of the islets of Langerhans. IDDM susceptibility exhibits a familial pattern with approximately half of the genetic predisposition

residing within the immune response region of the MHC. Daily insulin injections are absolutely necessary to prevent ketoacidosis and death. NIDDM, on the other hand, usually afflicts persons over the age of 40. Approximately 4% of the population is affected by NIDDM, and the majority of the patients are female. The clinical onset is usually chronic and the pathogenesis is not autoimmune. Although the disease is familial, the genetic susceptibility does not map into the MHC. Finally, insulin treatment is not always necessary. A careful diet can often control the symptoms.

2. Insulin-dependent diabetes in man. The etiology of IDDM is still unknown. It is hypothesized that multiple factors may trigger onset of IDDM in those susceptible to the disease, including: viral infection, stress, diet or immune system fluctuation in response to an infection at a non-pancreatic site. Seasonal variation in the onset of IDDM points toward a role for viral infection in the pathogenesis of IDDM (Christau et al., 1977). In particular, high titers of antibody against Coxsackie B-4 virus are detected in recent onset diabetics, and the seasonal peaks of new cases of IDDM correlate with those for Coxsackie virus infection (Gamble et al., 1969). Indeed, Coxsackie B-4 virus has been cultured from the pancreas of a child that died of acute IDDM, and the virus was able to induce IDDM in laboratory animals (Yoon et al., 1979). The population with IDDM is also highly enriched in patients

with congenital rubella (20%) and encephalomyocarditis (12%) and infection of laboratory animals with the rubella virus results in pancreatic abnormalities (Menser et al., 1978; Yoon et al., 1977). Finally, Epstein-Barr, varicella zoster, cytomegalovirus, polio and influenza virus infections have all been associated with an increased incidence of IDDM (Rayfield and Yoon, 1981; Jenson et al., 1980).

Before the discovery of insulin, the syndrome always resulted in ketoacidosis and death. Insulin injections now control ketoacidosis and permit a relatively normal life. The injections do not, however, prevent the debilitating long term complications such as increased incidence of myocardial infarction, renal failure, diabetic neuropathy and blindness, and propensity to develop gangrene, that make the syndrome the third leading cause of death (Rossini and Chick, 1980). The insulin pump, that automatically administers insulin in response to changes in blood sugar, has also failed to prevent long term complications from arising. There is still no treatment to prevent the onset of IDDM, and the current methods that have successfully resulted in remissions in animal models (such as injection of the immunosuppressive cyclosporine) are still highly controversial as therapeutic measures in man (Cahill and McDevitt, 1981; Rubenstein, 1988).

3. Genetics of susceptibility and resistance to IDDM in man. Epidemiologic studies clearly indicate that susceptibility and

resistance to IDDM are heritable conditions (Rimoin and Rotter, 1984). Indeed, nearly one-tenth of children having one diabetic parent and one-quarter of children having two diabetic parents will eventually present with the disease (Soeldner, 1982). Yet, it is unclear by what mode susceptibility is transmitted. There is only a fifty to seventy (70% when the twins are of the HLA-DR3/DR4 haplotype) percent concordance for IDDM in studies of monozygotic twins susceptible to the syndrome (Barnett et al., 1981).

The majority of the evidence suggests that several genes probably define susceptibility and resistance to IDDM (Rotter, 1981). These genes can be divided into those that reside within the major histocompatibility complex and those that do not. Several non-HLA markers, including the Kidd blood group antigen, have been associated with susceptibility to IDDM (Hodge et al., 1981; Bertrams, 1982).

Genes within the MHC, however, have received the bulk of attention from diabetologists and immunologists. The reasons for this attention are: 1)the genes within the HLA are estimated to control 50% of the susceptibility (easily the most clearly defined marker for the disease), 2)access to a vast library of immunologic and genetic probes for the HLA region, and 3)the correlation between the central role of the MHC in the immune system and the autoimmune component of IDDM.

The association of genetic susceptibility of IDDM to the MHC (HLA B8 and B15) was first described fifteen years ago

(Cudworth and Woodrow, 1975). Subsequently, with the advent of more precise immunologic probes, the disease association was mapped to the HLA-D or immune response region (Platz et al., 1981; Cudworth and Wolf, 1982). Indeed, greater than 95% of insulin-dependent diabetics bear the HLA-DR3 and/or HLA-DR4 haplotypes (Rotter et al., 1983; Wolf et al., 1983) Up to 3% of HLA-DR3/DR4 individuals will develop the disease, and 25% of HLA-identical siblings of diabetics will eventually contract the disease (Rotter et al., 1983; Gorsuch et al., 1982). Most recently, the HLA-D region association of IDDM has been mapped using restriction fragment length polymorphism (RFLP) analysis and oligonucleotide probing of polymerase chain reaction (PCR) amplified patient DNA. RFLP analysis first showed that IDDM was more closely associated with HLA-DQ_{beta} than HLA-DR, and most recently, the oligonucleotide/PCR analysis has identified a serine residue at position 57 of HLA-DQ beta allele that is enriched in insulin-dependent diabetics, and when substituted with asparagine results in resistance to IDDM (Owerbach et al., 1983; Todd et al., 1988). The serine at position 57 is also found in the normal population. Todd and coworkers speculate that position 57 of HLA-DQ beta is a critical residue in one of several susceptibility genes encoded in the HLA-D region. Site-directed mutagenesis of the HLA-DQ beta chain 3.2 allele has also allowed the identification of amino acid position 45 as a critical determinant that distinguishes the HLA-DQ beta chain

3.2 and 3.1 alleles (Kwok et al., 1989).

Although the correlation between non-aspartate at position 57 of HLA-DQ beta and IDDM is convincing among DR4 and DR2 Caucasians, it does not hold for DR7 Caucasians. In addition, the DRw9 haplotype encodes an aspartic acid at position 57 and is positively associated with IDDM in the Japanese (Awata et al., 1989; Ikegami et al., 1989; Sheehy et al., 1989).

In summary, IDDM exhibits a familial inheritance involving several predisposing genes. Some of these genes (one likely involving HLA-DQ beta position 57 in DR4 and DR2 individuals) are found within or in linkage disequilibrium with the MHC. These genes are estimated to determine half of the genetic predisposition to IDDM. The susceptibility gene(s) within the HLA-D region can act in a co-dominant fashion as is demonstrated by the excess of DR3/DR4 heterozygotes and direct analysis using the oligonucleotide/PCR technique. Other non-HLA genes (one closely linked to the Kidd blood group antigen on chromosome 2) are also involved in disease susceptibility. Clearly, the lack of complete concordance for disease in monozygotic twins implicates epigenetic factors in the pathogenesis of IDDM. Epidemiologic analysis and the work of Yoon and Notkins provide a strong argument that viral infection (with Coxsackie B-4 virus as the prime candidate) may in many cases represent a precipitating event (Yoon et al., 1979).

4. Histopathology of the diabetic pancreas. Von Meyenburg, nearly 5 decades ago, first described the infiltration of the islets of Langerhans by inflammatory mononuclear cells that he observed in autopsies of patients that had died of acute IDDM (Von Meyenburg, 1940). Subsequent studies have confirmed his observations but have shown that insulitis is often a transient phenomenon that is not always observed at the time of clinical presentation (Gepts, 1965; Junker et al., 1977; Bottazzo et al., 1983; Doniach and Morgan, 1973). Insulitis, when it is present, appears to be composed mainly of cytotoxic/suppressor cells with some T-helper cells (Bottazzo et al., 1983). By the time the patient is symptomatic, greater than 90% of the beta cells have been destroyed, while the other cells of the islet (e.g. alpha, delta) remain untouched (Gepts, 1980; Rahier et al., 1983). Both insulitis and beta cell destruction appear to initiate at localized regions of the pancreas with subsequent spread. Clinical IDDM presents when enough beta cells have been destroyed to obviate effective glucose homeostasis.

5. Humoral autoimmunity precedes IDDM. Bottazzo first described the presence of antibodies directed against islet cells in the sera of patients with IDDM (Bottazzo et al., 1974). Subsequently, it has become clear that three different classes of islet cell antibodies exist. Antibodies directed

against the cell surface of islets are present in the sera of the majority of cases of newly diagnosed IDDM (Lernmark et al., 1980). These antibodies disappear relatively quickly after the onset of IDDM with only one-quarter of diabetics harboring these antibodies two years after onset (Dobersen et al., 1980). On the other hand, these antibodies (taken from several discordant twins of insulin-dependent diabetics) have been detected several years before clinical presentation, and have been reported to immunoprecipitate a 64-kilodalton islet cell surface protein (Baekkeskov et al., 1982). These antibodies have also been reported to block insulin secretion, and have thus been implicated in two different pathways that might contribute to the pathogenesis of IDDM (Herold et al., 1984).

Antibodies against the islet cell cytoplasm have also been detected in the majority of new cases of IDDM (Del Prete et al., 1977; Lendrum et al., 1976; Bottazzo et al., 1980). These antibodies are rarely found in the normal population or in patients with autoimmunity against other endocrine organs (Irvine et al., 1977). These antibodies also disappear relatively quickly after clinical onset, but have been found to precede the onset of IDDM by as many as eight years (Irvine et al., 1980; Srikanta et al., 1983). Finally, cytotoxic antibodies directed against islet cells have been demonstrated in the sera of newly diabetic patients using complement-dependent cytotoxicity assays (Chandy et al., 1984; Idahl et

al., 1980; Huang and Maclaren, 1976). It has been hypothesized that these antibodies might function in a natural killer cell mediated destruction of islet cells (Pozzilli and DiMario, 1984).

In summary, it has been documented that a humoral response against the islet cells precedes IDDM in an impressively large number of cases studied. It is, however, unproven that this humoral response plays any direct role in the pathogenesis of IDDM. Indeed, a humoral response against islet cells does occasionally occur in normal individuals that never go on to develop IDDM (Spencer et al., 1984), and the long latency period between the first appearance of islet cell antibodies and clinical presentation argues against a "single-hit" role for the antibodies in disease pathogenesis. On the other hand, it appears unlikely that the humoral response is simply a consequence of some other primary lesion. The phenomenon occurs too early in the scheme of things, and long before insulitis. Perhaps the presence of a baseline level of humoral autoimmunity renders the individual susceptible to a second hit that might not normally result in disease pathogenesis.

6. Cell mediated autoimmunity in IDDM. Many of the studies on possible abnormalities in the number and function of lymphocyte subsets in IDDM have been contradictory due to differences in experimental protocols and variability in the

clinical status of the patients studied. For example, it is still a matter of debate whether a state of T lymphopenia exists in either the prediabetic or newly diabetic patient (Pozzili et al., 1983; Buschard et al., 1983; Selam et al., 1979; Cattaneo et al., 1976). In addition, data on the variation in the relative numbers of T lymphocyte subsets in diabetics are contradictory (Lernmark, 1984; Horita et al., 1982).

Certain findings, however, have become generally accepted. Helper/inducer (OKT4, Leu 3A) cell numbers are not changed in diabetics. Ia^+ T cells and B lymphocytes are found to be elevated, indicating a "sensitized" cellular immune system (Jackson et al., 1982; Bersani et al., 1981). Numerous studies indicate that suppressor T cell activity is impaired in diabetics, and this appears to be controlled by genes at or near HLA-DR as non-diabetic HLA-DR3/and or 4 share this feature (Buschard et al., 1980; Fairchild et al., 1982; Lederman et al., 1981; Gupta et al., 1982; Ambinder et al., 1982). Finally, lymphocytes from diabetics are defective in the autologous mixed lymphocyte reaction, and this likely results from an impaired ability to synthesize interleukin-2 (Gupta et al. 1983; Chandy et al., 1984). Taken together, it is clear that the cellular immune system of diabetics is abnormal both prior to and at the time of onset of IDDM. As is the case with the observed abnormalities in humoral immunity, the critical question is what role the abnormalities

in cellular immunity play in disease pathogenesis. Five sets of experimental data provide a strong argument for a critical role for cell mediated immunity in disease pathogenesis in man. First, computerized positron emission scanning of indium^{111} labeled peripheral blood lymphocytes reinjected into diabetic patients demonstrate that many of the lymphocytes "home" to the pancreas (Kaldany et al., 1982). Second, there has been one report that peripheral blood lymphocytes from an acutely diabetic patient can induce diabetes in nude mice (Buschard et al., 1978). Third, peripheral blood lymphocytes from diabetic children have been shown to kill human insulinoma cells (Huang and Maclaren, 1976). Fourth, pancreatic transplants between diabetic twins results in rapid insulitis in the grafted tissue and rapid destruction of the transplanted beta cells (Sutherland et al., 1984). Finally, the immunosuppressive drug cyclosporine has been shown to prevent the progression of IDDM when administered at an early stage (Stiller et al., 1984). Taken together, these studies provide compelling evidence for a direct role for cell mediated immunity in the pathogenesis of IDDM in man.

7. The nonobese diabetic mouse. Spontaneity of disease onset is obviously an important feature of IDDM in man. The NOD mouse develops a spontaneous form of diabetes that involves a classic insulitis (Makino et al., 1980). The murine syndrome

shares several features with human IDDM including: association with a particular haplotype of the MHC, profound cellular immune abnormalities, and the presence of islet cell antibodies preceding clinical presentation (Katooka et al., 1983). The islets are infiltrated primarily by activated T lymphocytes at the time of overt diabetes, and blockade of the cellular arm of the immune system by total body irradiation, anti-lymphocyte sera or immunosuppressive drugs prevents diabetes. Disease can be transferred passively to normal syngeneic mice with injections of peripheral blood lymphocytes or bone marrow (Katooka et al., 1983). The genetics of susceptibility appear to be similar to that in man in that several genes and environmental factors are thought to be required for expression of the syndrome. There is, however, a preponderance of females that succumb to the disease. The MHC of the NOD mouse is unusual in that it is a recombinant MHC ($H\text{-}2K^{d}/H\text{-}2D^{b}$) and does not encode a functional I-E molecule (Hattori, 1986). It is also unusual in that it bears the only I-A beta chain having a serine at position 57 (Todd et al., 1988). This supports the hypothesis that this residue is a critical susceptibility determinant in man. The identification of several recombinant haplotypes derived from the NOD and CTS or ICR mice will allow the mapping and in vivo assay of IDDM susceptibility determinant(s) within the NOD mouse MHC (Ikegami et al., 1989).

Association of susceptibility to IDDM with a particular

variable region segment of the beta chain of the T cell receptor was assessed by generating an NOD transgenic mouse carrying a rearranged T cell receptor beta chain gene cloned from an ovalbumin reactive T cell (Lipes et al., 1989). The transgene suppressed the utilization of the remaining V beta segments via allelic exclusion, but did not prevent the development of insulitis.

8. The Bio-Breeding Rat. The discovery, sixteen years ago, of a Wistar-derived rat that developed a spontaneous IDDM remarkably similar to the human syndrome caused great excitement among diabetologists and immunologists (Chappel and Chappel, 1983; Rossini et al., 1985; Fuks et al., 1988; Castano and Eisenbarth, 1990). Various sublines of the original BB/Ottawa line now exist, including diabetes resistant lines (BB/W or BB/N) and high incidence (BB/Hooded) or inbred lines. In the absence of exogenous insulin, the blood glucose levels in the BB rat rise to 500 mg/dl and the animals become severely ketoacidotic and die within a short period of time (Nakhooda et al., 1978; Marliss et al , 1982). Disease usally strikes during a "zone of onset", between 60 and 100 days of age, and afflicts between 10 and 75% of the animals depending upon the line and particular litter (Rossini et al., 1985). The syndrome occurs without a sexual bias and is not accompanied by obesity.

The fact that not all BB rats become diabetic suggests that environmental factors play an important role in disease pathogenesis. Surprisingly, there is no evidence in the BB rat model for a role for viral infection in disease pathogenesis. BB rats raised in gnotobiotic conditions still succumb to IDDM, and there are no viral particles evident in the islets of newly diabetic BB rats (Rossini et al., 1979; Rossini et al., 1985). These studies do not rule out a possible role for vertically transmitted viruses. It is clear that more work needs to be done in this important area.

Diets containing high levels of carbohydrate or fat do not seem to have an effect on the incidence of IDDM in the BB rat (Rossini et al., 1981). Diets high in protein, however, have been shown to increase the incidence of IDDM (Scott et al., 1987; Elliot and Martin, 1984). Diets in which casein is the only source of protein dramatically reduce the incidence of IDDM (Scott et al., 1985). Diet appears to influence the incidence of IDDM most profoundly just prior to or during weaning (Daneman et al., 1987; Issa-Chergui et al., 1988).

19. Genetics of susceptibility and resistance in the BB rat. Breeding studies involving the BB rat and non-susceptible strains of rats indicate that multiple genes define susceptibility for IDDM in the BB rat (Guttmann et al., 1983). Genetic susceptibility is transmitted in a functionally recessive manner, suggesting that some of the susceptibility

genes need to be present in duplicate for disease expression. The exception to this rule involved a F_1 mating between the BB and Buffalo rat. This cross generated the only F_1 animals that developed IDDM and suggested that the Buffalo rat also harbors a susceptibility gene. The value of the BB rat as a genetic model of IDDM became more obvious with the finding that diabetes absolutely required at least one chromosome bearing the $RT1^u$ haplotype of the rat MHC (Colle et al, 1981; Naji et al., 1981; Jackson et al., 1984). Gene(s) within the MHC were thus shown to be necessary but not sufficient to define susceptibility to IDDM. Two other independently segregating genetic loci have also been shown to be involved in genetic predisposition. One of these loci controls the profound T lymphocytopenia that is characteristic of the BB rat, and the other controls infiltration of the pancreas by lymphocytes (Guttmann et al., 1983). It is thought that these separate susceptibility genes and unknown environmental stimuli contribute to the eventual onset of IDDM. These three putative loci have now been separately placed on an ACI rat genetic background to generate animals congenic at these loci (R.D. Guttmann, personal communication). It is hoped that these animals will facilitate the identification of the non-MHC genes involved in genetic predisposition.

Some work has been done to elucidate the significance of the association of IDDM in the BB rat to the u haplotype. It was hypothesized that the BB rat u haplotype might bear a

unique IDDM-susceptibility gene (polymorphism) not found in other u haplotypes found in non-IDDM prone animals. Indeed, the mating of the BB rat with the non-diabetes prone WF rat (bearing a seroidentical u haplotype) did not result in any diabetic progeny in the F_1 (Colle et al., 1986). One group reported I-A_{alpha} RFLPs that distinguished the MHCs of diabetes-prone animals from diabetes-resistant BB/N animals (Buse et al., 1984; Buse et al., 1985). It has since become clear that these RFLPs do not represent a "diabetogenic" variant of the u haplotype, as the BB/N strain has been shown to be contaminated with MHC sequences from the ACI and another unknown rat (Buse et al., 1985). Analysis of the class II polypeptides of the BB and WF rats, by two-dimensional gel electrophoresis and mapping of tryptic peptides, failed to show any differences between the two proteins (A. J. Goldner-Sauve, McGill University Ph.D. thesis, 1986). Furthermore, various immunologic assays for a unique polymorphism in the BB rat $RT1^u$ haplotype have failed to indicate such a polymorphism (Goldner-Sauve et al., 1985). Specifically, congenic animals bearing either the BB or WF rat u haplotypes are non-responsive in mixed lymphocyte cultures and tolerate cardiac grafts between each other (R.D. Guttmann, personal communication).

Chapters 2-4 of this thesis will detail our analysis of the association of IDDM in the BB rat with the u haplotype of the MHC.

10. Modulation of MHC gene expression in the prediabetic and newly diabetic pancreas. Sites of inflammation, including the diabetic pancreas, often have increased levels of MHC antigen expression. This phenomenon has prompted the hypothesis that aberrant expression of these molecules might precede organ specific autoimmunity, and might in fact trigger the autoimmune response by presenting autoantigen to the immune system (Hanafusa et al., 1983; Bottazzo et al., 1983b). It has been suggested that aberrant expression of MHC antigens might be triggered by localized production of lymphokines in response to viral infection. To test whether this scenario occurs in the diabetic pancreas, two issues need to be resolved. First, the temporal order of modulation in MHC gene expression needs to be defined in relation to other known parameters that precede the onset of IDDM. Second, the inducibility of MHC molecules by cytokines needs to be investigated *in vitro* on the target cell type, the beta cell. The endocrine cells of the pancreas normally express low levels of class I antigen and do not express class II antigens (Faustman et al., 1980). The pancreata of recent onset insulin-dependent diabetics are reported to exhibit enhanced levels of class I MHC antigen expression and aberrant expression of class II MHC molecules (Bottazzo et al., 1985; Foulis and Farquharson, 1986; Foulis et al., 1987) These studies claim that aberrant class II expression occurs on the

islet beta cells, prior to insulitis. In the BB rat, one group reports that class II antigen expression on the vascular epithelium precedes diabetes and insulitis, while de novo class II expression is not observed on beta cells until there is a full-blown insulitis (Dean et al., 1985). In our laboratory, we have never observed a class II positive beta cell in prediabetic or newly diabetic BB rats (see Chapter 5). The work of Pipeleers and coworkers (1987) might explain these discrepancies. Their data suggest that Ia-positive, insulin-positive cells are mononuclear cells that have phagocytosed debris from ongoing beta-cell destruction. In fact, these cells are present in the normal pancreas of diabetes-resistant animals. They, therefore, suggest that further studies using additional markers are necessary to definitively state whether or not beta cells express class II molecules prior to or during the course of IDDM.

A recent report analyzing the temporal order of events preceding IDDM suggests that the first abnormality seen in BB rats is the hyperexpression of class I MHC antigens throughout the pancreatic islets (Bone et al., 1990). The enhanced class I MHC expression was followed by accumulation of ED1+ macrophages, and finally by lymphocytic infiltration. Aberrant class II MHC expression was never observed on endocrine cells.

Chapter 5 of this thesis will detail our analysis of MHC gene expression in the pancreata of prediabetic and newly

diabetic BB rats and age-matched WF controls.

In vitro studies on the inducibility of MHC antigen expression on isolated islets or rat insulinoma cells (RINm5F) have also been contradictory. Some workers have been able to induce class II antigen expression on isolated islets using gamma-interferon (Markmann et al., 1987; Walker et al , 1986, Wright et al., 1987), while others have not (Campbell et al., 1985; Campbell et al., 1986; Pujol-Borrell et al., 1986). Others have reported that class II induction can be acheived using both tumor necrosis factor and gamma-interferon (Pujol-Borrell et al., 1986). When rat insulinoma cells are used as beta cell analogs, the results are once again contradictory. Some workers cannot induce class II antigen expression using either crude lymphokine preparations (from conA stimulated splenocytes) or gamma-interferon (with doses as high as 1000 units/ml culture fluid), [Campbell et al., 1986]. Others report that they can induce class II antigen expression on a minor population of these same cells using doses of gamma-interferon as low as 10 units/ml (vanVliet et al., 1987).

Chapter 6 of thesis will detail our analysis of the molecular biology of gamma-interferon and crude lymphokine mediated induction of MHC gene expression on RINm5F (RIN5F) cells.

11. Transgenic models of targeted (aberrant) MHC gene expression. Several transgenic mouse lines have been

generated that either aberrantly express class II molecules or express enhanced levels of class I antigen on islet beta cells (Allison et al., 1988; Sarvetnick et al., 1988; Bohme et al., 1989). Many of these mice spontaneously develop an insulin-dependent diabetes that results from beta cell destruction. It is now clear, however, that the beta cell destruction is not immune-mediated, as there is no insulitis and no rejection of transplanted islets. Overproduction of MHC class I or II molecules appears to be directly cytocidal to the beta cells. Sarvetnick and coworkers also produced a transgenic mouse line in which the beta cells produce gamma-interferon. These mice also develop an IDDM, preceded by an intense insulitis. Transplanted islets are destroyed in this model. MHC class I molecules are overexpressed in the islets of these mice but MHC class II molecules remain undetectable.

12. Histopathology of the pancreas in diabetic and prediabetic BB rats. IDDM in the BB rat results from the destruction of the pancreatic beta cells. The other cells of the islet (A, D and PP) are also decreased in the BB rat (Seemayer et al., 1982). Insulitis is a standard feature of IDDM in the BB rat (Nakhooda et al., 1977; Seemayer et al., 1982) and also occurs with significant frequency in normoglycemic BB rats and in some rats that have been undergoing insulin treatment for a prolonged period of time. Infiltration by mononuclear cells is not restricted to the islets, as aggregates of infiltrating

cells are observed around ductules and acinar tissues. Insulitis precedes overt diabetes by as long as ten days (Logothetopoulos et al., 1984). The cellular infiltrate is composed primarily of activated T lymphocytes with few if any B lymphocytes (Like et al., 1982). Both helper-inducer ($OX19^+$, $W3/25^+$) and cytotoxic-suppressor cells ($OX19^+$, $OX8^+$) are present in the infiltrate. The number of macrophages and natural killer cells present in the pancreas is also reported to increase prior to IDDM (Bottazzo et al., 1985). Occasionally, a high percentage of eosinophils are found in the infiltrate, and mirrored by an increase in the numbers of peripheral blood eosinophils (Like et al., 1979; Prowse et al., 1986; Elder and Maclaren, 1983; Kurner et al., 1986). It has been hypothesized that the eosinophils might contribute to beta-cell damage via an antibody-mediated hypersensitivity reaction (Prowse et al., 1986).

13. Humoral immunity in the BB rat. Islet cell surface and complement fixing antibodies are detectable in most BB rats between 40 and 100 days of age (Dyrberg et al., 1983; Dyrberg et al., 1984). Their presence precedes the onset of diabetes and insulitis, and correlates with the incidence of IDDM in various sublines of the BB rat (Pollard et al , 1983). Humoral immunity against islet cells appears to reach a maximal level at the onset of IDDM (Laborie et al., 1985). Interestingly, these antibodies immunoprecipitate a 64

kilodalton protein from normal Wistar Furth islets, a protein of identical size to that immunoprecipitated from human islets using patient sera (Dyrberg et al., 1984; Baekkeskov et al., 1984). In contrast to the situation in man, autoantibodies to lymphocytes and several endocrine organs also appear prior to IDDM (Like et al., 1982c; Elder et al., 1982, Dyrberg et al., 1984). These antibodies do not, however, have any detectable effect on the function of these other cell types.

14. Abnormalities in T cell numbers in the BB rat. One of the most striking features of IDDM in the BB rat is the profound T-cell lymphopenia that is found in the peripheral blood, the lymph nodes and the spleen (Jackson et al., 1981; Poussier et al., 1982; Bellgrau et al., 1982; Naji et al., 1983; Elder and Maclaren, 1982; Jackson et al., 1983). The T lymphopenia affects both T helper ($W3/25^+$) and cytotoxic ($OX8^+$) T cells, although the effect on the cytotoxic T cells is the more profound (Elder and Maclaren, 1982; Jackson et al., 1981; Poussier et al., 1982; Jackson et al., 1983; Naji et al., 1983; Greiner et al., 1986; Naji et al., 1983b; Woda et al., 1986). Only 1-5% of the peripheral blood lymphocytes are estimated to be cytotoxic/suppressor T cells. One particular subset of T lymphocytes, bearing the differentiation antigen RT6, is completely absent in the BB rat (Greiner et al., 1986). This alloantigen is found on the majority of peripheral blood thymocytes, and the depletion of this subset

is thought to account for most (if not all) of the observed T lymphopenia in BB rats. It is still unknown why this particular subset of T cells is missing in the BB rat It is hypothesized that a defective gene or genes affects the normal differentiation of this subset of T cells (Angelillo et al., 1988). A recent report suggests that T lymphopenia is not required for disease onset. Several lines of BB rats in the Worcester colony developed IDDM at a low incidence (3%) in the absence of T lymphopenia (Like et al., 1986). Similar lines have also been developed in the Seattle colony (Herold et al , 1989). This suggests that while T lymphopenia greatly increases the incidence of IDDM, it is not necessary for expression of the full-blown syndrome.

In the midst of the usual T lymphopenia, however, there are increased numbers of Ia^+ "activated" T lymphocytes in the peripheral blood of prediabetic and newly diabetic BB rats (Francfort et al., 1985). The numbers of activated T lymphocytes are highest during the first week of hyperglycemia. Intrathymic lymphocyte numbers are normal, as are the absolute numbers of B lymphocytes (Elder and Maclaren, 1983; Jackson et al., 1983; Naji et al., 1983b).

15. Abnormalities in T cell function in the BB rat. In addition to the abnormalities of T cell number, there is significant evidence that the T cells of the BB rat are functionally abnormal. T lymphocytes in the peripheral blood

and the lymphoid organs have a reduced capacity to proliferate in the presence of Concanavalin A (Con A), (Elder and Maclaren, 1983; Jackson et al., 1983; Prud'homme et al., 1984; Woda and Padden, 1986). Similarly, lymphocytes respond poorly to phytohemagglutinin (PHA) and to pokeweed mitogen (Prud'homme et al., 1984). Lymphocytes from the BB rat are also poor responders in the mixed lymphocyte culture, and the BB rat exhibits only a weak rejection response to allogeneic tissues (Elder and Maclaren, 1983; Bellgrau et al., 1982; Naji et al., 1983a). Interleukin-2 (IL-2) production in response to Con A stimulation is also reduced in the BB rat, and cannot be reversed by the addition of exogenous IL-2 (Prud'homme et al., 1984; Woda and Padden, 1986). Several lines of evidence indicate that the abnormal T cell responsiveness in the BB rat does not originate from a defect in the T lymphocyte, but rather a suppressive effect provided by the macrophage. First, Prud'homme and coworkers (1984) showed that the addition of splenocytes from a BB rat suppressed the proliferation and IL-2 production of WF T cells in response to several mitogens. This suppressive effect could be completely abrogated by depleting the BB splenocyte preparation of macrophages or by adding the prostaglandin synthetase inhibitor indomethacin. Second, Woda and Padden (1986) were able to sort T cells from the BB rat and showed that these cells were normal in their ability to proliferate and produce IL-2 in response to mitogens. The mechanism by which

macrophages from the BB rat suppress T cell function is unknown.

Finally, BB rats appear to have a blunted ability to mount a cytotoxic immune response. Very few functional cytotoxic T cells are produced against virally infected cells, and it is controversial whether islet beta-cell cytotoxic T cells exist (Woda and Padden, 1987; Dean et al., 1985).

16. T cell lymphopenia and dysfunction in the context of a normal thymus. Abnormalities in the number and function of T cells immediately suggests that a primary immunologic defect in the BB rat may reside in the thymus. Indeed, thymus transplantation can prevent IDDM in the BB rat (Georgiou and Bellgrau, 1989). Prothymocytes undergo "thymic education," where they are thought to interact with MHC antigens. This interaction leads to the deletion of autoreactive T cell clones, and thus is a major force in the shaping of the T cell repertoire. From a gross anatomical view, the BB rat thymus is normal in morphology and cellular composition (Jackson et al., 1983). MHC class I and II antigen expression is normal both quantitatively and spatially (N. Parfrey, personal communication). A thymus transplanted from a BB rat into an irradiated IDDM-resistant animal does not transfer T cell abnormalities or disease to the recipient (Francfort et al., 1985b). Conversely, a thymus taken from an IDDM-resistant animal will not protect an IDDM-prone animal from developing T

cell abnormalities or disease. These studies indicate that the BB rat thymus is normal, and suggest that the T cell abnormalities originate in the prothymocyte. Neonatal thymectomy prevents disease, as the putative autoreactive prothymocyte requires a thymus to mature into a T cell clone with the capability to participate in disease pathogenesis (Like et al., 1982d).

17. Passive transfer studies and isolation of autoreactive T cell clones. It is controversial whether splenocytes from diabetic BB rats can transfer disease to athymic animals (Nakhooda et al., 1981; Rossini et al., 1983). ConA activated splenocytes from newly diabetic BB rats can induce disease in young BB rats prior to their age of spontaneous onset, and in WF rats or cyclophosphamide-treated heterozygotes bearing one u haplotype (Koevary et al., 1983; Like et al., 1985; Koevary et al., 1985). The requirement for conA activation suggests that the disease inducing cell is a T cell, as does the MHC restriction of the phenomenon. T cell lines that specifically react with the islet beta cell have been isolated from the spleen and pancreas of newly diabetic BB rats (Prud'homme et al., 1984b). These T cells exhibit a T helper cell phenotype and proliferate (and produce IL-2) in the presence of islet cell membranes and histocompatible antigen presenting cells (Prud'homme et al., 1987). Due to the instability in reactivity of T cell lines, T cell clones have recently been

derived from newly diabetic BB rats (Prud'homme et al., 1986). These clones also exhibit the T helper cell phenotype. The reactivity of both the T cell lines and clones could be inhibited by monoclonal antibodies directed against the RT1.D but not RT1.B class II product (Prud'homme et al., 1987). None of these clones or lines have been tested for their ability to transfer disease or insulitis.

18. Natural killer cells and their potential role in IDDM. OX8$^+$, anti-asialo GM1$^+$, OX19$^-$, natural killer cells are increased in prediabetic and newly diabetic BB rats in comparison to diabetes resistant strains (Woda and Padden, 1986; Woda and Biron, 1986). Several lines of evidence suggest that these NK cells may play a role in the pathogenesis of IDDM. First, conA stimulation of splenocytes isolated from diabetic BB rats results in increased cytotoxicity of the non-MHC matched RINm5F rat insulinoma cell line (RT1^u) in <u>in vitro</u> assays. Non-MHC restricted killing is a hallmark of NK cells, while activated T cells require the appropriate MHC molecules for killing. Second, it has been demonstrated that the increased numbers of NK cells in diabetes prone BB rats translates into increased NK activity (MacKay et al., 1986). These same authors also demonstrated that islet cells from a variety of MHC types could be killed by these NK cells. Other cell types were not susceptible to NK cell-mediated killing. The effector cells were clearly NK

cells as they killed the tumor cell lines G1-TC and YAC-1 *in vitro*, and were blocked by anti-asialo GM1 serum. YAC-1 cells efficiently competed out killing of RINm5F cells, and the efficiency of lysis was enhanced when the source of effector cells was enriched for the low density fraction of splenocytes separated on a Percoll gradient. This fraction would be enriched for monocytes, large lymphocytes and NK cells. Perhaps the most convincing evidence suggesting a role for NK cells in the pathogenesis of IDDM comes from the *in vivo* studies where injection of antibodies directed against the OX8 or asialo-GM1 determinants prevents the onset of IDDM (Like et al., 1986b; Woda et al., 1987b). The mechanism by which NK lysis might be targeted to islet beta cells *in vivo* remains elusive. Traditionally, specific NK lysis would also involve antibody-dependent cell-mediated cytotoxicity (ADCC). The early humoral response to islet beta cells might therefore direct the specific NK lysis. There has, however, been no evidence to support this hypothesis.

19. Cytokines, are they cytotoxic to beta cells? Several *in vitro* studies have demonstrated that a number of cytokines can be cytotoxic to beta cells. The pI7 form of interleukin-1, produced predominantly by NK and dendritic cells, is directly cytotoxic to islet cells (Mandrup-Poulsen et al., 1986; Bendtzen et al., 1986; Mandrup-Poulsen et al., 1987). Moreover, gamma-interferon and tumor necrosis factor have been

shown to act synergistically with IL-1 in cytolysis of islet cells in vitro (Pukel et al., 1988). There is, however, no direct evidence that these cytokines are cytotoxic to islet beta cells in vivo. One study, which demonstrated that administration of silica prevented IDDM in BB rats, suggests that macrophages are necessary for the pathogenesis of IDDM, and might suggest that cytokines produced by macrophages might exert their effect via direct cytolysis of beta cells (Oschilewski et al., 1985). A recent study indicates that macrophage infiltration of the pancreas is the first cellular phenomenon that occurs prior to IDDM, and is required for subsequent lymphocytic infiltration (Hanenberg et al., 1989).

20. Prevention of IDDM via immunotherapy. IDDM has been prevented in the BB rat using several immunotherapeutic approaches. These interventions both stem from previous data implicating, and provide new evidence for, the involvement of several types of immune cells in the pathogenesis of IDDM. Antiserum or monoclonal antibody (OX19) against pan-T cell determinants can reverse hyperglycemia in prediabetic BB rats and prevent the onset of IDDM in non-symptomatic animals (Like et al., 1982; Like et al., 1986b). Replacement of bone marrow cells in neonatal BB rats with donor cells taken from histocompatible diabetes-resistant animals reduces the incidence of IDDM (Naji et al., 1981; Naji et al., 1983). Moreover, neonatal thymectomy prevents IDDM in the BB rat

(Like et al., 1982d). Transfusion of whole blood, splenocytes or lymphocytes from histocompatible diabetes-resistant animals can prevent the onset of IDDM (Rossini et al., 1985). Most recently, the capacity to prevent the onset of IDDM has been attributed to the W3/25$^+$ helper/inducer lymphocytes (Castano and Eisenbarth, 1990). Among these cells, those that bear the RT6 marker appear to be critical in determining susceptibility to IDDM. Diabetes-resistant BB rats succumb to diabetes when RT6$^+$ T lymphocytes are depleted from the circulation (Greiner et al., 1987). Furthermore, anti-asialo-GM1 treatment, while preventing diabetes in the BB rat, cannot block the onset of diabetes in diabetes-resistant BB rats depleted of RT6$^+$ cells (Woda et al., 1987).

21. Cyclosporine treatment of IDDM in the BB rat. Cyclosporine A (CsA), a drug routinely used in the immunosuppression of patients that have undergone transplants, has been reported to either prevent or blunt the onset of IDDM when administered orally on a daily basis to young prediabetic BB rats (Laupacis et al., 1983; Jaworski et al., 1986; Yale et al., 1987). Sustained cyclosporine treatment is not free of risk, however, as continued treatment often leads to glucose intolerance in the recipient animals (Yale et al., 1985; Yale et al., 1987). If cyclosporine treatment is initiated significantly before the zone of onset of diabetes, subsequent withdrawal from the treatment regimen does not result in

appearance of the syndrome (Like et al., 1984; Jaworski et al., 1986). If the treatment regimen is initiated later, a reduction in the treatment rate to twice weekly can be sustained without a significant increase in the appearance of the syndrome (Brayman et al., 1987). Such a regimen would thus minimize the serious side effects that daily cyclosporine treatment would present. Finally, the combined treatment of newly diabetic BB rats with cyclosporine and anti-IL-2 receptor monoclonal antibody has been shown to restore normoglycemia to greater than 70% of the animals (Hahn et al., 1987). These data in the BB rat have prompted clinical trials in man that are now in progress.

The prevention of IDDM in BB rats by administration of cyclosporine appears to act by inhibiting the recruitment of ED1+ macrophages that normally follows hyperexpression of class I MHC antigens (Bone et al., 1990). In the absence of infiltration by macrophages, subsequent recruitment of lymphocytes is also blocked. Paradoxically, hyperexpression of class I MHC antigens by the islets is not inhibited by cyclosporine treatment.

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In this chapter, we demonstrate via molecular genotyping of multiple intercrosses between the Biobreeding, Lewis and Buffalo rats that the susceptibility determinant for insulin-dependent diabetes mellitus (IDDM) encoded in the u haplotype of the rat MHC (RT1) is a dominant trait. This finding is in agreement with the mode of inheritance observed in human IDDM where the disease segregates with HLA-DR4 and occurs in individuals heterozygous for this haplotype. The data also suggest a dose effect, where animals homozygous for the u haplotype exhibit an increased relative risk for the disease. This is agreement with the increased relative risk of HLA-DR4/DR3 heterozygotes in man. These data, then, are further evidence for the extensive similarities between the MHC association of IDDM in man and the BB rat.

CHAPTER TWO.

A SINGLE DOSE OF THE MHC-LINKED SUSCEPTIBILITY DETERMINANT ASSOCIATED WITH THE RT1u HAPLOTYPE IS PERMISSIVE FOR INSULIN DEPENDENT DIABETES MELLITUS IN THE BB RAT

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ABSTRACT

Syndromes of insulin-dependent diabetes have been described in mouse, rat and man. In all three, the presence of one or more specific alleles of the major histocompatibility complex is a prerequisite for appearance of the disease. In the BB rat, diabetes is associated with the $RT1^u$ haplotype. We have performed a series of intercrosses of diabetic BB rats with normal Lewis and Buffalo rats and examined the offspring of all litters producing at least one diabetic animal. 45 of the 250 rats that developed diabetes were heterozygous for the $RT1^u$ haplotype by serotyping. Furthermore, diabetic rats heterozygous by serotyping at the RT1A class I loci were also heterozygous at the RT1B and RT1D loci of the class II region and did not show evidence of a recombinant haplotype when examined by Southern blot analyses using molecular probes for class I and class II genes. Diabetic rats heterozygous or homozygous for $RT1^u$ were phenotypically indistinguishable with respect to age of onset and severity of disease. Therefore, in the rat, as in the human, a single dose of the high risk allele at the major histocompatibility complex is sufficient for the development of insulin-dependent diabetes mellitus if other susceptibility factors and the appropriate environmental factors are in place.

INTRODUCTION

Syndromes of insulin-dependent diabetes mellitus (IDDM), in which the histopathology of the pancreas reveals infiltration by cells of the immune system, have been described in mouse (1), rat (2), and man. In all three, the presence of one or more specific alleles of the major histocompatibility complex (MHC) is a necessary, though not a sufficient prerequisite for appearance of the disease. In the rat, IDDM is associated with the $RT1^u$ haplotype (3). Breeding studies using an MHC recombinant strain in which there has been a recombination event between the class I and class II loci of RT1 have permitted us to conclude that u alleles at the MHC coding regions to the right of the class I RT1.A region and to the left of the RT1.C class I region are necessary but not sufficient for expression of overt diabetes (4). It has been suggested, however, that because IDDM is more common in animals homozygous for the u haplotype than in those heterozygous for u, that a "diabetogenic gene" within the MHC functions in a recessive fashion to permit expression of disease when the other genetic and environmental factors necessary for the disease are present (5). In our breeding studies, we have regularly encountered animals heterozygous for the u allele in which IDDM develops, indicating that the

MHC associated IDDM susceptibility determinant is a dominant trait (6). Since assignment of genotype had been made on the basis of serotyping at the RT1.A locus present on red blood cells, the possibility remained that such animals were heterozygous at RT1.A but homozygous at the class II regions as a consequence of an undetected recombination event. Thus, the present study was undertaken to determine the genotype of the class II and the non-RT1.A class I loci by restriction fragment length polymorphism analysis of genomic DNA isolated from several animals in the breeding studies. We show that the diabetic rats heterozygous by serotyping at the RT1.A class I locus are also heterozygous at the RT1.B and RT1.D loci of the class II region and do not show evidence of a recombinant haplotype. Furthermore, we demonstrate that although the relative risk for development of disease is greater for animals homozygous for the u haplotype of RT1 than for those carrying a single copy of the permissive haplotype, this increment in relative risk is not large. Furthermore, the diabetic syndrome is no different in animals homozygous from those heterozygous for the $RT1^u$ haplotype.

MATERIALS AND METHODS

Breeding studies: animals were maintained and bred as previously described (7). Lines derived from Lewis ($RT1^l$) by BB rat (Lew x BB) crosses were maintained by brother-sister matings for 6 generations and those from Buffalo ($RT1^b$) by BB rat (Buf x BB) crosses for 12-14 generations. Diabetic males were preferentially used for breeding. We have analyzed data on all of the progeny from mating pairs producing at least one diabetic offspring. All of the progeny were serotyped at the class I loci as previously described (8). Odds ratios were calculated according to the method of Woolf and combined according to the method of Mantzel-Haenzel (9).

DNA probes: For RFLP analysis of class I genes, we used a 248 base pair PstI fragment from the class I Balb/c cDNA clone pH2IIa as a hybridization probe (10). A 4.7 kilobase BamHI/EcoRI fragment from cosmid clone $H\text{-}2^d$ 24.2 was used to detect the I-A alpha chain homolog and a 6 kilobase EcoRI fragment from cosmid clone 41.1 to detect the I-A beta chain homolog. A 1.2 kilobase BamHI fragment from cosmid clone 8.4 was used to detect the I-E beta chain homolog (11). Sheared, denatured rat genomic DNA was added to the prehybridization solution (at a concentration of 5 ug/ml) prior to

hybridization with the beta chain probes to mask repetitive elements in the blotted rat DNA that cross-hybridize with repetitive elements in the probes. All of these probes were the kind gift of L. Hood, California Institute of Technology, Pasadena, CA.

RESULTS

There were 15 mating pairs in the LEW ($RT1^l$) x BB ($RT1^u$) crosses producing litters in which at least one animal developed IDDM. These pairs produced a total of 150 progeny of which 96 typed $RT1.A^{u/u}$, 40 were $RT1.A^{u/l}$, and 14 were $RT1.A^{l/l}$ The odds ratio for developing IDDM in the u/u animals as compared with the u/l animals was 1.40 (p=0.64). There were 122 mating pairs in the Buf ($RT1^b$) x BB ($RT1^u$) crosses which produced litters with at least one rat developing IDDM. These pairs produced a total of 741 offspring of which 538 typed $RT1.A^{u/u}$, 158 were $RT1.A^{u/b}$, and 45 were $RT1.A^{b/b}$. The odds ratio for development of IDDM in the $RT1^{u/u}$ offspring compared with the $RT1^{u/b}$ animals in these crosses was 1.63 (p=0.02). The combined odds ratios for the two groups was 1.59 with 95% confidence limits of 1.08-2.34 (p=0.02). This represents the relative likelihoods of development of IDDM in a homozygous ($RT1^{u/u}$) animal versus one carrying one u haplotype ($RT1^{u/x}$). Animals not carrying at least one u haplotype did not develop disease.

The diabetic syndrome was indistinguishable in the animals homozygous and heterozygous for the $RT1.A^u$ allele. The male to female ratio in the (Lew x BB) homozygotes was 11:11 and in the heterozygotes was 4:3. The corresponding

figures in the (Buf x BB) crosses were 90:93 and 19.19 respectively. The age of onset was also similar. Thus, the combined ages of onset in the two sets of crosses were 95±2 days for the homozygotes and 98±14 days for the heterozygotes

Because of the paucity of reports in the literature of animals heterozygous for the u allele becoming diabetic, we were concerned that early in our breeding studies we might have fixed a recombination event between the RT1 A locus (whose products were detected by serotyping) and the class II loci whose products appear to confer disease susceptibility. We therefore examined genomic DNA of diabetic animals typing RT1.$A^{u/b}$. Figure 1 shows Southern blot analyses of DNA from diabetic rats serotyped as u/b heterozygotes as well as DNA from BB and Buffalo control reference strains. The DNA was digested with BglII and probed with class II MHC probes for I-A alpha (panel A), I-A beta (panel B) and I-E beta (panel C). Lane 1 in each panel contains DNA from a BB rat, lane 2 from a Buffalo rat and lanes 3-5 contain DNA from three diabetic offspring serotyping as RT1.$A^{u/b}$. In each instance, the diabetic offspring showed two bands co-migrating with the parental bands as expected for a heterozygote. They did not show any novel bands nor were there any bands absent that might indicate a recombinant event. A similar analysis is evident in Figure 2 in which the RFLP study utilized a 248 bp PstI fragment from the class I Balb/c cDNA clone pH2IIa as a hybridization probe. There are a large number of hybridizing

bands as expected for a class I probe which detects class I type genes flanking the class II region of the rat MHC. Arrows labeled a-c indicate bands unique to the BB rat genome (lane 1) whereas arrows d-h indicate bands unique to the Buffalo rat (lane 2). Again, the serologic heterozygote diabetic offspring in lanes 3-5 demonstrate all the bands from each of the parental types and do not show novel or missing bands.

DISCUSSION

The two sets of breeding studies produced a large series of progeny for study. We examined the offspring of those mating pairs which produced at least one diabetic animal in order to ensure that the appropriate genetic predisposing factors required in addition to the permissive MHC for disease expression were segregating in those breeding pairs. We found forty-five diabetic animals that were heterozygous at the MHC and thus apparently carrying a single dose of the MHC-linked, $RT1^u$ associated susceptibility determinant. However, a recombinant MHC haplotype would not necessarily be noted by the routine serotyping which detects RT1.A products Therefore, Southern blots were performed using probes for the murine I-A and I-E loci. These demonstrated that rats that were serotyped u/b at the class I RT1.A locus are in fact also heterozygous at the class II RT1 B and RT1 D loci. These data indicate that the MHC associated determinant carried by the $RT1^u$ haplotype can act in a dominant fashion. This is similar to the findings in human IDDM. Thus, Rotter et al. interpret population data of HLA-DR frequencies in diabetics as incompatible with a simple recessive mode of inheritance of the MHC-linked susceptibility determinants (12). Similarly, an analysis of diabetic human pedigree data carried out by

MacDonald et al. leads to the conclusion that the disease is inherited primarily via a single dose of HLA-DR4 (13). Finally, of 39 IDDM patients reported by Todd et al. whose DQ beta chain genotype were examined by molecular typing, 10% were found to be heterozygotes for the putative susceptibility determinant at residue 57 of the DQ beta chain (14).

The odds ratio of 1.59 indicates that there may be a dose effect for the MHC-associated susceptibility determinant. Therefore, although an animal heterozygous for this determinant is clearly capable of developing the disease, such a likelihood is increased by two copies of the determinant to a small but significant extent. This is again reminiscent of the human data that indicate the highest relative risk for the HLA-DR4/HLA-DR3 compound heterozygote (15). There are at least two types of general explanations of the increased relative risk of the homozygote at class II. The first refers to the presumed mode of action of immune response genes, usually displaying a dominant mode of inheritance for the responder phenotype (16). This is consistent with the putative function of class II gene products in antigen presentation in which "correct" presentation could be carried out by the single copy of the heterozygote; however, the probablility of this event would presumably be enhanced by the homozygous state. This has in fact been noted in studies of Ir genes in which the F1 animal shows an immune response intermediate between those of the dominant responder and non-

responder parental strains (16).

The second possibility is that there are two MHC-linked susceptibility determinants; a single copy of one of these (analogous to DR4) is permissive for disease occurrence and the presence of the second determinant (analogous to DR3) increased the likelihood of insulin-dependent diabetes mellitus. We cannot definitively discriminate these two hypotheses on the basis of these breeding studies. Nevertheless, in the rat, as in the human, a single dose of the high risk allele is sufficient for diabetes to develop if other susceptibility factors and the appropriate environmental factors are in place.

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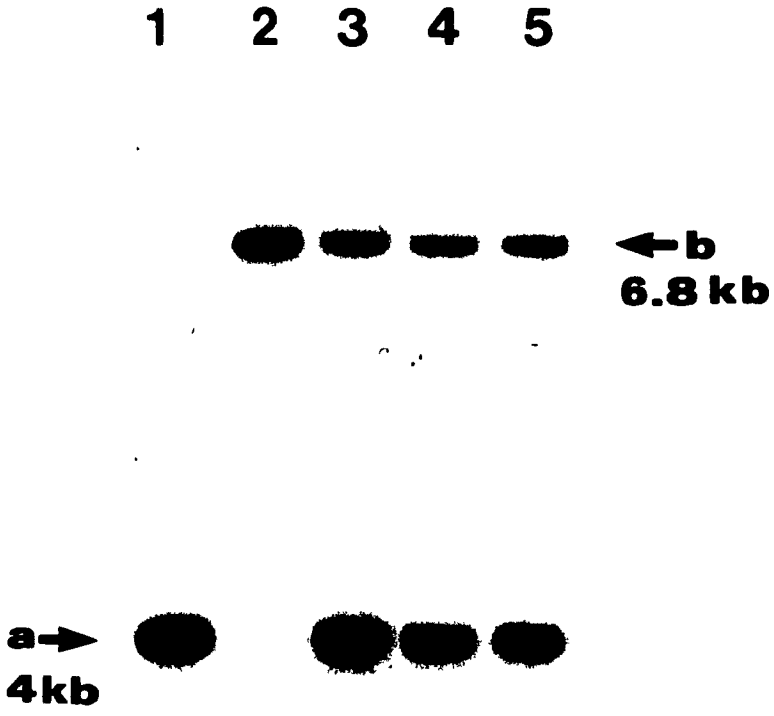
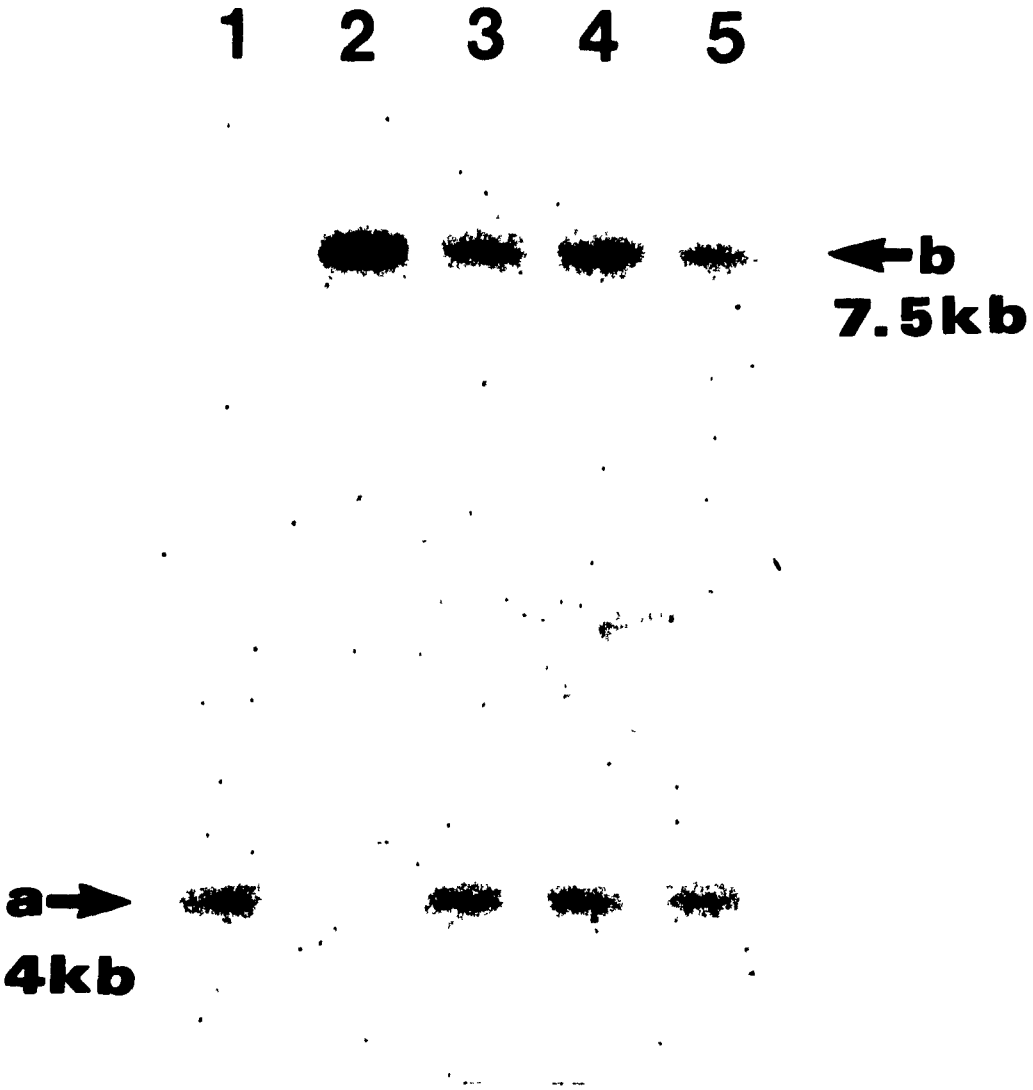
Table 1. RT1A genotypes of offspring

	(Lew × BB) crosses RT1A					(Buf × BB) crosses RT1A				
	DM	total	uu	ul	ll	DM	total	uu	ub	bb
From mating pairs producing homozygous DM	22	140	95	33	12	183	561	520	30	11
From mating pairs producing heterozygous DM	7	15	4	9	2	38	180	18	128	34
Minus one litter having both		5	3	2	0	–	–	–	–	–
Totals	29	150	96	40	14	221	741	538	158	45

FIGURE 1. Southern blot analyses of DNA digested with BglII and probed with class II MHC probes for I-A alpha (panel A), I-A beta (panel B) and I-E beta (panel C). Lane 1 in each panel contains DNA from a BB rat, lane 2 from a Buffalo rat and lanes 3-5 contain DNA from three diabetic animals serotyping as RT1.$A^{u/b}$. In each instance the arrow "a" denotes a band unique to the BB rat whereas arrow "b" denotes a band unique to the Buffalo strain. Each of the three diabetic offspring showed two bands co-migrating with the parental bands as expected for a heterozygote animal. They did not show any novel bands nor were there any bands that might indicate a recombinant event.


1 2 3 4 5
b
6.8 kb
a
4kb


1 2 3 4 5
b
7.5kb
a
4kb

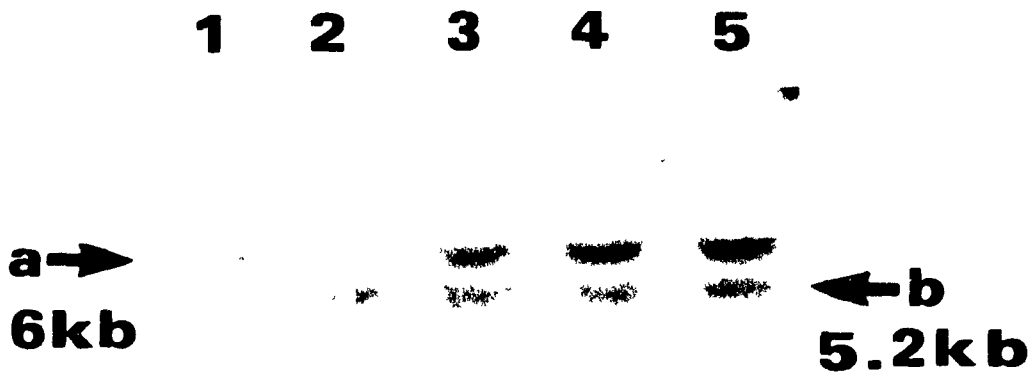
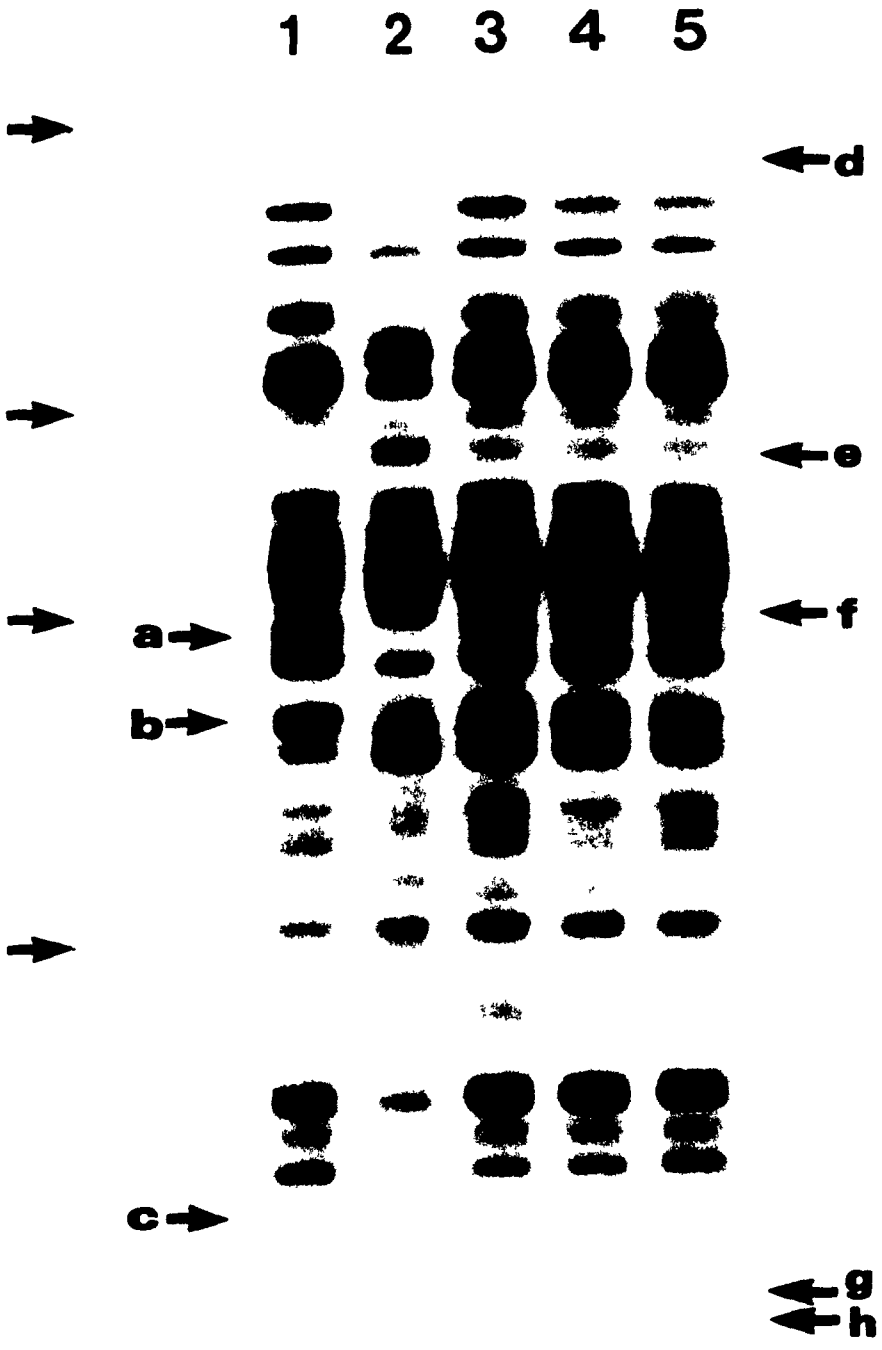

1 2 3 4 5
a
6kb
b
5.2kb

FIGURE 2. Southern blot analyses of DNA digested with BglII and probed with a class I MHC gene probe. Lane 1 in each panel contains DNA from a BB rat, lane 2 from a Buffalo rat and lanes 3-5 contain DNA from three diabetic animals serotyping as RT1.$A^{u/b}$. There are a large number of hybridizing bands as expected for a class I probe which detects class I genes flanking the class II region of the rat MHC. Arrows labeled a-c indicate bands unique to the BB genome (lane 1) whereas d-h indicate bands unique to the Buffalo rat (lane 2). The serologic heterozygote diabetic offspring in lanes 3-5 demonstrate all the bands from each of the parental types and do not show novel or missing bands.


1
2
3
4
5
d
e
f
a
b
c
g
h

Having determined the penetrance of the MHC encoded susceptibility determinant(s) for IDDM in the previous chapter, we set out to map the region(s) of the MHC involved in genetic predisposition. We also set out to determine whether the susceptibility determinant(s) were unique to the u haplotype of the BB rat by asking whether u haplotypes from other disease-free strains could permit the development of IDDM when placed on the genetic background of the BB rat. In this chapter we demonstrate that the susceptibility determinants for IDDM are not found in the RT1.A locus of the MHC. We also present data that strongly suggest that u haplotypes from other disease-free strains can support the development of IDDM in the context of the BB rat genetic background. These findings support the hypothesis that the susceptibility determinant(s) in the BB rat MHC are not unique to that strain. A second finding of this study involves the issue of an IDDM resistance gene. Previous breeding studies between the BB rat and the seroidentical, disease-free Wistar Furth rat demonstrated that the incidence of diabetes was not significantly higher than in crosses between the BB rat and non-RT1u strains. These results suggested that an IDDM resistance gene might reside within the Wistar Furth MHC. Molecular genotyping allowed the identification of diabetic animals homozygous for the Wistar Furth MHC, suggesting that the resistance gene is located elsewhere in the Wistar Furth genome.

CHAPTER THREE.

SUSCEPTIBILITY AND RESISTANCE GENES TO INSULIN-DEPENDENT DIABETES MELLITUS IN THE BB RAT

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ABSTRACT

Insulin-dependent diabetes mellitus (IDDM, type I) is an autoimmune disorder exhibiting a strong association with particular haplotypes of the major histocompatibility complex (MHC). We have previously shown that the u haplotype of the rat MHC (RT1) is absolutely required for expression of IDDM in the BB rat model of the disease. To define the precise regions of the RT1 contributing to disease occurence and to address the mechanism by which the associated haplotype participates in disease pathogenesis, we have transferred recombinant haplotypes bearing the IDDM-associated MHC in defined regions onto the BB rat genetic background. In this report, we present data from two breeding studies utilizing the r8 haplotype ($RT1.A^{a}B^{u}D^{u}E^{u}C^{u}$) that demonstrate that 1) the RT1.A locus is not involved in the disease association, 2) the MHC genes determining disease susceptibility are not unique to the BB rat, and 3) IDDM resistance genes are found outside the MHC. We also present evidence that the immunoregulatory defect characteristic of BB rats enhances the incidence of IDDM although it is not absolutely required for disease expression. We were able to track the transmission of the recombinant haplotypes in diabetic progeny using a combination of monospecific alloantisera and restriction fragment length analysis using locus-specific MHC gene probes.

INTRODUCTION

The major histocompatibility complex (MHC) is a tandemly organized multigene member of the immunoglobulin gene superfamily that codes for cell-surface, heterodimeric glycoproteins that play a major role in antigen presentation and cell-cell interaction (1-3). The class I and II genes of the MHC code for the polypeptide chains (save the beta2 -microglobulin chain of the class I antigen) that compose the class I and II histocompatibility antigens. Class I antigens are expressed on most cells of the body albeit in varying degrees while class II antigen expression is normally restricted to cells of the immune system. Both classes of MHC antigens function in the recognition of foreign antigens by the immune system. In this capacity, class I antigens restrict antigen recognition by $CD8^+$ lymphocytes while class II antigens restrict $CD4^+$ lymphocyte recognition. Although there is a high degree of sequence homology between counterpart genes in the MHCs of different species, the genetic organization of the MHC varies from species to species. The rat MHC (RT1) resembles the mouse MHC (H-2) in that the class II loci (RT1 B and D) are flanked by the class I RT1.A locus (centromeric) and the class I RT1.E and RT1.C loci (telomeric, 4).

Numerous autoimmune diseases exhibit strong associations

with particular haplotypes of the MHC (4,5). Insulin-dependent diabetes mellitus (IDDM, Type I diabetes) is an autoimmune disorder that results from the T-cell mediated destruction of the insulin-producing beta cells of the islets of Langerhans (6). The syndrome exhibits a polygenic mode of inheritance with the MHC contribution estimated to account for 50% of the heritability (7). In man, greater than 95% of insulin-dependent diabetics are of the HLA-DR3 and/or -DR4 serotypes (as compared to 45% of the normal population) and in the BB rat model of the disease at least one u haplotype of the RT1 is a requirement for disease expression (8,9).

There are two scenarios that might explain the MHC association of IDDM. A diabetogenic mutation might have occurred within the associated haplotype or the relevant mutation(s) might have ocurred outside the MHC, promoting disease due to a strong genetic predisposition conferred, perhaps only partially, by the associated haplotype. We have addressed this issue in the BB rat model of IDDM by using recombinant haplotypes bearing the associated u haplotype in defined regions of the MHC Specifically, the r4 ($A^uB^uD^uE/C^a$) and r8 ($A^aB^uD^uE/C^u$) haplotypes have been placed on the BB rat genetic background by breeding and the transmission of the recombinant haplotypes tracked in the resulting diabetic progeny by Southern blot hybridization with locus specific MHC probes (10-13). The u regions in these recombinant haplotypes were derived from BB remote animals. We reasoned that this

approach would provide a direct *in vivo* test to determine whether diabetogenic potential is unique to the BB rat *u* haplotype.

In this report we describe two breeding studies using the *r8* haplotype. In the first, a high disease incidence hooded diabetic rat (BB derived) was mated with a non diabetes prone PVG.r8 rat (a PVG rat congenic at the RT1 for the *r8* recombinant haplotype). F2 animals of all three possible RT1.A serotypes (a/a, u/a, and u/u) became diabetic. As previously reported, disea e parameters in these animals were identical to those in the BB rat (14). We present here the genetic structures of the MHCs from selected animals from this breeding study that clearly demonstrate the identity of the RT1.$A^{a/a}$ diabetic rat MHC to the PVG.r8 MHC and the homozygosity of the *r8* haplotype in these diabetic animals. In the second breeding study, we mated a diabetic animal homozygous for the *r8* haplotype (generated in the first breeding study) with a non-diabetes prone Wistar Furth rat. The Wistar-Furth rat bears the *u* haplotype at RT1 and is seroidentical at RT1 to the BB rat. F2 animals of all three possible RT1 A genotypes became diabetic Here we present the incidence of IDDM in the three groups of progeny and the molecular genotyping data at the RT1. The results presented in this paper as well as our findings with the *r4* haplotype (S.J. Ono, T. Seemayer, R.D. Guttmann, A. Fuks and E Colle, manuscript submitted) demonstrate that multiple BB-remote *u*

haplotypes can promote IDDM when placed on a BB rat genetic background and argues against a diabetogenic mutation within the BB rat MHC.

MATERIALS AND METHODS

BREEDING. The breeding scheme followed to produce the diabetic rats homozygous for the r8 haplotype has been described (14). Briefly, they were the result of a cross between male hooded diabetic rats (RT1.$A^uB^uD^u$) from the Ottawa colony and females of the PVG.r8 strain obtained from Drs. G.W. Butcher and J.W. Howard, Cambridge, U.K. (11). This strain is ($A^aB^uD^u$). Diabetic rats homozygous for the r8 haplotype were bred with r8 homozygous littermates to establish a line of r8 diabetic rats. We used diabetic males homozygous for the r8 haplotype to mate with a female Wistar Furth (WF, Microbiological Associates, Walkersville, MD) The resulting 10 F1 rats were mated and produced 94 F2 animals Rats were housed three to a cage and were weighed twice weekly. Animals which failed to gain weight between weighings were tested for glucosuria. Blood glucose of 16.6 mmol/l confirmed the presence of insulin-dependent diabetes mellitus (IDDM).

ANTIGEN TYPING. Animals were typed for antigens at the RT1.A locus by serotyping using monospecific alloantisera as previously described (14). Animals with the r8 haplotype typed a at the A locus; animals with the WF haplotype typed u at the A locus. In addition, we bled all rats at 70 to 80

days of age and determined the total white count in a Coulter counter. Finally we used 5 x 10^6 peripheral blood lymphocytes to test responsiveness to 2.5 ug concanavalin A (conA) as previously described (14).

STATISTICAL ANALYSIS. Data on total white counts and conA responsiveness were analyzed using one-way analysis of variance (SPSS, Inc. Chicago, IL).

NUCLEIC ACID PROBES. For RFLP analysis of class I genes we used a 248 bp PstI fragment from the class I Balb/c cDNA pH2IIa as hybridization probe. A 6 kb EcoRI fragment from cosmid clone 41.1 was used to detect I-A beta chain homolog and a 1.2 kb BamHI fragment from cosmid clone 8.4 to detect the I-E beta chain homolog. Sheared, denatured rat genomic DNA was added to the prehybridization solution (at a concentration of 5 ug/ml) prior to hybridization with the beta-chain probes to mask repetitive elements in the blotted rat DNA that cross-hybridize with repetitive elements in the beta-chain probes. All of these probes were the kind gift of L. Hood, California Institute of Technology, Pasedena, CA (12,13).

DNA EXTRACTION AND SOUTHERN BLOTTING. High molecular weight DNA was extracted from spleens removed from ether-anesthesized animals. Spleens were first minced and then forced through a

wire-sieve using a glass plunger. The resulting cell suspension was then diluted in 1 ml phosphate buffered saline and 10 volumes of 0.5 M EDTA (pH 8.0), 100 ug/ml proteinase K, and 0.5% Sarcosyl. This suspension was then lysed overnight in a 55 C water bath. Nucleic acid was then extracted from proteins by three phenol extractions (equal volume phenol), two phenol/chloroform/isoamyl alcohol extractions, and two chloroform extractions The nucleic acid was then dialyzed against 4 liters of 50 mM Tris-HCl (pH 8.0), 10 mM EDTA and 10 mM NaCl with several changes. RNA was then removed by treating the nucleic acid preparation with 100 ug/ml RNase at 37 C for 3 hours followed by a phenol/chloroform extraction The DNA was then equilibrated by extensive dialysis in 10 mM Tris-HCl (pH 8.0), 1mM EDTA. For Southern blotting, 20 ug of DNA was digested to completion by two incubations with the restriction endonuclease BglII at a concentration of 3 units/ug input DNA. The digested DNA was then concentrated by ethanol precipitation using ammonium acetate and resolved on 0.8% agarose gels for 24 hours at 40V in 0.4 M Tris-acetate buffer. After ethidium bromide staining and photography, the separated DNA was transferred to Zeta-probe membranes (Biorad, Rockville Center, New York) by Southern blotting The blots were then preannealed for 16 hours and then hybridized for 20 hours at 47 C in a solution containing 50% formamide, 5X sodium chloride sodium citrate (SSC), 0.004% Ficoll, 0.004% bovine serum albumin, 0 004% polyvinylpyrrolidone, 20 mM $NaPO_4$

buffer (pH 6.5), 0.2% denatured salmon sperm DNA/ml, 0.1% sodium dodecyl sulfate (SDS), and 10% dextran sulfate. The filters were then washed 2X 15 minutes in 2X SSC at room temperature, 2X 30 minutes in 2X SSC, 1% SDS at 65 C, and 2X 1 hour in 0.1X SSC, 0.5% SDS at 55 C. The filters were then exposed to Kodak XK-1 film with DuPont Lightning Plus intensifying screens at -80 C for 10 to 48 hours.

RESULTS

GENETIC STRUCTURE AND TRANSMISSION OF CLASS I GENES. We examined the genetic structures of the class I genes of selected animals from the PVG.r8 x Hooded cross (Figure 1). Hybridization of BglII digested high molecular weight DNA from these animals with the class I cDNA probe pH2IIa identifies approximately 19 to 27 restriction fragments bearing a high degree of homology to class I genes Only 3 bands differentiate the class I genetic structure of the Hooded rat from that of the PVG.r8 rat (89% identity). On the other hand, at least 12 fragments differ between the ACI ($RT1^a$) and PVG.r8 class I genetic structures (55% identity, although this figure is artificially high due to multiple bands indistinguishable by size although likely distinct by sequence) shared between the u and a haplotypes) All three distinguishing bands are present in diabetic animals serotyping $RT1.A^{a/u}$ (lane 4). Band a (approximately 7 kb) is present in the Hooded, WF and $RT1.A^{u/u}$ serotyping animals but absent in the PVG.r8 or $RT1.A^{a/a}$ serotyping animals Therefore, the loss of this fragment is associated with the .r8 recombinant event and is a valuable marker for the RT1 A locus. Bands B (approximately 8 kb) and c (approximately 5 5 kb) are present in the PVG r8, ACI, and $RT1.A^{a/a}$ or $^{a/u}$ serotyping animals. These fragments, then, map to the $RT1.A^a$ locus. The conservation of these differences and the lack of

evidence for a recombinant event during the breeding (no new polymorphisms) in RT1.$A^{a/a}$ diabetic F2 animals (lane 2) demonstrates the successful transmission and homozygosity of the r8 haplotype in diabetic animals produced in the PVG.r8 x Hooded cross. Figure 2 shows the class I genetic structures of selected animals from the RT1.$r^{a/r8}$ x WF cross. The class I RFLP patterns of the WF and Hooded rats are identical. As a result, the same 3 restriction fragments distinguish between the WF and RT1.r8 class I genetic structures. Notably, band a is present and bands b and c absent in the class 1 RFLP patterns of two diabetic F2 progeny that serotype RT1.$A^{u/u}$ (lanes 3 and 4). These data demonstrate that these diabetic progeny are homozygous for the WF RT1.

ANALYSIS OF CLASS II GENES. Hybridization of BglII digested genomic DNA from selected animals from the two crosses with the I-A beta chain probes (Figure 3) detects a 4 kb fragment in all of the breeding study animals (Hooded, PVG.r8, RT1.$A^{a/u}$ diabetic, RT1.$r^{a/r8}$ diabetic, WF and F2 diabetic) and a 13 kb fragment in the ACI animals. Hybridization of these same DNA preparations with the I-E beta chain probe (Figure 4) detects five polymorphic fragments in toto. One of these, an 8.5 kb fragment is common to all the animals. Two, at 6 kb and 5.1 kb are distinct for the breeding study animals while the remaining two at 16.5 kb and 4.3 kb are distinct for the DNA from the ACI rat. In our experience, the 5.1 kb fragment

exhibits weaker homology to the hybridization probe as compared to the other fragments as it does not persist after high stringency washes. The results from figures 3 and 4 demonstrate that all the breeding study animals bear the PuuPu haplotype at RT1.B beta and RT1.D beta and show no evidence for a recombinational event introduced during the breeding. We have previously determined that the r8 haplotype bears an intact RT1.B^{u} alpha chain and that there is no evidence for recombination at that locus in F2 RT1.A$^{u/u}$ diabetic progeny from the PVG.r8 x Hooded cross (S.J. Ono, unpublished observations). In summary, the class I and II RFLP analysis of the genetic structures of selected animals from these two breeding studies demonstrate the successful transmission and homozygosity of 1) the r8 haplotype in the RT1.A$^{u/u}$ diabetic animals in the PVG.r8 x Hooded cross and 2) of the RT1^{u} haplotype in the RT1.A$^{u/u}$ diabetic animals in the R11.A$^{r8/r8}$ x WF cross.

PHENOTYPIC CHARACTERISTICS OF THE BREEDING DATA. Detailed phenotypic analysis of the PVG.r8 x Hooded breeding study has been previously reported (14). For the RT1.$^{r8/r8}$ x WF breeding study, the distribution of RT1 A locus genotypes amongst the 94 F2 rats did not differ from the expected 1.2:1 ratio (30 u/u, 40 u/a, 24 a/a). Diabetes developed in 7 of 94 F2 animals (7.2%). Three of the diabetic animals typed u/u at the RT1.A locus.

DISCUSSION

We and others have previously demonstrated that at least one u haplotype at the BB rat MHC is an absolute requirement for the development of spontaneous IDDM in the BB rat (8,9). To determine what regions of the associated MHC contribute to disease susceptibility and to address the mechanism by which the associated haplotype contributes to disease pathogenesis, we have performed breeding studies that place recombinant haplotypes (r4 and r8) onto the BB rat genetic background. These recombinant haplotypes bear $RT1^u$ sequences derived from BB-remote animals (10,11).

We have previously reported the RT1.A serotyping of the F2 and backcross progeny from the mating of a PVG.r8 rat to a Hooded rat (BB-derived, 14). The presence of $RT1.A^{a/a}$ diabetic progeny demonstrated that the RT1.A locus is not involved in the IDDM association to the u haplotype. In this study, we present the class I and II genetic structures of the PVG.r8 and Hooded rat MHCs and demonstrate the identity of the MHC genetic structures from the PVG.r8 and $RT1.A^{a/a}$ diabetic animals. Analysis of the class II genes of the PVG.r8 rat and the $RT1.A^{a/a}$ diabetic progeny from this mating demonstrate that the R8 recombination occurred between RT1.A and RT1.B beta. The lack of evidence for a second recombination in the $RT1.A^{a/a}$ diabetic progeny with any of the MHC probes used in this study demonstrates the successful transmission and

Table I compares the total white counts and the conA responsiveness between the WF x PVG r8 cross and the R8 x Hooded diabetic cross. The white counts were higher in the (WF x R8) F2 non-diabetic animals ($p<.001$) and this was reflected in the slightly higher white counts amongst the diabetic rats resulting from this cross although this latter difference was not statistically significant. The responsiveness to conA stimulation was not different in the 2 groups (Table 2).

homozygosity of the r8 haplotype in these animals.

In the second breeding study presented in this paper (where we mated an RT1.r8 diabetic animal with a non-diabetes prone WF rat) we identified diabetic progeny whose class I and II genetic structures were identical to those of the parental WF rat. Thus, we demonstrate that two independent BB-remote $RT1^u$ contributions can permit IDDM on a BB background. Recently, we have demonstrated that a third BB-remote $RT1^u$ (r4) is also permissive for IDDM when placed on a BB background (S.J. Ono, T. Seemayer, R.D. Guttmann. A. Fuks and E. Colle, manuscript submitted). These results indicate that the IDDM susceptibility genes in $RT1^u$ map between RT1.A and RT1.C but are not unique to the BB rat. These results correspond to those of Todd and coworkers who have found that although susceptibility to IDDM in maps to HLA-DQ beta, no unique class II sequences are found exclusively in IDDM patients (15). Thus, in rat and man IDDM is not due to mutant MHC class II genes but is likely restricted to certain wild-type class II alleles. In contrast, the NOD mouse appears to have a unique I-A beta subsitution at position 57 not found in any other published I-A beta sequence (15,16).

In previous crosses where WF rats have been mated with BB rats, we have noted that the incidence of diabetes in the progeny is only slightly higher than in litters from crosses between BB and non-$RT1^u$ animals (17). This suggested the possibility of an IDDM-resistance gene within the MHC. The

identification of diabetic animals homozygous for the WF derived $RT1^u$ in the second breeding study suggest to us that the putative WF IDDM-resistance genes are located outside the MHC.

We have previously reported that BB rats and diabetic rats derived from them have an immunoregulatory defect characterized by decreased number of total white cells, decreased total blood lymphocytes, decreased numbers of peripheral blood T lymphocytes, decreased responsiveness of peripheral blood lymphocytes to conA stimulation, decreased mitogenic responses in mixed lymphocyte reaction, a decreased ability to reject skin grafts and depleted populations of lymphocytes marked by the monoclonal antibodies OX19 and those directed against RTϵ, (18). Although the presence of this immunoregulatory defect is important in increasing the incidence of disease, Like has reported the occurrence of spontaneous IDDM in non-lymphopenic rats with normal mitogenic responses in animals of the BB resistant lines (19) In the F2 studies which we have done, we usually note 1 or 2 diabetic animals with T lymphocytes or conA responses in the normal range. This is true in the present study where 1 animal with a total white count of 7000 and a conA response of 43683 cpm developed IDDM at 79 days of age. However, 6/7 diabetic rats had conA responses of less than 18000 cpm. Thus the presence of an immunoregulatory defect greatly enhances the incidence of disease. It is pointed out that, as in other F2 studies,

there are many animals (19/94) which have low conA responses but which do not develop disease, suggesting the presence of other genetic and/or environmental factors.

In summary, we conclude that IDDM susceptibility gene(s) map between RT1.A and RT1.C and that these genes are not unique to the BB rat. The ability of the WF MHC to permit IDDM further supports this conclusion and suggests that IDDM-resistance gene(s) are found outside the MHC. Finally, the immunoregulatory defect characteristic of BB-derived animals, although not absolutely necessary for expression of IDDM, enhances the incidence of the syndrome.

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Table I

COMPARISON OF PHENOTYPIC CHARACTERISTICS IN TWO CROSSES

Cross	WF x RT1.r8* (n=94) x±sem	PVG.r8 x Hooded* (n=82) x±sem	
WBC all F2 cells/mm^3	11863±394	6561±249	p=<.001
WBC IDDM cells/mm^3	7009±699	5655±1377	ns
conA all F2 cpm	52403±3804	64146±5628	ns
conA IDDM cpm	7124±6098	12603±5306	ns
age at onset IDDM days	90.8±6.8	101.2±7.3	ns
Male:Female	4/3	4/3	

Table II

PHENOTYPIC CHARACTERISTICS OF IMMUNOREGULATORY DEFECT WF X RT1.R8 DIABETIC

ConA Response	IDDM+	IDDM-	Total	%Total
<18000 cpm	6	19	25	26%
>18000 cpm	1	68	69	74%
Total WBC				
<7000 cells/mm^3	4	10	14	14%
>7000 cells/mm^3	3	77	80	86%

FIGURE 1. Breeding Study I. Southern blot of the genetic structures of class I MHC genes in DNA extracted from the Hooded (lane 1) and PVG.r8 (lane 2) animals. Lanes 3 and 4 were loaded with DNA extracted from diabetic progeny from the mating of the Hooded and PVG.r8 animals Lane 5 was loaded with DNA extracted from an ACI rat. Band a is specific for the intact $RT1^u$ haplotype genetic structure (a faint band at that position appears in incompletely digested DNA from the PVG.r8 rat but does not persist after extended digestion, e.g. Figure 2 lane 2). Bands b and c are specific for the .r8 haplotype. The animal in lane 3 serotyped $RT1.A^{a/a}$ and the animal in lane 4 $RT1.A^{u/a}$. The arrows on the far left are molecular weight markers corresponding to the fragments of HindIII digested lambda DNA. The genomic DNA samples were digested with BglII and hybridized with random hexamer primed pH2IIa probe at a specific activity of 1 x 10^8 cpm/ml.

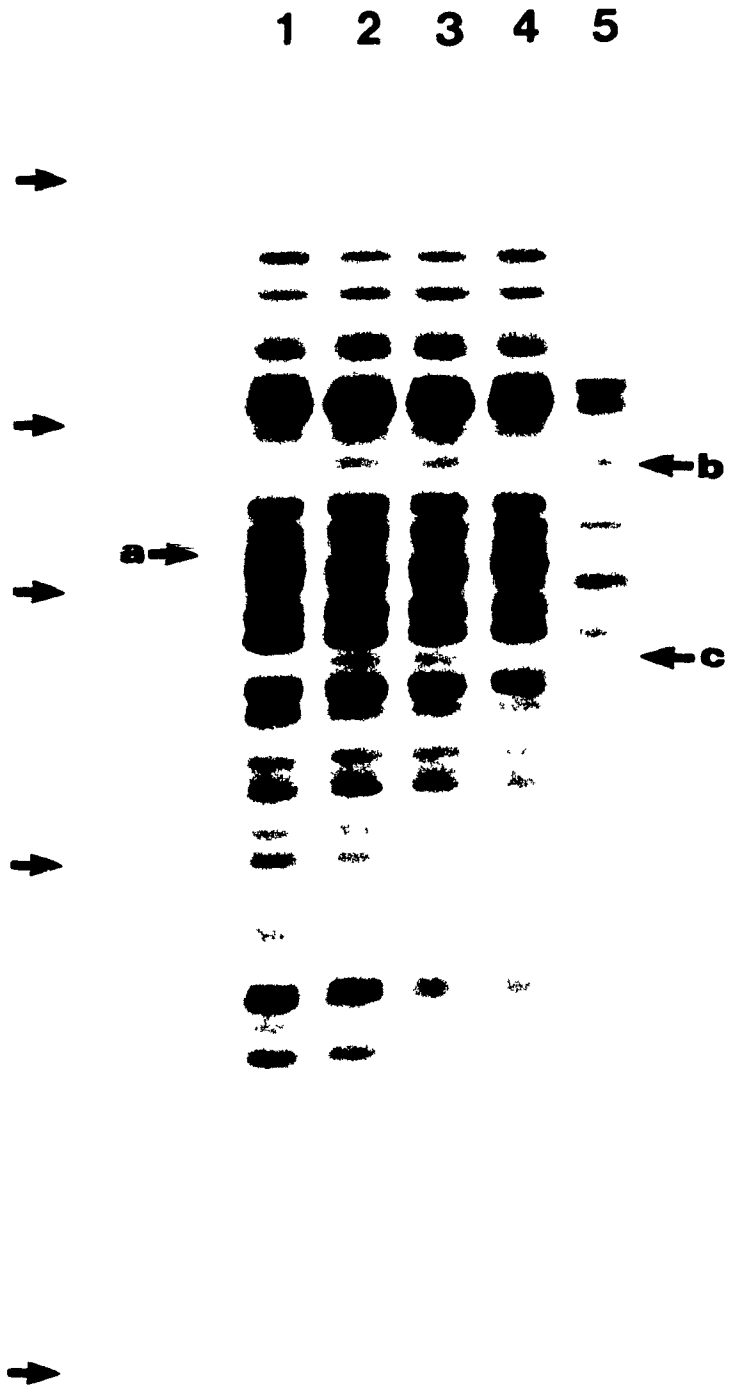

1
2
3
4
5
a
b
c

FIGURE 2. Breeding Study II. Southern blot of the genetic structures of class I MHC genes in DNA extracted from a WF (lane 1) and an RT1.$A^{a/a}$ diabetic rat (lane 2). Lanes 3 and 4 were loaded with DNA extracted from diabetic progeny from the mating of the RT1.$A^{a/a}$ diabetic rat and the WF rat. The animals in lanes 3 and 4 serotype RT1.$A^{u/u}$. Lane 5 was loaded with DNA extracted from an ACI rat Band a is distinct for the $RT1^u$ haplotype, and bands b and c for the .r8 haplotype. The enzyme and hybridization probe were as in Figure 1.

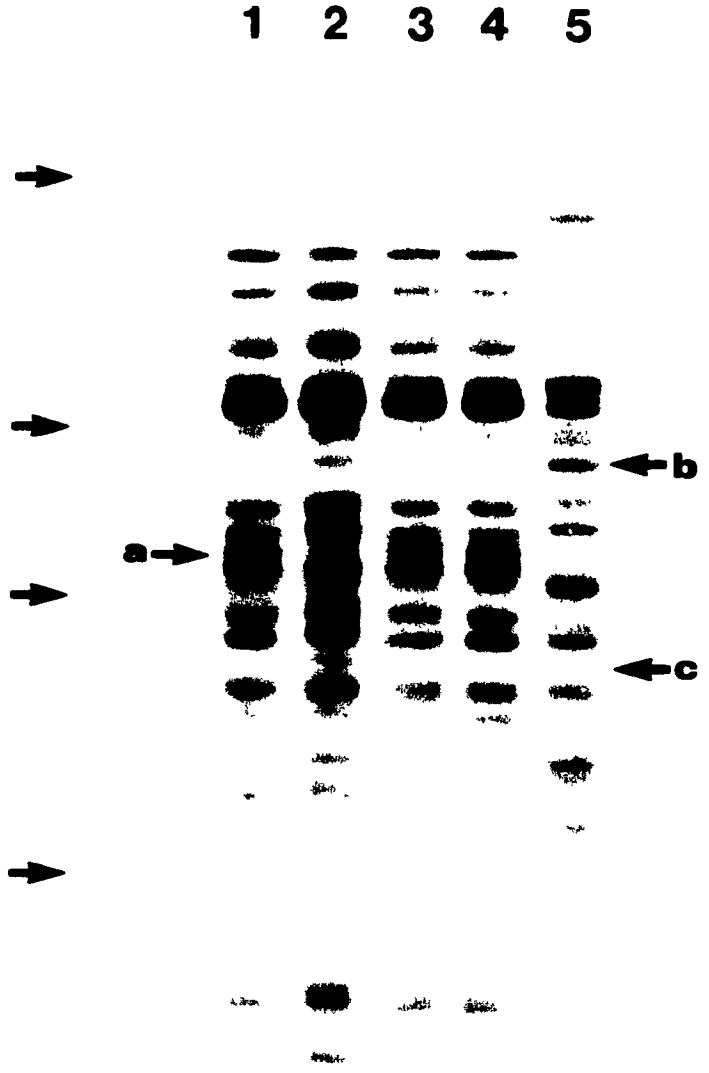

1
2
3
4
5
a
b
c

FIGURE 3. Blot hybridization of the I-A beta chain homolog in DNA from animals from the two breeding studies. Lanes 1-5 correspond to lanes 1-5 in Figure 1, and lanes 6-10 correspond to lanes 1-5 in Figure 2. The restriction endonuclease is BglII and the random hexamer labeled 41.1 fragment. The 4 kb band is shared among the breeding partners and their progeny and is distinct from the 13 kb ACI fragment.

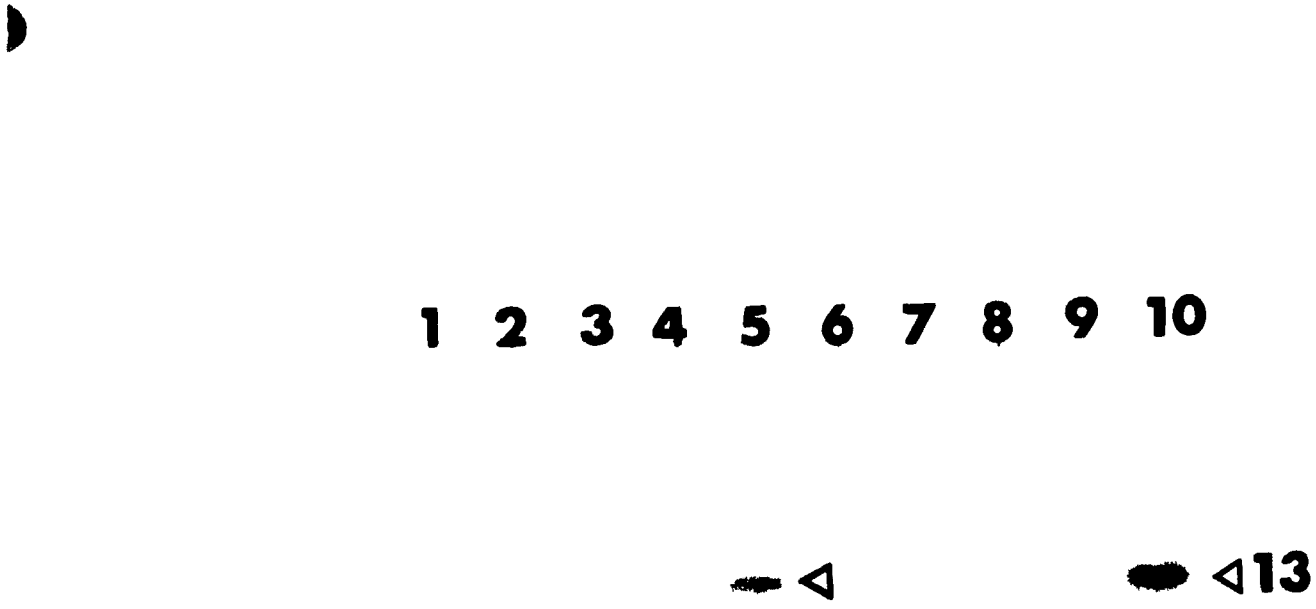
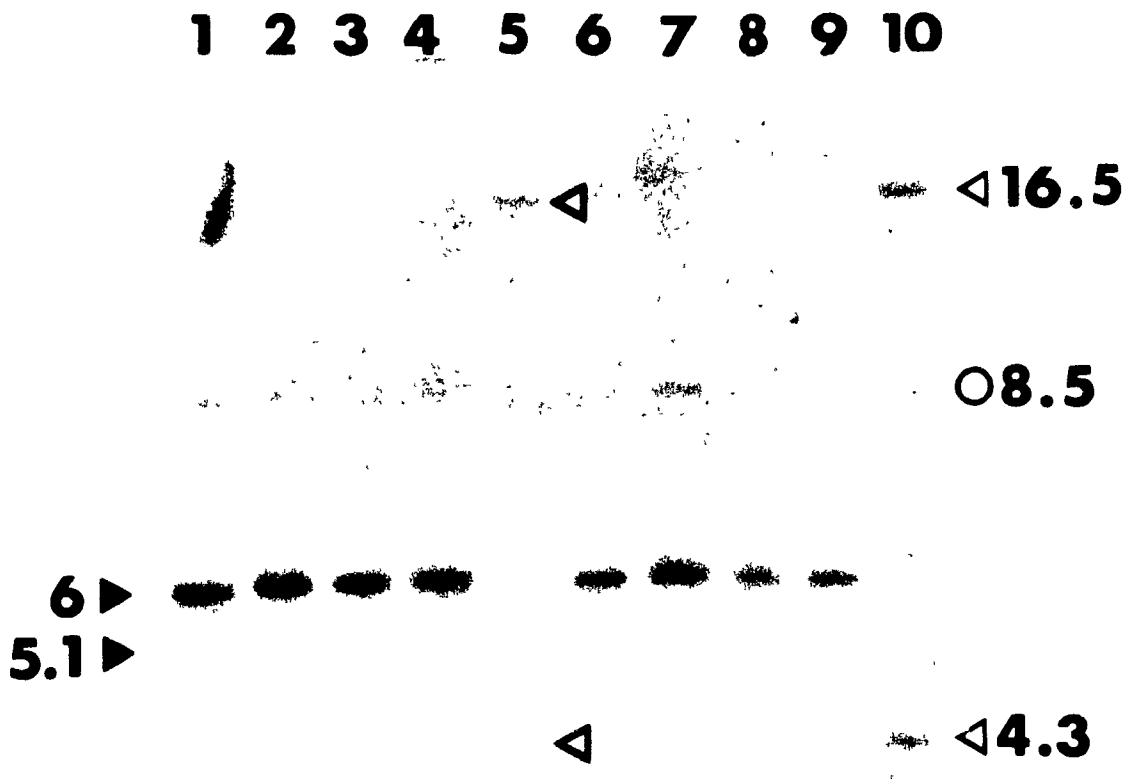

1 2 3 4 5 6 7 8 9 10
13
4

FIGURE 4. Blot hybridization of the I-E beta chain homolog in DNA extracted from animals from the two breeding studies. Lane assignments are as in Figure 3. The restriction endonuclease is Bgl II and the hybridization probe the random hexamer labeled 8.4 fragment. The 6.0 and 5.1 kb fragments are shared among the breeding partners and their progeny. The 5.1 kb fragment shares less homology with the hybridization probe than the 6.0 kb fragment as it does not persist after high stringency washes (data not shown). An 8.5 kb non polymorphic fragment is shared in all these animals The 16.5 and 4.3 kb fragments are specific for the ACI rat.


1 2 3 4 5 6 7 8 9 10
16.5
8.5
6
5.1
4.3

In the previous chapter we determined that the RT1.A locus is not involved in genetic predisposition for IDDM. In this chapter we demonstrate that the RT1.C locus is also not required for disease onset. Taken together, these two chapters indicate that the susceptibility determinants for IDDM reside within the immune response (class II) region of the rat MHC. In addition, this study identified a third source for a u haplotype from a disease-free strain that could support the development of the syndrome. The mapping of disease association to the immune response region was critical as the disease maps to the HLA-D region in man and the I-A locus in the NOD mouse. The consistent mapping of susceptibility determinant(s) for IDDM to the immune response region in different species strongly suggests a shared and critical component for the pathogenesis of IDDM. One striking feature of the diabetic progeny lacking the u haplotype at the RT1 C locus was the rare occurence of insulitis (infiltration of the pancreatic islets by inflammatory cells), a hallmark in the pathology of IDDM. These experiments, then, map a gene or genes required for insulitis to the region corresponding to the Qa/Tla region of the mouse MHC (H-2) or in linkage disequilibrium with that region They also suggest that insulitis may not be a necessary requirement for IDDM, prompting a second look at existing hypotheses on the pathogenesis of IDDM.

CHAPTER FOUR.

ASSOCIATION OF SUSCEPTIBILITY TO SPONTANEOUS DIABETES MELLITUS IN THE RAT WITH GENES OF THE MAJOR HISTOCOMPATIBILITY COMPLEX

This chapter has been published in Diabetes, 37:1438, 1988.

ABSTRACT

The present study was designed to map the diabetes susceptibility gene(s) associated with the rat major histocompatibility complex (MHC), RT1. We have crossed spontaneously diabetic male rats bearing the recombinant $RT1^{r8}$ haplotype with female rats of the ACI 1^{r4} congenic strain. Three diabetic rats were determined to be homozygous for the r4 haplotype by serotyping. The absence of recombination within the MHC was determined by analysis of restriction fragment length patterns of the diabetic animals and the parental strains. This study, in conjunction with previous breeding studies, maps the diabetes susceptibility gene(s) to the right of the RT1.A locus and to the left of the RT1 C locus. A low incidence of diabetes in the F2 (4.5%) emphasized the multifactorial nature of the susceptibility The presence of depressed responsiveness of peripheral blood lymphocytes to concanavalin A stimulation increases the prevalence of the overt disease. An unusual feature of the diabetic syndrome in this study is the sparse or absent pancreatic lymphocytic inflammatory response with true insulitis being a rare finding.

INTRODUCTION

The major histocompatibility complex (MHC) is a group of closely linked loci that code for cell surface glycoproteins that play a major role in the specificity of antigen recognition. Class I antigens, 44,000-M_r membrane-bound glycoproteins bound by noncovalent linkages to beta$_2$-microglobulin molecules encoded on a different chromosome, are present on most cells of the body. These molecules restrict antigen recognition predominantly by cytotoxic T cells. Class II antigens, consisting of two membrane-bound glycoproteins, and alpha-chain of 31,000-34,000 M_r and a beta-chain of 26,000-29,000 M_r, are present on B lymphocytes and other cells of the immune system that present antigen to regulatory T cells. Class II products restrict antigen recognition predominantly by regulatory T cells. Both class I and class II products are codominantly expressed.

There is considerable homology of the gene products of the MHC region between species. However, the organization of the complex is not always the same. The organization of the rat MHC, designated RT1, is similar to that of the mouse in that the class II loci (B and D) are flanked by the class I A locus and the class I E locus. A third region of Class I antigens, the C locus, is separated from the E locus by a region which governs some aspects of growth and development in

the rat. This C locus may be homologous with the Qa/Tla region of the mouse The A locus is identified by serotyping, the E/C loci have been identified by graft rejection and weak alloantigenic reactions. The RT1 B locus is homologous with the human HLA-DQ region and the mouse I-A region The RT1.D locus is homologous to the human HLA-DR region and the mouse I-E region (1).

Previous breeding studies by ourselves (2) and others (3) have shown that the presence of at least one u haplotype of the rat RT1 region is a necessary, though not sufficient, condition for the development of the spontaneous insulin dependent diabetic syndrome. Using rats bearing the $RT1^{r^a}$ ($A^aB^uD^u$) haplotype we have previously shown that the u allele of the class I A locus is not necessary for susceptibility to the development of spontaneous diabetes as long as the genes to the right of the A locus code for the permissive u alleles (4). In the present study, we have crossed these diabetic rats which are homozygous for the $RT1^{r^a}$ haplotype with rats bearing another RT1 recombinant haplotype, the $RT1^{r^4}$ ($RT1.A^uB^uD^uC^a$) haplotype, to determine if the diabetic susceptibility gene(s) map to the left of the C region of the RT1. This appears to be the case since homozygous r4 animals were found among the diabetics. The animals resulting from this cross are of future interest because, despite the presence of the immunoregulatory defect seen in other diabetes prone animals, the pancreas prior to and at the onset of

diabetes is almost devoid of lymphocytic infiltration and frank insulitis is a rare finding.

MATERIALS AND METHODS

BREEDING. The breeding scheme used to produce the diabetic rats homozygous for the RT1^{r8} haplotype has been described (4). Briefly, they were the result of a cross between male Hooded diabetic rats (RT1.A^{u}B^{u}D^{u}) from the Ottawa colony and females of the PVG.r8 (A^{u}B^{u}D^{u}) strain obtained from Drs G W Butcher and J.C. Howard, Cambridge University, U K (5) Diabetic rats homozygous for the r4 haplotype were bred with r8 homozygous littermates to establish a line of RT1^{r8} diabetic rats. We used diabetic males homozygous for this r8 haplotype to mate with a female AC1.1^{r4} (RT1.A^{u}B^{u}D^{u}C^{r4}, 6). Eight F1 animals were intercrossed to produce 175 F2 animals In addition, an F1 female was backcrossed to a diabetic male homozygous for the r8 haplotype to produce the first BC generation (BC1). The single heterozygous (r4/r8) diabetic male from BC1 was then intercrossed with 2 female heterozygous littermates to produce the first intercross of the backcross (BCIC1). Finally, a heterozygous (r4/r8) diabetic male of the F2 was mated with a homozygous littermate (r4/r4) to produce two litters of F3 animals.

Rats were housed thre to a cage and were weighed twice weekly. Animals which failed to gain weight between weighings were tested for glucosuria. Blood glucose values of >16 6 mmol/l confirmed the presence of insulin-dependent diabetes

mmol/l confirmed the presence of insulin-dependent diabetes mellitus (IDDM) Diabetic animals were treated with protamine zinc insulin. Animals were killed at 160 days.

ANTIGEN TYPING. Animals were typed for antigens at the RT1.A locus by serotyping using monospecific alloantisera as previously decribed (7). Animals with the $RT1^{r8}$ haplotype typed a at the A locus; animals with the $RT1^{r4}$ haplotype typed u at the A locus. In addition, we bled all rats at 70 to 80 days of age and determined the total white count in a Coulter counter Finally, we used 5 x 10^6 peripheral blood lymphocytes to test responsiveness to 2.5 ug concanavalin A (conA), as previously described (4).

PATHOLOGICAL ANALYSIS. Pancreatic tissue was taken either at the time of autopsy at 160 days or by biopsy, as previously described (7). Briefly, tissue was taken from the tail of the pancreas except as noted in the text. The tissue was fixed in Bouin's solution and twelve step sections from each block were stained with hematoxylin-phloxine-saffron. Insulin and glucagon were identified by an indirect immunoperoxidase method, as previously described (8). All sections were read coded without knowledge of the clinical status of the animal.

DNA PROBES. For restriction fragment length polymorphism (RFLP) analysis of class I genes we used a 248 bp PstI

fragment from the class I Balb/c cDNA pH2IIa as hybridization probe (9). A 6 kb EcoRI fragment from cosmid clone 41 1 was used to detect the I-A beta chain homolog and a 1.2 kb BamHI fragment from cosmid clone 8.4 to detect the I-E beta chain homolog (10) Sheared, denatured rat genomic DNA was added to the prehybridization solution (at a concentration of 5 ug/ml) prior to hybridization with the beta-chain probes to mask repetitive elements in the blotted rat DNA that cross hybridize with repetitive elements in the beta-chain probes. All of these probes were the kind gift of L. Hood, California Institute of Technology, Pasadena, CA. These DNA probes derived from the mouse have previously been successfully used by us and by others (1,8) to examine the corresponding homologs in the rat.

DNA EXTRACTION AND SOUTHERN BLOTTING. High molecular weight DNA was extracted from spleens removed from ether-anesthesized animals. Spleens were first minced and then forced through a wire-sieve using a glass plunger. The resulting cell suspension was then diluted in 1 ml phosphate buffered saline and 10 volumes of 0.5 M EDTA (pH 8.0), 100 ug/ml proteinase K, and 0.5% Sarkosyl. This suspension was then lysed overnight in a 55 C water bath. Nucleic acid was then extracted from proteins by three phenol extractions (equal volume phenol), two phenol/chloroform/isoamyl alcohol extractions, and two chloroform extractions. The nucleic acid was dialyzed against

4 liters of 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 10 mM NaCl with several changes. RNA was then removed by treating the nucleic acid preparation with 100 ug/ml RNase at 37·C for 3 hours followed by a phenol/chloroform extraction. The DNA was then equilibrated by extensive dialysis in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. For Southern blotting, 20 ug of DNA was digested to completion by two incubations in the restriction endonuclease BglII at a concentration of 3 units/ug input DNA. The digested DNA was then concentrated by ethanol precipitation using ammonium acetate and resolved on 0.8% agarose gels for 24 hours at 40V in 0.4 M Tris acetate buffer. After ethidium bromide staining and photography, the separated DNA was transferred to Zeta-probe membranes (Biorad, Rockville Center, NY) by Southern blotting (11). The blots were preannealed for 16 hours and then hybridized for 20 hours at 47·C in a solution containing 50% formamide, 5 x sodium chloride/ sodium citrate (SSC), 0.04% Ficoll, 0.004% BSA, 0.004% polyvinylpyrrolidone, 20 mM $NaPO_4$ buffer (pH 6.5), 0.2% denatured salmon sperm DNA/ml, 0.1% sodium dodecyl sulfate (SDS), and 10% dextran sulfate. The filters were then washed 2 x 15 minutes in 2 x SSC at room temperature, 2 x 30 minutes in 2 x SSC, 1% SDS at 65·C, and 2 x 1 hour in 0.1 x SSC, 0.5% SDS at 55 C. The filters were exposed to Kodak XK-1 film with DuPont Lightning Plus intensifying screens at -70·C for 10 to 48 hours.

STATISTICAL ANALYSIS. Data on total white counts and conA responsiveness were analyzed using one-way analysis of variance (SPSS, Inc. Chicago, IL).

RESULTS

The distribution of genotypes in the F2 population did not differ from the expected 1:2:1 ratio (Table I). The overall incidence of diabetes in the F2 was 4.5%. The mean age of onset of diabetes was 121 days. In the F2 there was one diabetic animal homozygous for the $RT1^{r4}$ ($RT1.A^{u}$) haplotype (Table I). In addition we identified 2 additional diabetic animals, one in the intercross of the backcross and one in the F3 which typed RT1 u/u at the A locus. Restriction fragment length patterns demonstrated that these animals were identical with the ACI.1^{r4} parental strain and thus ruled out the possibility of a recombination event in these animals (Figure 1). Lane 3 is from a PVG.r8/ACi.1^{r4} diabetic heterozygote, lane 4 from a diabetic r4 homozygote. The absence of bands a, d, and e in lane 4 clearly show that this is a r4 homozygous animal. Conversely, the presence of all 10 bands in lane 3 indicate that this is a r4/r8 heterozygote. The identitiy of the patterns seen with probes hybridizing with the B and D loci (Figures 2 and 3) confirms that the r4 haplotype is indistinguishable from the r8 haplotype at the class II loci and that the recombination in the r4 haplotype maps to the right of the region coding for class II products. The patterns for these two haplotypes (r4 and r8) which are u at the class II region are clearly differentiated from the

class II a pattern of the ACI strain.

F2 rats becoming diabetic had lower total white counts (6052±1171 (SEM) vs 9289±265 cells/mm^3) and lower responses of peripheral blood lymphocytes to conA stimulation (12956±4671 vs 34886±2748 cpm) than did nondiabetic rats.

We had histologic material on 150 of the 167 non diabetic littermates. Nine animals (6%) showed pancreatic lymphocytic infiltration. When present, the infiltrate was very sparce and was often located within the acinar tissue. Tissue was available on 7 of the 8 F2 diabetics. In two animals tissue was taken within 1 week of detection of diabetes, in the remaining 5 within 2 days of detection. We saw infiltration of islets with mononuclear cells in only 2 animals biopsied on the day of detection. In the others we saw either islets which were fairly large, densely eosinophilic, and composed entirely of cells staining for glucagon but not for insulin with immunochemistry (Figure 4) or disintegrating islets with only a limited inflammatory response in the surrounding tissue. We worried that the process had already passed the tail of the pancreas, which is our usual site of tissue procurement, so we killed two animals on the day of detection of diabetes and obtained tissue from both head and tail. The histology was similar in the two regions In the BC1 diabetic, tissue was obtained 30 days after detection and showed islets containing only glucagon without inflammation. In the remaining three BCIC1 and F3 animals the histology

examined on the day of detection was similar to that seen in the F2. In one of these animals we had a biopsy taken 30 days prior to detection which was devoid of inflammatory cells (Figure 5a). Sections taken at the time of diagnosis revealed a few lymphocytes surrounding the ductules (Figure 5b) and distorted islets containing cells staining only for glucagon (Figure 5c). Thus, a true insulitis was seen in only 2 of the 10 diabetic animals in which tissue was available within one week of detection of disease.

DISCUSSION

Previous studies in the rat have indicated an association between the gene products of the rat MHC region, RT1, and the susceptibility to the development of spontaneous diabetes (2,3). We have previously shown that the genes of the A locus are not necessary for this susceptibility (4). The present study shows that genes in the C region and to the right of that region are also not required for susceptibility. The RT1C gene products which have been detected by inducing subacute or chronic skin graft rejection are biochemically similar to class I antigens (12) The genes located at RT1.C cross hybridize with mouse cDNA probes for class I genes. However, they differ from RT1.A determined class I antigens in that they have a more limited tissue distribution, appearing predominantly on B and T lymphocytes and subpopulations of macrophages (13). The RT1.C region appears to be much "longer" than RT1.A, in that about 80% of restriction fragments revealed by hybridization with class I MHC probes can be assigned to the C locus and 20% to the A locus (13). The $RT1^{r4}$, or <u>wr1</u> haplotype was described by Kohoutova et al. (6) on the basis of weak skin graft rejection. More recently, Gunther et al. have reported differences in the RFLP patterns which distinguish this haplotype from both the u and the a haplotypes (13). However, the exact site of the recombination

event is not known. Gill has estimated the distance between RT1.A and RT1.C to be 2-3 cM (1).

All of the genes encoding the class II MHC products are between the RT1.A and RT1.C regions, and thus the occurrence of IDDM in homozygous r4 rats is fully consistent with the hypothesis that the class II products confer disease susceptibility. This is agreement with studies in which the mitogenic response to islet antigens of T-cell clones isolated from the pancreata of newly diabetic rats can be abrogated by antiserum directed against the class II gene products (14) and with experiments which showed a decreased incidence of diabetes in rats treated with anti-RT1.D^u antisera (15)

This study also provides evidence that the recombination event in the $RT1^{r4}$ haplotype is, in fact, to the right of the B and D regions since no differences in restriction fragment length polymorphisms were seen when DNA from the r4 and the r8 haplotypes were compared with probes recognizing the I A beta and I-E beta chain homologs (RT1.B beta and RT1.D beta, respectively).

In addition, this study demonstrates that the region between RT1.A and RT1.C from yet another u haplotype can confer susceptibility to IDDM, since we have previously shown that not only the u haplotype from the BB rat strain, but also that from the standard inbred Wistar Furth and that from the PVG.r8 (originally derived from the AO strain) are also permissive for diabetes.

The incidence of diabetes was the same as that which we have seen in other F2 studies (4.5%). In general, the animals, both diabetic and non-diabetic, had lower conA responses of their PBL than did the animals in other F2 studies. However, the animals which became diabetic had conA responses significantly lower than those of non-diabetic animals.

An unexpected finding in this study was the minimal lymphocytic infiltration of the pancreas. In previous F2 studies we have noted infiltration of lymphocytes in the pancreas of a significant number of the non-diabetic littermates of diabetic animals. The most commonly encountered lesion is the periductular infiltrate. Even in the end-stage pancreas it is not uncommon to find, in addition to the typical shrunken islet, persistent foci of lymphocytes around ductules. We have previously reported a prevalence of this lesion of 10% in 83 F2 non-diabetics in a (Lewis x BB cross) at 120 days (2), and in 32% of 101 non-diabetic F2 in a (Buf x BB cross) at 65-90 days (7). We have unreported prevalence figures of 20% for the F2 animals in the (Hooded diabetic x PVG.r8) cross at 160 days (4). Thus the prevalence of 6% of non-diabetic F2 animals in the present study is the lowest we have seen. We recognize that the focal nature of the infiltrates makes any estimate of their prevalence a minimal one. However, since the sampling technique is the same in each study, the probability of finding such lesions in

the 12 sections which we routinely examined from each animal will be higher in those rats with the most widespread process

Because this finding was unexpected we did not have biopsies on most of these animals at an age prior to the expected age of onset. However, biopsies done on the day of detection or shortly thereafter also had an attenuated inflammatory response. We have reported that when the pancreas is examined 1-3 days post-detection mononuclear cells were documented in several islets of each animal as well as in the peri-ictal region (8). This continues to be our experience. Recent studies using BB?Ottawa rats maintained and detected according to the same protocol as the animals in this study show a true infiltrative insulitis in over 75% of cases biopsied within a few days of detection. In the r4 x r8 cross, mononuclear cells within islets were seen in only 2 of the 10 animals in which tissue was available within 1 week of detection. In the other 8 rats the biopsies contained islets which consisted either of disintegrating cells or of cells staining only for glucagon with a limited inflammatory response in the surrounding tissue. This picture is sufficiently at variance with our previous experience to seem worthy of note. We recognize that we may have missed the florid insulitis because of timing of the histologic examination. For this reason, in a few of the last litters of the study we did perform biopsies of the animals at 73 days The one rat of the biopsied group which developed diabetes had

a normal biopsy 30 days prior to detection and only a minimal infiltrate in a pancreas with degranulated islet cells at the time of destruction.

Since the pancreatic lesions were similar in animals with all three possible RT1 genotypes of this particular cross it is unlikely that genes within the B/D/E region are responsible for the attenuated inflammatory response. It is more likely that other genes in the ACI background strain are modifying the immune reaction. We have data from an F2 study of an (ACI x BB) cross (unreported because of breeding difficulties which failed to generate sufficient numbers of animals) in which only 1 of 32 F2 animals had any type of pancreatic infiltration.

These observations are clearly preliminary. Breeding studies, including the backcross of the complete diabetic phenotype onto the ACI.^{u} congenic line, which we have developed, are in progress. Furthermore, we are not suggesting that the milder inflammatory lesion means that the diabetic process in these animals is not immune mediated. In this regard the recent study of Haynes et al. (16) is of interest They demonstrated that the presence of L3T4 (helper/inducer) cells was necessary for the development of a virally induced diabetes in BALB/c mice (a model in which thymic dependent mechanisms are important) even though the histologic picture shows degranulation and necrosis of islets without lymphocytic infiltrates. Thus our findings raise the

possibility that, even though the triggering of beta cell destruction may be immune mediated, there may be strain specific differences in the degree and extent of the subsequent inflammatory response.

Thus, the data presented maps the RT1 associated diabetes susceptibility genes to an area to the right of the class I RT1.A locus and to the left of the RT1.C locus. The low incidence of diabetes in the F2 generation is similar to that seen in other studies, again emphasizing the multifactorial nature of the susceptibility to IDDM. The presence of depressed responsiveness to conA stimulation increases the prevalence of overt disease. An unusual feature of the diabetic syndrome in this study is the sparse or absent pancreatic lymphocytic inflammatory response with a true insulitis being a rare event.

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TABLE I

Genotypes of Animals

Cross		Number	RT1.A Locus		
			u/u (r4/r4)	u/a (r4/r8)	a/a (r8/r8)
F1 x F1	All F2	175	50	78	47
(u/axu/a)	IDDM F2	8	1	5	2
F1 x r8DM	All BC1	11		8	3
(u/axa/a)	DM BC1	1		1	
BC1xBC1DM	All BCIC1	34	7	18	9
(u/axu/a)	DM BCIC1	2	1	1	
F2 x F2DM	All F3	10	6	4	
(u/uxu/a)	IDDM F3	1	1		

FIGURE 1. Southern blot of the genetic structures of class I MHC genes in DNA extracted from the PVG.r8 (lane 1) and ACI.1^{r4} (lane 2) animals. Lanes 3 and 4 were loaded with DNA extracted from diabetic progeny from the mating of the PVG r8 (diabetic) and ACI.1^{r4} animals. Lane 5 was loaded with DNA extracted from an ACI rat. The bands labeled a-e are specific for the r8 class I MHC genetic structure and those labeled f-j are specific for the r4 class I MHC restriction fragment pattern. The arrows on the far left are molecular weight markers corresponding to the fragments of HindIII digested lambda DNA The DNA was digested with BglII and hybridized with random hexamer primed pH2IIa probe with a specific activity of 1 x 10^6 cpm/ml

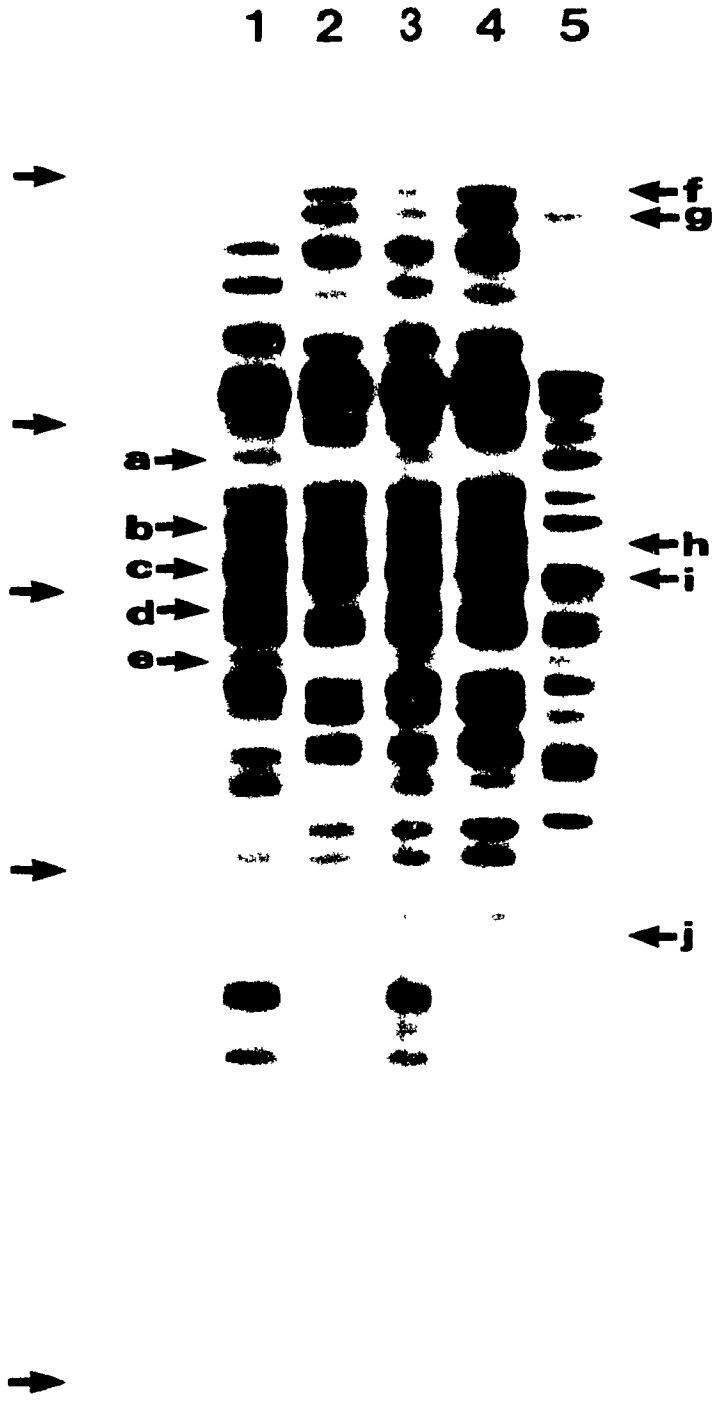

1
2
3
4
5
a
b
c
d
e
f
g
h
i
j

FIGURE 2. Blot hybridization of the I-A beta chain homolog in DNA from animals from the ACI.1^{r4} x PVG.r8 mating. The lane assignments are as in Figure 1. The restriction endonuclease is BglII and the hybridization probe the random hexamer labeled 41.1 fragment. The 4 kb band is shared among the breeding partners and their progeny and is distinct from the 12 kb ACI fragment.

1 2 3 4 5

<13

4>

FIGURE 3. Blot hybridization of the I-E beta chain homolog in DNA extracted from animals from ACI^{r4} x PVG r8 mating. Lane assignments are as in the previous figures. The restriction endonuclease is Bgl II and the hybridization probe the random hexamer labeled 8.4 fragment. The 6.0 and 5.1 kb fragments are shared among the breeding partners and their progeny The 5.1 kb fragment shares less homology with the hybridization probe than the 6.0 kb fragment as it does not persist after higher stringency washes (data not shown). An 8 5 kb nonpolymorphic fragment is shared by all the animals. The 16.5 and 4.3 kb fragments are specific for the ACI rat.

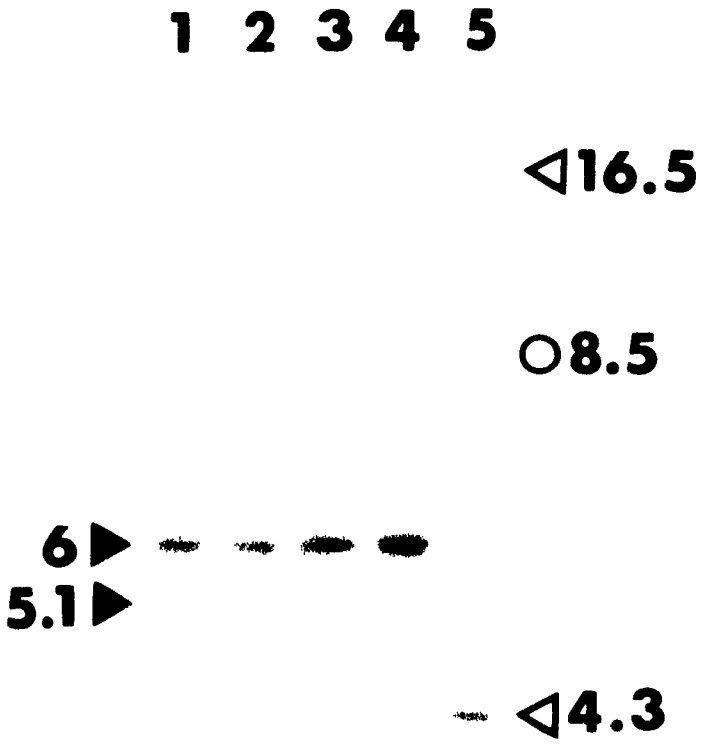
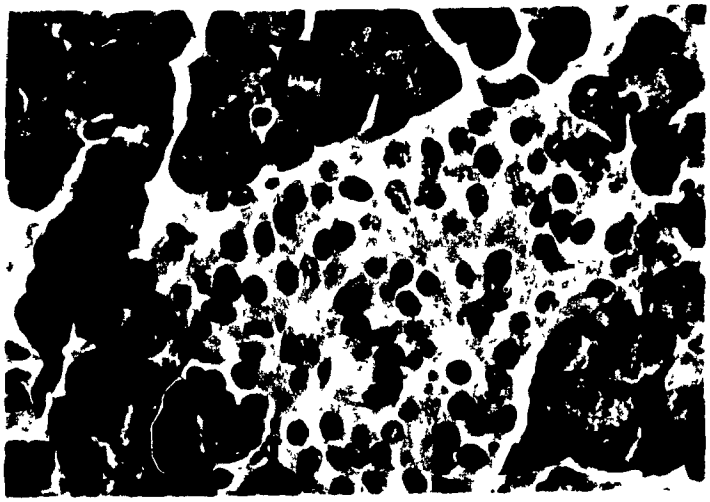
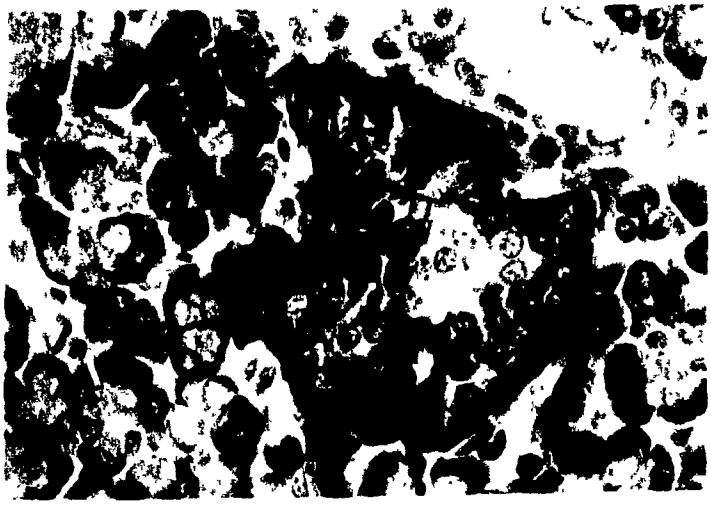

1 2 3 4 5
16.5
8.5
6
5.1
4.3

FIGURE 4. a) Pancreatic islet devoid of cellular infiltrate (hematoxylin-phloxine-saffron, x400). b) Islet stained for glucagon (indirect immunoperoxidase technique, x400). c) Islet stained for insulin (indirect immunoperoxidase technique, x400).

A



B



C

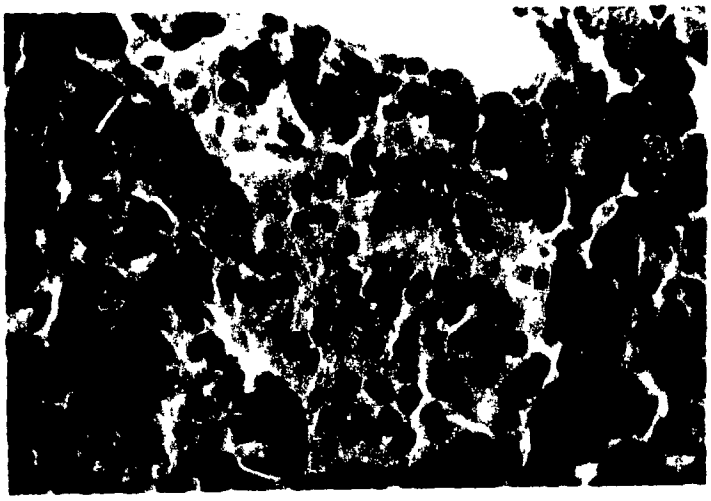
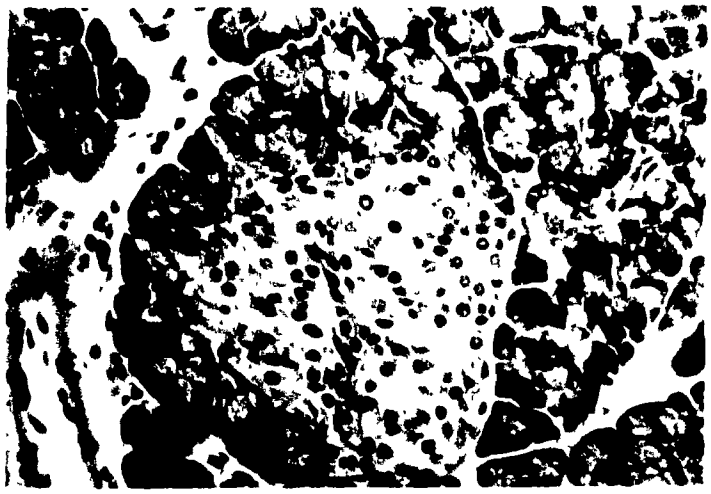
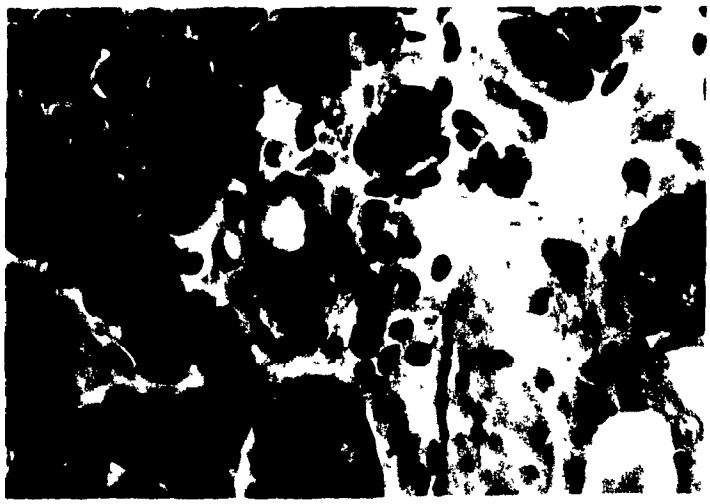


FIGURE 5. a) Normal islet from animal 30 days prior to onset of glycosuria (hematoxylin-phloxine-saffron, x200) b) Scant periductular mononuclear cell infiltrate (hematoxylin-phloxine-saffron, x400). c) Distorted islet devoid of mononuclear cell infiltrate (hematoxylin-phloxine-saffron, x400).

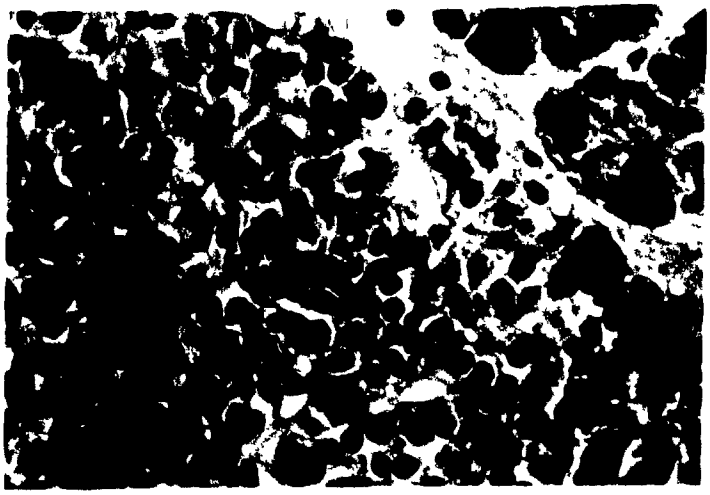
A



B



C



The experiments described in the preceding chapters localized the susceptibility determinant(s) for IDDM that are encoded in the u haplotype of the MHC to the immune response region. They also demonstrated that these determinants can act in a dominant fashion in the context of other disease promoting genes and appropriate environmental factors. The experiments indicate that the susceptibility determinants within the immune response region are not unique to the u haplotype of the BB rat. Genetic mapping experiments in mouse man and rat have shown that the genes encoding the class II antigens of the MHC are located within the immune response region. Indeed, particular alleles of the class II antigens are highly enriched in those predisposed for autoimmunity in general and IDDM in particular. It is still unclear, however, what the significance of this association is in terms of the molecular basis of disease pathogenesis. The Bottazzo laboratory has suggested that the association of autoimmune disease to class II antigens might reflect the aberrant expression of these antigens on the target cell, i.e. the pancreatic beta cell in IDDM. To address this hypothesis, we analyzed the expression of MHC antigens and genes in the pancreata of prediabetic and newly diabetic rats

CHAPTER FIVE.

INSULIN-DEPENDENT DIABETES MELLITUS IN THE BB RAT: ENHANCED MHC CLASS I HEAVY CHAIN GENE EXPRESSION IN PANCREATIC ISLETS

This chapter has been published in Diabetes, 37:1411, 1988.

ABSTRACT

Modulation in major histocompatibility complex (MHC) gene expression correlates with the inflammatory reactions that occur during graft rejection and autoimmune disease. We analyzed the expression of class I and II MHC genes in the pancreatic islets of prediabetic and newly diabetic BB rats by immunochemistry of tissue sections and northern blotting of RNA extracted from isolated islets. We show here that enhanced levels of MHC class I heavy chain RNA are present in pancreatic islets prior to overt inflammation and the onset of insulin-dependent diabetes mellitus (IDDM) in the spontaneously diabetic BB rat. Immunohistochemical analysis reveals enhanced class I antigen expression throughout the pancreatic islets of newly diabetic animals but no induction of class II antigen on any endocrine cells within the islet. Varying degrees of inflammatory infiltrate are observed in the sections exhibiting enhanced class I antigen expression or in nearby serial sections. Southern blot analysis reveals no restriction fragment length polymorphism or amplification of the endogenous class I heavy chain genes as compared to that of seroidentical disease-resistant Wistar Furth rats. I A and I-E alpha hybridizing RNA appear *de novo* prior to overt diabetes although concomitantly with T cell receptor beta

chain and gamma-interferon gene hybridizing RNA and after MHC class I heavy chain RNA enhancement is observed. These data indicate the possibility that enhanced class I heavy chain gene expression might play a role in the progression of IDDM.

INTRODUCTION

MHC class I antigens are cell surface glycoproteins that serve as recognition structures for cytotoxic T lymphocytes (1). MHC class I antigens are essentially ubiquitously expressed although their concentration on different cell types varies widely (2). Substantial evidence exists that the level of MHC class I antigen on a target cell is an important determinant in the effective presentation of foreign antigen to the immune system. First, there are increasing data that transformed cells appear to express very low levels of MHC class I antigen (3). These cells are highly metastatic and are presumably able to evade immune surveillance. A number of laboratories have shown that manipulation of such cells to enhance MHC class I gene expression either by transfection of an additional class I gene or by induction with gamma interferon abrogates their tumorigenicity and metastatic potential (4,5) Second, sites of allograft rejection or autoimmune reactions have been documented to exhibit enhancement of class I gene expression (6-10) In these cases, modulation in MHC class I expression appears to be quantitative rather than qualitative since the target cells often express a low density of MHC class I antigen

Insulin-dependent diabetes mellitus is an autoimmune disease involving multiple genetic as well as environmental

factors (11) We and others have shown that autoimmunity against the insulin-producing beta cells of the islets of Langerhans requires specific alleles within the MHC (12-14). This requirement is shared by the human syndrome and the two animal models of spontaneous IDDM. the Biobreeding or BB rat and the non obese diabetic or NOD mouse. The association of a number of autoimmune disorders with the MHC has stimulated the hypothesis that inappropriate expression of MHC antigens on target cells might function as a disease trigger through presentation of autoantigen (15) To date, the emphasis of investigation has been on the potential role of inappropriate MHC class II gene expression in IDDM Here we have analyzed the expression of both MHC class I and II genes at the level of RNA abundance in isolated islets from biopsies of a series of prediabetic BB and age-matched diabetes-resistant WF rats and at the level of cell surface antigen expression by immunohistochemistry. In these studies we killed BB and WF rats at 30, 40 and 50 days of age to compare the levels of MHC gene expression in isolated islets and corresponding sections of prediabetic animals to those of disease resistant animals. These time points were chosen as they precede the usual period of onset of IDDM in the BB rat (16). Newly diabetic BBDP animals of approximately 100 days of age were sacrificed to determine the levels of MHC gene expression in the islets of these animals. We also document the appearance of T cell receptor beta chain and gamma-interferon RNA as indicies of

infiltration of the islets by activated T lymphocytes and other inflammatory cells that complement our microscopic findings of infiltration.

MATERIALS AND METHODS

ANIMALS. BB, Wistar Furth (WF), BBUF and ACI rats were obtained from established colonies at the McGill University/Montreal Children's Hospital Research Institute and the Royal Victoria Hospital. The BB rats were originally from the Biobreeding Laboratories and were generously provided by Dr. Pierre Thibert of the Department of Health and Welfare Canada. WF rats were originally from Harlan Sprague-Dawley (Walkersville, Maryland). BBUF animals were from a line generated by Dr E. Colle at the Montreal Children's Hospital. The BB, BBUF and WF rats bear the u haplotype at the rat MHC, RT1. The ACI rat bears the a haplotype. The diabetes-prone BB rats used in this study did not exhibit signs of overt diabetes at the time of study. The diabetic BBUF rats used in this study were newly diabetic (within 24 hours after IDDM was diagnosed). All animals were fed Purina Chow ad libitum.

ISOLATION OF ISLETS. Pancreata from age-matched BB and WF animals and newly diabetic BBUF animals were removed from ether-anesthesized animals (three or four animals per time point per strain). Pancreata wer then pooled according to type and enriched for islets. Briefly, islets were dispersed by sequential incubations in collagenase and aprotinin as previously described (17). The purified islets were then

collected and used immediately for RNA preparation.

NUCLEIC ACID ISOLATION. Islet preparations were washed three times with 10 mls of ice-cold phosphate buffered saline. The samples were then resuspended in 10 mls of 5M guanidine thiocyanate, 10 mM EDTA, 50 mM Hepes pH 7.6 and 0.5% 2 mercaptoethanol followed by homogenization in a Brinkman Polytron for two minutes. The resulting homogenates were then passed through cheesecloth sieves to remove large particulate matter and centrifuged at 12,062g for twenty minutes at 4 C Supernatants were then collected and Sarkosyl added immediately to a final concentration of 4%. Aliquots of the supernatants were then layered over 5 7 M CsCl containing ethidium bromide in SW 50 1 polyallomer ultracentrifuge tubes and spun overnight at 50,000g. Buoyant high molecular weight DNA was retained. Pelleted RNA was rinsed with sterile water, ethanol precipitated using ammonium acetate and then resuspended in 4M urea, 50 mM Hepes pH 7.5, 10 mM EDTA.

SOUTHERN AND NORTHERN BLOTTING DNA was digested to completion using 5 units of restriction enzyme per ug of DNA, electrophoresed at 100 V for eight hours on a 0.7% agarose gel in Tris borate buffer and transferred to Gene Screen Plus hybridization membranes according to manufacturer's protocols 10 ug of total RNA was electrophoresed at 80 V for 14 hours on a 0.7% agarose gel containing 40 mM MOPS

a 0.7% agarose gel containing 40 mM MOPS (morpholinepropanosulfonic acid) pH 7.0, 10 mM sodium acetate, 1 mM EDTA and 2.5 M formaldehyde. RNA was transferred to Zeta-probe membranes according to manufacturer's conditions. Membranes were prehybridized and hybridized according to manufacturer's specifications. The northern blots presented in this paper are from the same membrane probed with the various radiolabeled gene probes. Rat MHC class I heavy chain transcripts and genes were detected using the murine H-2 cDNA pH2IIa. Rat MHC class II alpha chain transcripts were visualized using the I-A alpha clone p24.2 and the I-E alpha clone 32.11. All MHC probes were obtained from L. Hood, California Institute of Technology. Rat T-cell receptor beta chain transcripts were detected using the murine cDNA clone RBL-5 #70 donated by T. Mak, Ontario Cancer Institute. Gamma-interferon message was detected using clone pGM8 donated by J. Hiscott, McGill University. The beta-actin cDNA was obtained from the American Type Culture Collection, Rockville, MD.

IMMUNOHISTOCHEMISTRY. Frozen pancreatic tissue was sectioned; 5 um cryostat sections were fixed in acetone for ten minutes onto glass slides. Mouse anti-rat monoclonal antibodies (MoAbs: tissue culture supernatants) OX6 (class II directed) and OX18 (class I directed) were used to detect MHC products. These MoAbs were obtained from Dimension Laboratories, Sera Lab, Mississauga, Ontario. Cellular binding of these

method using peroxidase-conjugated F(ab')2 fragment-goat anti mouse IgG (F(ab')2 fragment-specific) obtained from Cooper Biomedical Inc., Malvern, PA. Polyclonal antibody (guinea pig anti-bovine insulin) [Miles Scientific, Rexdale, Ontario] and peroxidase conjugated goat anti-guinea pig IgG (Dimension Laboratories) were utilized to detect insulin. Primary and secondary antibodies were incubated on the slides for 90 minutes at room temperature. The concentration of antibodies used varied between 1/10 for OX6, 1/200 for OX18, and 1/500 for guinea pig anti-bovine insulin. All antisera were diluted in PBS containing 1% BSA and 10% Wistar rat serum to minimize non-specific staining. Positive controls for insulin staining consisted of normal pancreatic tissue. For MHC gene products, spleen sections were employed. Negative controls were represented by pancreatic and spleen sections in which the primary antibody had been omitted

RESULTS

MHC RNA EXPRESSION. In this study we analyzed the time course of modulation of MHC gene expression documented during autoimmunity against the islet beta cell in IDDM. We extracted total RNA from islet preparations from the experimental animals at the ages indicated. These RNAs were then analyzed by northern blotting for the expression of beta-actin and MHC class I heavy chain genes (Figures 1A and B). Hybridization with the beta-actin probe confirmed that equivalent amounts of islet RNA from the different biopsies were loaded on the gels. All islet RNA preparations contained MHC class I heavy chain RNA. The levels of MHC class I heavy chain RNA increased with time in both diabetes-prone BB and diabetes-resistant WF rats. Strikingly, while the levels of MHC class I heavy chain RNA were equivalent in the resistant and prone islet RNA preparations from biopsies taken at 30 days of age, at 40 days of age there was a significant enhancement in MHC class I heavy chain RNA from the diabetes-prone animals. This enhancement became even more dramatic at 50 days of age. In islet RNA from the newly diabetic BBUF rats there was an elevated level of MHC class I heavy chain RNA in comparison to islet RNA from diabetes-resistant 60 day old WF rats. Importantly, the diabetes-prone BB rats used in this study (aside from the newly diabetic animals) did not exhibit signs of overt IDDM at time of biopsy. Densitometry

of northern blots (Figure 2) indicates that enhancement in MHC class I heavy chain RNA in the isolated islets from the diabetes-prone animals is approximately two-fold higher than in non-diabetes-prone WF rats at 40 days of age and approximately three-fold higher at 50 days of age. Islet RNA from the newly diabetic BBUF animals maintains a two-fold enhancement in MHC class I heavy chain RNA as compared to islet RNA from 60 day old control rats. As the RNA preparations at each time point were extracted from islets pooled from a number of animals (n=15 per strain, n=3 or 4 per time point) the relative abundance of a particular transcript represents a mean of the group. Calculation of standard deviation is not possible as not enough RNA can be extracted for analysis from a single animal.

To determine the levels of MHC class II alpha chain gene expression in the islets of prediabetic BB rats we analyzed RNA from the islet biopsies by northern blotting followed by hybridization with alpha chain probes from the I A and I E loci of the murine H 2 complex (Figures 3A and B). None of the disease-resistant WF or 30 or 40 day old BB rat islet RNA preparations contained detectable MHC class II alpha chain RNA. RNA from the 50 day old BB and newly diabetic BBUF islet biposies conatined detectable MHC class II alpha chain transcripts homologous to both I A alpha and I-E alpha probes. The levels of MHC class II alpha chain expression were significantly lower than the level of MHC class I heavy chain

expression in these samples. Finally, the levels of MHC class II alpha chain expression were greater in the newly diabetic BBUF rat islets than in those of the 50 day old diabetes-prone BB rat. In summary, we observe the de novo appearance at 50 days of age of transcripts homologous to both the I-A and I-E alpha chain genes in islet RNA preparations from diabetes-prone animals showing no signs of overt diabetes.

IMMUNOCHEMISTRY OF PANCREATIC SECTIONS. Staining to detect class I MHC antigens showed either totally negative or very slightly positive beta cells in prediabetic BB animals and Wistar Furth age-matched controls. There was no detection of de novo class II antigen expression on any endocrine cells within the islets of prediabetic or newly diabetic animals. Newly diabetic BBUF animals demonstrated markedly enhanced class I staining on endocrine cells In these cases, varying degrees of inflammatory infiltrate were demonstrable in the same section or in nearby serial sections. Class II staining in the newly diabetic animals was positive only on dendritic cells dispersed within the islet and on mononuclear cells at sites of a cellular inflitrate (Figure 4A-D).

EXPRESSION OF T CELL RECEPTOR BETA CHAIN AND GAMMA-INTERFERON GENES AS INDICIES OF INFILTRATION OF ISLETS BY T LYMPHOCYTES. We and others have previously described in immunohistochemical studies that a massive pancreatic lymphocytic infiltration of

the islets of Langerhans precedes IDDM (18,19) As activated T lymphocytes express both MHC class I and II genes, we wanted methods to monitor this infiltration. In addition to microscopic analysis of pancreatic tissue sections, we analyzed RNA from the islet biopsies for transcripts homologous to the murine T cell receptor beta chain and gamma interferon genes as indicies of T lymphocytic infiltration (Figures 5A and B). None of the islet RNAs from the diabetes resistant WF rats contained RNA homologous to the T cell receptor beta chain or gamma-interferon gene probes Homologous RNA to both probes was also absent in the 30 and 40 day old BB rat-derived islet RNA. Very low levels of T cell receptor beta chain and gamma-interferon gene transcripts were detected in the 50 day BB and newly diabetic BBUF islet preparations. The levels of both transcripts are slightly greater in the newly diabetic islet RNA compared to the 50 day BB preparation. These results demonstrated then, that T cell receptor beta chain and gamma-interferon gene transcripts appeared concomitantly in the islet RNA preparations from the 50 day BB rats. Furthermore, the de novo appearance of these transcripts coincided exactly with the de novo appearance of MHC class II alpha chain transcripts and occurred after the enhancement in MHC class I heavy chain expression.

COPY NUMBER AND GENETIC STRUCTURE OF ENDOGENOUS MHC CLASS I GENES. As enhancement in MHC class I heavy chain gene

expression was the first detectable change in MHC gene expression prior to diabetes we set out to investigate the cause of this enhancement. One possibility was that the enhancement was due to a mechanism inherent to the class I genes of the BB rat. Either amplification or translocation of an endogenous class I gene could result in enhanced expression of class I genes. We digested high molecular weight DNA isolated from the islet preparations from the pancreatic biopsies with the restriction endonuclease EcoRI, Southern blotted the restriction fragments and annealed the immobilized DNA with a class I cDNA probe. We were interested to determine the copy number of the endogenous class I genes from the diabetes-prone and -resistant animals and found that the copy number of the endogenous class I genes was unchanged in the diabetes-prone as compared with the resistant animals at each time point prior to diabetes (Figure 6). Thus, gene amplification of one or a number of MHC class I genes was ruled out as an explanation for enhancement of MHC class I heavy chain gene expression In addition, this result did not reveal a MHC class I restriction fragment length polymorphism distinguishing the diabetes-prone from diabetes-resistant animals. Thus, there are no obvious differences between the genetic structures of the MHC class I genes of diabetes-prone and -resistant animals that might explain overexpression of those genes in the islets of diabetes-prone animals.

DISCUSSION

IDDM in both man and the BB rat is a spontaneous syndrome that occurs in a population of genetically predisposed individuals and results from the autoimmune destruction of the beta cells of the endocrine pancreas. Substantial research has investigated the questions of how the syndrome is triggered and why autoimmunity is targeted to beta cells. Initially, a number of investigators used sera from diabetic humans and rats to identify potential autoantigens on pancreatic islets. These studies revealed both islet cell surface and cytoplasmic antigens detectable in pancreatic sections from normal and diabetes-prone sources (20). Antibody to these antigens was present prior to overt diabetes although selectively in sera from IDDM-prone sources Baekkeskov and Lernmark have identified a potential islet autoantigen of 64 kilodaltons using this approach (21). Eisenbarth and colleagues have taken a second approach that stems from their hypothesis that the target autoantigen(s) are likely to be islet specific differentiation antigens. They have prepared a number of monoclonal antibodies to islet preparations that show varying degrees of specificity for the endocrine pancreas (22). Both of these approaches hold promise as a means of identifying target autoantigen(s)

Identifying the target autoantigen(s), however, would not solve the problem of disease initiation unless the

autoantigen(s) were expressed de novo prior to disease. As genetic predisposition for IDDM is to a large extent determined by the MHC and as the MHC antigens are required for the presentation of foreign antigen to the immune system, it has been attractive to consider the MHC antigens as potential triggers of autoimmunity in IDDM Barclay and Mason initially suggested inappropriate MHC class II expression as a mechanism for autopresentation in general and Bottazzo and coworkers have continued this line of investigation with extensive work on a number of autoimmune diseases including IDDM (15,23). Bottazzo and coworkers have found in both man and the BB rat a de novo expression of immunohistochemically defined MHC class II antigens on insulin-producing beta cells coincident with or prior to IDDM, respectively (24,25). Here we present the results from our immunohistochemical analysis of MHC gene expression in sections of pancreas from prediabetic and newly diabetic animals and the first analysis of MHC gene expression in isolated islets at the level of RNA abundance.

We observe markedly enhanced levels of MHC class I antigen throughout the pancreatic islets of newly diabetic animals (Figure 4B). Sections from prediabetic BB or any of the diabetes-resistant WF rats show little or no staining with the class I monoclonal antibody (see Figure 4A, this is in agreement with the finding that islet cells normally exhibit a class II negative, class I low expressor phenotype [26]; note the comparatively high levels of class I antigen on the

inflammatory cells and ductual epithelium in Figure 1B) Varying degrees of inflammatory infiltrate are always observed either in the same section exhibiting enhanced class I antigen expression or in nearby serial sections This strongly suggests that the enhanced class I antigen expression we observe is a consequence of the inflammatory infiltrate and is not involved in the initial recruitment of insulitis It is likely that inflammatory cells induce the enhanced expression of class I antigen on islets via cytokines that they secrete such as gamma-interferon or tumor necrosis factor alpha. Southern blot analysis of islet DNA using a class I cDNA probe does not reveal amplification or translocation of endogenous class I genes (Figure 6) and is further evidence that enhancement in class I gene expression does not result from a mechanism inherent to the class I genes of the BB rat. In multiple biopsies from prediabetic or newly diabetic animals, we have never observed the de novo induction of class II antigen expression on any cells within the islet (Figure 4C; Class II positivity is limited to infiltrating cells within the islet (Figure 4C) Class II positivity is limited to infiltrating cells or dendritic cells within the body of the islet. Staining of serial sections with anti insulin monoclonal antibodies clearly demonstrate that both insulin positive and -negative cells within the newly diabetic islet exhibit strong class I cell-surface staining (Figure 4D)

Northern blot analysis of RNA isolated from islets

purified from these animals demonstrates enhanced levels of class I heavy chain RNA in both prediabetic and newly diabetic animals in comparison to age-matched control WF rats (Figure 1B). This enhancement occurs prior to our observation of de novo MHC class II transcripts that appear coordinately with transcripts homologous to the T cell receptor beta chain and gamma-interferon genes (Figures 3 and 5). The coordinate appearance of these genes and our inability to detect any class II antigen expression in islets from prediabetic or newly diabetic animals strongly suggests that the infiltrating cells are the major source of de novo class II transcript.

Our observation of enhanced levels of class I heavy chain RNA in islet preparations from newly diabetic animals is in agreement with our observation of enhanced class I antigen expression on the islets of these animals. A portion of this enhancement in class I RNA is likely attributable to the high level of class I gene expression in infiltrating inflammatory cells (Figure 4B) and the remainder representing enhancement in the islet cells proper.

The early enhancement in class I RNA that we observe in prediabetic animals at 40 and 50 days of age is intriguing. As we have noted, immunohistochemical analysis does not detect an enhancement in class I antigen expression in the islets from these same animals. We suggest three explanations. First, the time course of class I antigen enhancement seen relative to class I mRNA could reflect a normal delay in

antigen expression relative to mRNA expression Second, it is possible that the elevated levels of class I RNA in prediabetic animals does not in fact result in enhanced levels of class I antigen expression due to a nonlinear relationship between the level of class I transcript and class I antigen (suggesting a posttranscriptional level of control). Alternatively, the discrepancy could be an artifact of the experimental protocol The design of this work was such that the vast majority of the pancreatic tissue examined was used in the preparation of islets for the extraction of RNA for northern analysis. Only a small piece (representing less than one-tenth of the total tissue mass) of the pancreas from each animal was frozen for subsequent immunohistochemical analysis Thus, the results of the northern analysis represent a broader sample range and a less topologically restricted set of islets tested for potential modulation in MHC gene expression than that used in the immunohistochemical analysis. As a consequence, enhanced class I RNA in prediabetic animals may be an indication that enhancement in class I gene expression is occuring on islets at a site removed from that examined by immunohistochemistry. This is not a trivial point as autoimmunity against the beta cell appears to initiate at localized regions of the pancreas with subsequent spread and thus implies that the triggering process might also progress in this fashion (27). In this scenario, the early enhancement in class I RNA would represent a localized induction of class

I gene expression due to early insulitis. That enhanced class I antigen expression on islets is finally observed in the newly diabetic animal may simply reflect that insulitis has at this point spread throughout the pancreas. In vitro studies by ourselves and others (28,29) on the inducibility by gamma-interferon of class I gene expression in the rat insulinoma cell line RIN5F argue against a significant level of posttranscriptional control in the induction of class I genes. Thus, we feel the third explanation is the most likely.

Our observation of enhanced levels of class I RNA prior to our detection of de novo class II, T cell receptor beta chain and gamma-interferon transcripts and prior to the appearance of inflammatory cells might be interpreted to suggest that this enhancement preceded not only overt disease but also insulitis. Alternatively, the initial inflammatory infiltrate might be too small in scale to produce a detectable T cell receptor beta chain or gamma-interferon RNA signal in the hybridization analysis although large enough to induce elevated levels of class I gene expression. That we only observe enhancement in class I antigen expression near a site of insulitis suggests to us that the second scenario is more probable.

Our inability to detect de novo class II antigen expression on any cells within the islets of prediabetic or newly diabetic animals (even those islets exhibiting peripheral insulitis, Figure 4C) is in disagreement with

previously reported findings (24,25). A recent paper by Pipeleers and coworkers might explain this discrepancy (30). These investigators rigorously analyzed the few Ia positive cells found within normal islets and report that these were mononuclear cells of nonendocrine origin that had phagocytosed fragments of damaged endocrine cells. These authors suggest that the Ia-positive, insulin-positive cells in the pancreas of newly diabetic individuals and rats may very well be these mononuclear cells that have phagocytosed debris from ongoing beta-cell destruction. They thus suggest additional markers are necessary to test whether there are in fact class II expressing beta-cells in the prediabetic or diabetic pancreas. There are several reasons that we feel that class II positive beta-cells do not exist either in the prediabetic or newly diabetic pancreas. In addition to the results presented in this paper, we fail to detect class II positive beta cells when we induce an acute diabetic syndrome by passive transfer of conA activated T lymphocytes from diabetic BB rats (Issa Chergui et al., in press), and we and others cannot induce class II antigen expression on insulinoma cells unless we use unphysiologically high doses of crude cytokine preparation or gamma-interferon (28,29).

In sharp contrast, enhanced class I gene expression on islets is a consistent finding in pancreatic sections from newly diabetic animals that arise either spontaneously or after passive transfer of activated T lymphocytes. Class I

gene expression is also easily inducible on insulinoma cells or isolated islets using low levels of crude cytokine preparation or gamma-interferon alone (28,29). Thus, the accumulated evidence from *in vivo* and *in vitro* studies is a compelling reason to consider enhanced MHC class I heavy chain expression as a potentially important factor in IDDM. Indeed, enhanced MHC class I heavy chain expression could contribute to the expression of IDDM via qualitative or quantitative means. Some of the enhanced class I antigens might include non-classical class I molecules not normally expressed on all cells yet inducible by cytokines. Alternatively, increased concentrations of classical class I antigens might facilitate the recognition of target cells by cytotoxic lymphocytes. The mounting evidence in other systems that the concentrations of MHC class I antigen on a cell plays a major role in determining its immunogenicity illustrates the feasibility of a similar role in autoimmunity in general and in IDDM in particular.

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FIGURE 1. A) Northern blot of total RNA purified from islet preparations from pooled pancreatic biopsies hybridized with a nick-translated beta-actin cDNA. Lane 1: RNA prepared from islets from 30 day WF rats, lane 2: 30 day BB rats, lane 3: 40 day WF rats, lane 4: 40 day BB rats, lane 5: 50 day WF rats, lane 6. 50 day BB rats, lane 7: 60 day WF rats, lane 8: newly diabetic BBUF animals of approximately 100 days of age.
B) Northern blot of total islet RNA probed with the MHC class I heavy chain cDNA pH2IIa. RNA analyzed in all northern blots presented in this paper are from the same islet preparations.

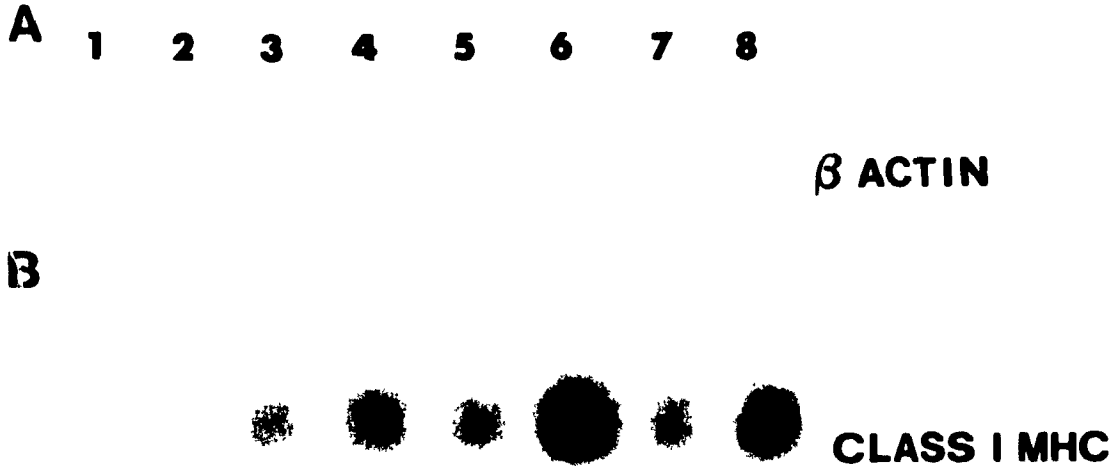

A
1 2 3 4 5 6 7 8
β ACTIN
B
CLASS I MHC

FIGURE 2. Quantitation of MHC class I heavy chain RNA. The autoradiograms in Fig. 1 were scanned with a densitometer to determine the intensity of the bands corresponding to MHC class I heavy chain and beta-actin RNAs. The amount of MHC class I heavy chain RNA in each sample was determined relative to that for beta-actin RNA in the sample. The results are expressed in arbitrary units. Circles mark the relative abundance of class I heavy chain RNA in the islets of diabetes-prone BB and newly diabetic BBUF rats while the triangles mark the relative abundance in WF rats.

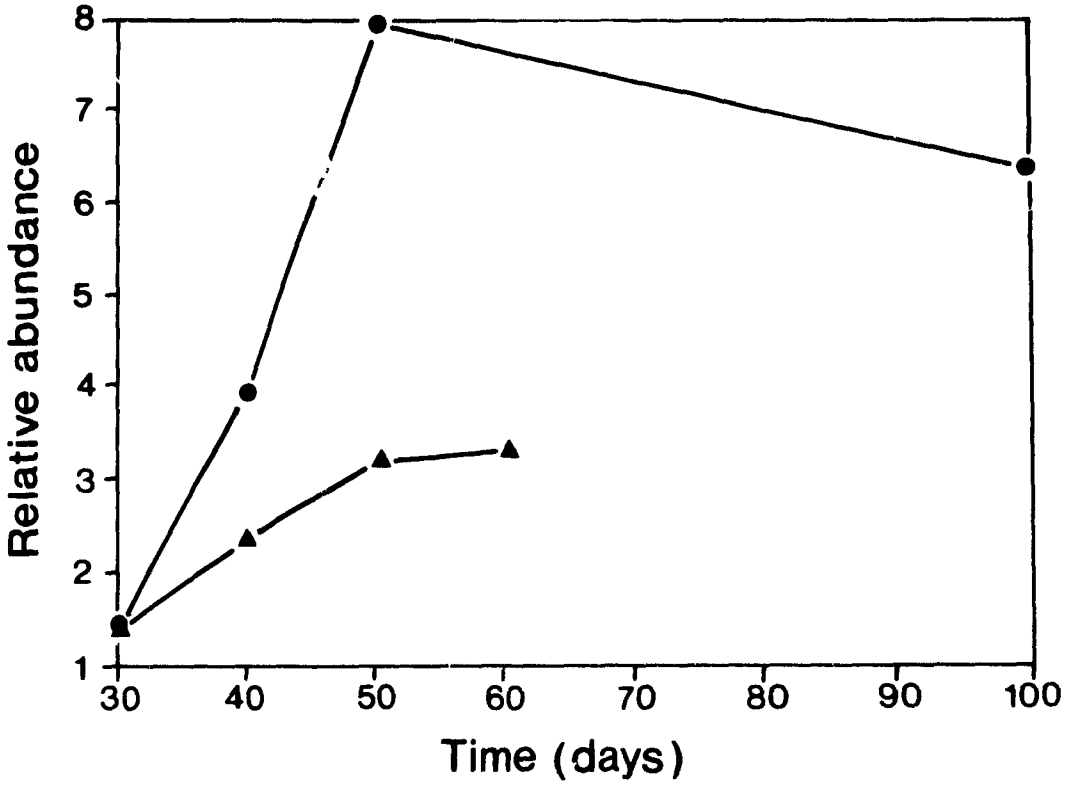

Relative abundance
8
7
6
5
4
3
2
1
30
40
50
60
70
80
90
100
Time (days)

FIGURE 3. A) Northern blot of total islet RNA probed with the MHC class II I-A alpha probe p24.2. B) Northern blot of total islet RNA probed with the MHC class II I-E alpha probe p32.11. Lane assignments are as in Figure 1.

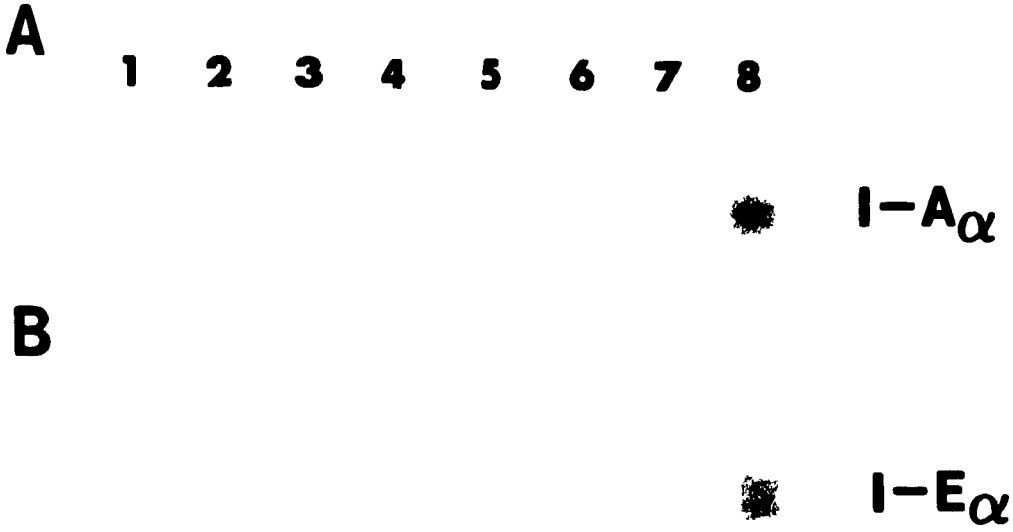

A
1 2 3 4 5 6 7 8
I-Aα
B
I-Eα

FIGURE 4. A) Photomicrograph of a pancreatic islet from a pre-IDDM animal stained with the anti-class I monoclonal antibody OX18. (indirect immunoperoxidase, x200). B) Photomicrograph of pancreatic islet from newly diabetic BBUF animal stained for class I MHC antigen. Enhanced levels of class I antigen occur throughout the islet on endocrine and exocrine cells. The cellular infiltrate on the left edge of the islet is strongly class I positive. (OX18, indirect immunoperoxidase, x200). C) Photomicrograph of same islet stained for class II MHC antigens. Positive staining is restricted to cellular infiltrate along the left edge of islet and dendritric cells. (OX6, indirect immunoperoxidase, x200). D) Photomicrograph of same islet stained for insulin. (Insulin, indirect immunoperoxidase, x200).

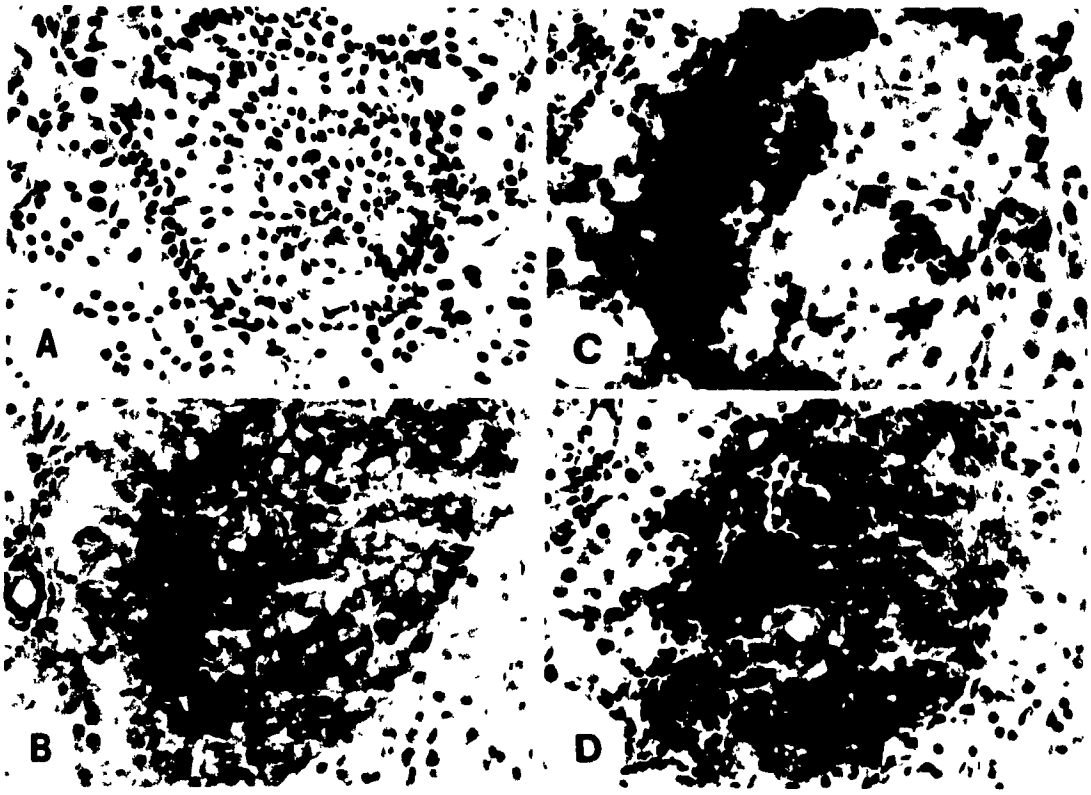

A
C
B
D

FIGURE 5. A) Northern blot of total islet RNA probed with the murine T-cell receptor beta chain cDNA RBL-5 #70. B) Northern blot of islet RNA probed with the gamma-interferon cDNA pGM8. Lane assignments are as in Figure 1.

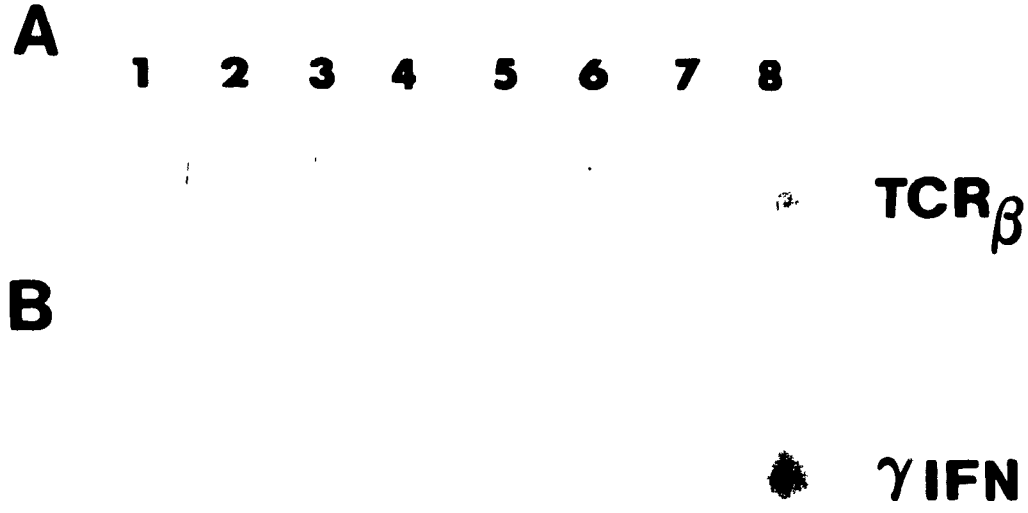
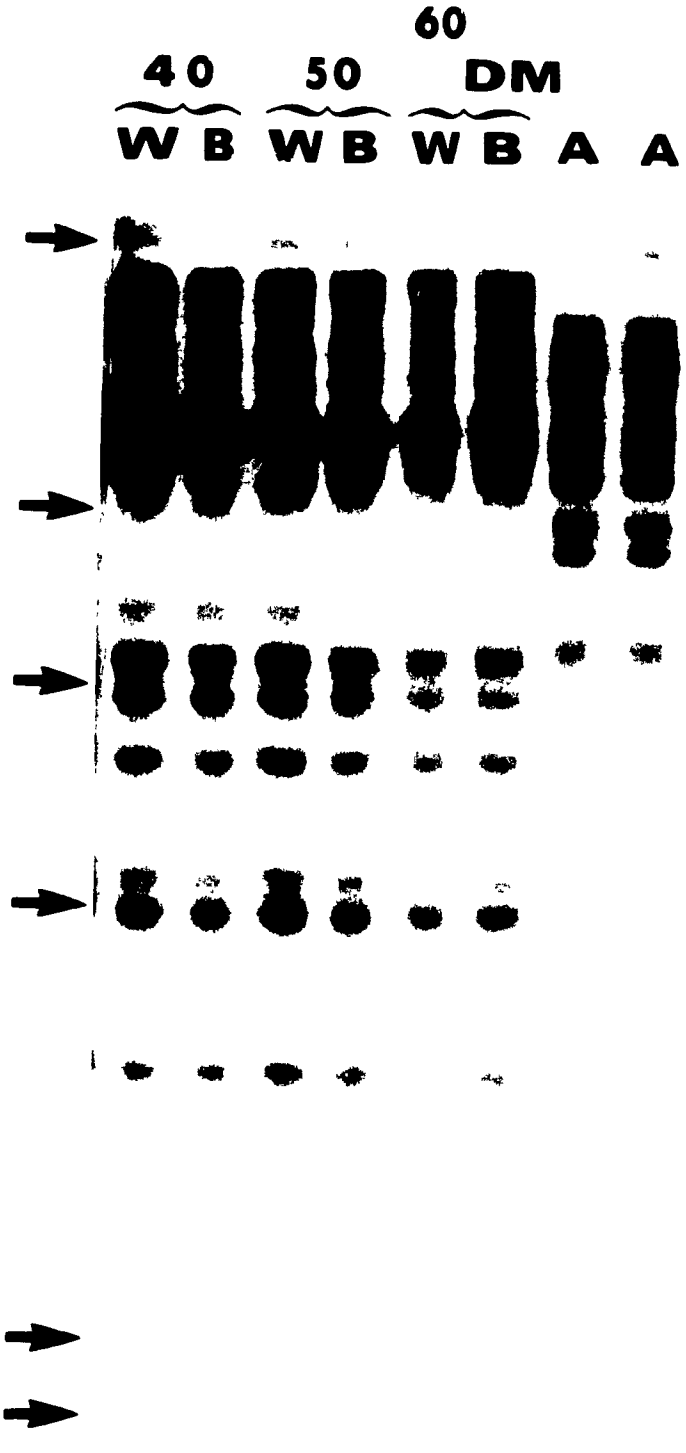
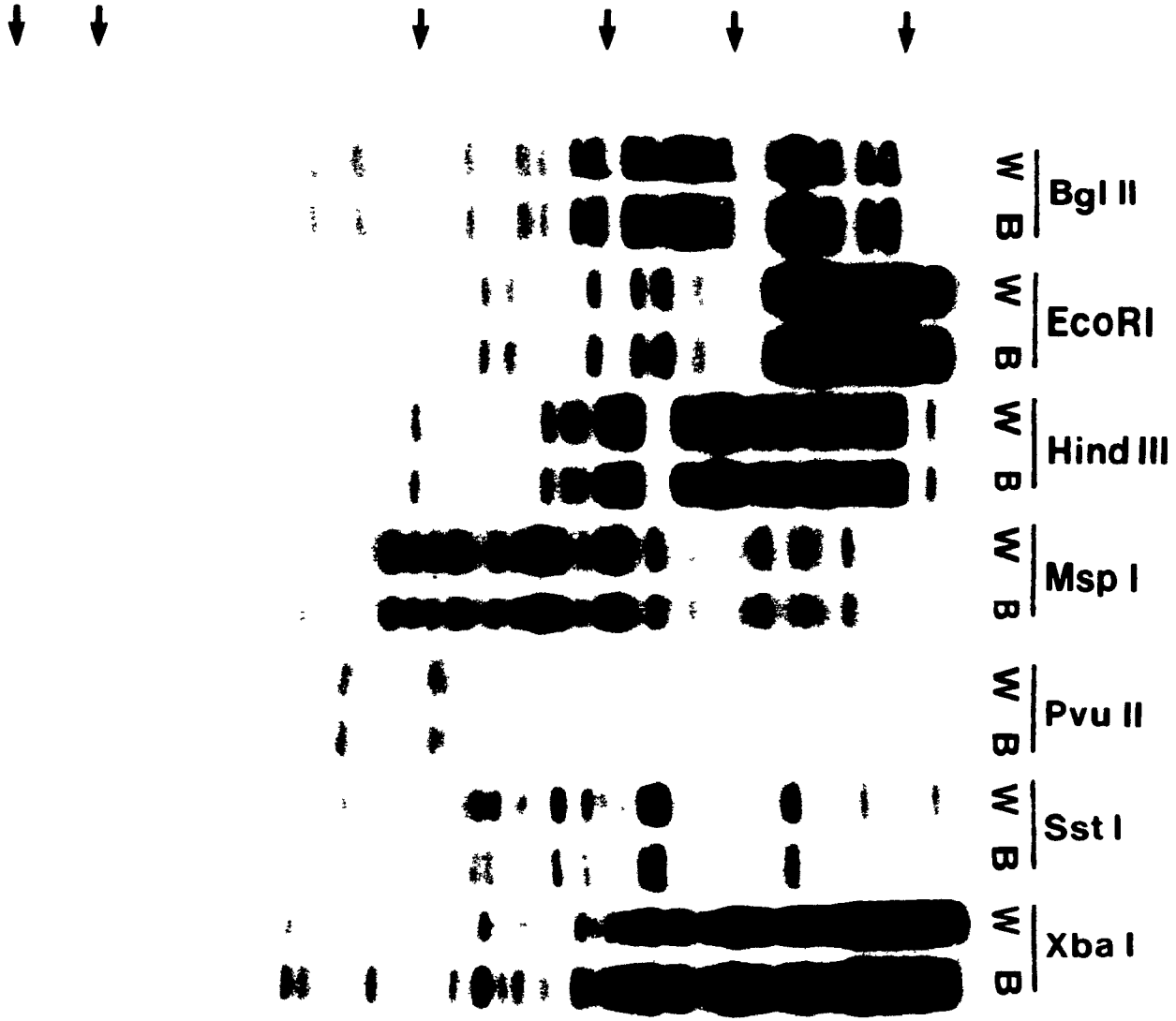

A
1 2 3 4 5 6 7 8
TCRβ
B
γIFN

FIGURE 6. Panel A: Southern blot of high molecular weight DNA purified from isolated islets from the same pancreatic biopsies used for the RNA purification. The numbers above the blot indicate the age in days of the animals from which the DNA was extracted, and W or 3 refers to Wistar Furth or BB rats. The lane labeled DM refers to DNA extratcted from the newly diabetic BBUF rats of approximately 100 days of age. A refers to DNA isolated from the spleens of ACI rats that bear the a haplotype at the RT1 and serve as haplotype-distinct comparisons for copy number analysis. The genomic DNAs in this experiment were digested with EcoRI and the Southern blots were probed with the MHC class I heavy chain cDNA pH2IIa. The arrows at the left refer to HindIII digestion fragments of bacteriophage lambda DNA.

Panel B: Restriction fragment length polymorphism analysis of genomic DNA isolated from BB and Wistar Furth rat spleens probed with the MHC class I heavy chain probe. No RFLPs were detected using multiple enzymes.


60
40
50
DM
W B W B W B A A


W B
Bgl II
W B
EcoRI
W B
Hind III
W B
Msp I
W B
Pvu II
W B
Sst I
W B
Xba I
W B

The experiments described in the previous chapter demonstrated that the first discernible modulation in MHC gene expression in the pancreata of prediabetic and newly diabetic rats is an enhancement in MHC class I heavy chain expression In contrast, class II antigen expression was never detected in prediabetic or newly diabetic animals. These data strongly suggest that aberrant expression of class II antigens on pancreatic beta cells does not play a central role in the pathogenesis of IDDM in the BB rat. The results do, however, suggest that enhanced class I gene expression might play a role in disease pathogenesis First, enhanced class I gene expression is an early phenomenon in the time course of disease onset, and second, it is a consistent finding. These findings are important in view of the studies involving transgenic mice, where forced expression of either class I or class II antigens on the pancreatic beta cell results in the induction of diabetic syndromes. Our findings during the time course of spontaneous IDDM suggest that the class I transgenic mice may have more relevance to the pathogenesis of IDDM than aberrant class II antigen expression. In the following chapter, we have investigated the inducibility of MHC genes and antigens on the rat insulinoma cell line RINSF using both crude lymphokine preparations and recombinant gamma interferon.

CHAPTER SIX.

GAMMA-INTERFERON INDUCES THE TRANSCRIPTION AND DIFFERENTIAL EXPRESSION OF MHC GENES IN THE RAT INSULINOMA CELL LINE RINm5F

This chapter has been published in Diabetes (38:911, 1989).

ABSTRACT

We have investigated the kinetics of gamma interferon induced expression of the class I and II major histocompatibility complex (MHC) genes in the rat insulinoma cell line RIN5F. Treatment of RIN5F cells (which are normally class II negative, class I low expressors) with 1 unit/ml recombinant rat gamma-interferon resulted in a fifteen fold enhancement in MHC class I antigen expression but no detectable induction of class II antigen expression 48 hours postinduction. This enhancement in class I antigen expression was a dose-dependent phenomenon and was preceded by a dose dependent increase in class I specific RNA Both class I and II genes were induced at the transcriptional level as determined by northern blotting and in vitro nuclear transcription assays, but exhibited strikingly different induction kinetics. Supernatants from conA stimulated splenocytes had a similar inductive effect on MHC gene expression. Preincubation of RIN5F cells in the hypomethylating agent 5-Azacytidine enhanced the ultimate level of class I RNA and antigen induced by a given amount of conA supernatant. Incubation in the presence of cyclosporine A did not inhibit class I induction. RIN5F cells, then, exhibiting a class I interferon response positive, class II

response negative phenotype 1) are additional evidence that enhanced class I antigen expression on beta cells may play a mechanistic role in autoimmune IDDM and 2) represent a valuable system for probing the similarities and differences in the interferon mediated induction pathways for class I and II MHC genes.

INTRODUCTION

The major histocompatibility complex (MHC) encodes primarily two general classes of polymorphic cell surface molecules which play a central role in immune regulation and function (1). The class I antigens are composed of a heavy chain found in noncovalent association with the extra-MHC encoded beta2-microglobulin The class II or Ia antigens are composed of noncovalently associated alpha-beta heterodimers. Class I antigens are expressed on most cells albeit in varying degrees while class II antigen expression is normally restricted to cells of the immune system Both classes of MHC antigen function in the presentation of foreign antigen to the immune system. In this capacity, class I antigens restrict antigen recognition by $CD8^+$ lymphocytes while class II antigens restrict $CD4^+$ lymphocyte recognition.

There are increasing data that indicate that quantitative variation in MHC antigen expression on a given cell can have a profound influence on its immunogenicity Numerous human and animal tumors exhibit greatly reduced levels of class I antigen (2). It is hypothesized that this may account for the ability of these tumors to evade immune surveillance Enhancement of these levels by transfection of a class I gene abrogates the tumorigenicity and metastatic potential of such tumor cells. Modulation in cell surface MHC antigen

tumor cells. Modulation in cell surface MHC antigen expression is also a common feature during the early stages of organ specific autoimmunity (3). Enhanced expression of class I antigen and aberrant expression of class II antigens have been reported on the beta-cells of the pancreatic islets of Langerhans (the target cell) in insulin-dependent diabetes mellitus (IDDM). Both enhanced class I antigen expression and aberrant class II antigen expression have been hypothesized to play a role in the initiation and/or progression of this syndrome.

The interferons constitute a family of hormone-like bioregulators with pleiotropic function (4). Immune or gamma-interferon is produced by activated T lymphocytes and can induce the expression of a number of genes including those within the MHC. Indeed, this role as an <u>in vivo</u> modulator of MHC expression is the focus of much current investigation as 1) gamma-interferon appears to have the capacity to abrogate the tumorigenicity of certain transformed cells and 2) gamma-interferon either alone or in conjunction with other lymphokines is considered a likely agent contributing to autoimmune inflammation.

As the ability of gamma-interferon to modulate MHC gene expression exhibits a tissue-specific phenotype, it is a phenomenon whose molecular genetics need be elucidated in each target cell of interest. We have previously reported finding enhanced levels of MHC class I heavy chain gene expression but

determined by immunohistochemistry of tissue sections and northern blotting of RNA extracted from isolated islets (5, Ono, S.J. et al., in press). In this paper we have investigated the effects of crude lymphokine preparations and recombinant rat gamma-interferon on MHC gene expression in the rat insulinoma cell line RIN5F (6). This insulin producing pancreatic beta cell line was originally derived from a naturally occurring rat insulinoma. This cell line expresses low levels of class I antigen and does not express class II antigens In this regard the cell line exhibits a similar phenotype to normal pancreatic beta cells and hence is a useful model for the investigation of MHC product expression and induction (7).

MATERIALS AND METHODS

CELLS The RIN5F cells were obtained from Dr. P. Poussier of the McGill Nutrition and Food Science Centre and maintained in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum. 1.1g/500 ml of HEPES, 5 x 10^3 units/100 ml of penicillin, 5 x 10^3 units/100 ml of streptomycin, 146 mg/500 ml glutamine and 0.1 mg/ml pyruvate. Cells were maintained at approximately 50% confluence and split 1:4 the night before experiments to ensure that cells were in a logarithmic phase of growth at the time of induction Prior to induction, cells were pelleted by centrifugation, washed in 15 volumes fresh complete media and seeded at a density of 2 x 10^6 cells per well in six-well tissue culture plates

REAGENTS Concanavalin A and 5-Azacytidine were purchased from Sigma Chemical Co. Cyclosporine A and recombinant rat gamma-interferon were kind donations from Sandoz and Hoffman LaRoche Pharmaceutical Corporations respectively. Cyclosporine A was dissolved in dimethyl sulfoxide.

MONOCLONAL ANTIBODIES. The OX6 and OX18 monoclonal antibodies were obtained from Dimension Laboratories, Sera Lab. OX6 reacts with rat Ia or class II MHC antigens whereas OX18

reacts with rat class I MHC molecules. P3 does not cross react with either class I or II MHC molecules and is routinely used by our laboratory as a control for nonspecific binding Fluorescein-conjugated affinity purified F(ab')2 fragment goat or rabbit antimouse IgG (F(ab')2 fragment specific) was obtained from Cappel Laboratories. Antisera were diluted in Hank's balanced solution containing 10% bovine serum albumin.

CULTURE CONDITIONS RIN5F cells were induced using either supernatant from conA stimulated rat splenocytes or recombinant rat gamma-interferon at the concentrations indicated Crude lymphokine preparations (CLP) were prepared by removing spleens from ether-anesthesized ACI rats Splenocytes were dissociated by forcing the spleens through a sterile wire sieve with a glass plunger. The cells were then washed and placed in culture media containing conA at a concentration of 0.04 ug/ml After incubation for 24 to 30 hours, splenocytes were removed from the media by centrifugation and the supernatant frozen in 15 ml aliquots All of the CLP preparations used in these experiments were from the same conA stimulation To induce RIN5F cells with either CLP or gamma-interferon, various amounts of inducer were introduced to individual wells containing the aliquoted cells The plates were then returned to the incubator and replicate aliquots removed at set time points postinduction for RNA extraction and flow cytometric analysis Expanded

cell culture conditions were used to extract enough RNA for northern blot analysis.

FLOW CYTOMETRIC ANALYSIS. Surface immunofluorescence staining of individual cells was analyzed by flow cytometry. Samples of 1 x 10^6 cells were incubated for 30 minutes at 4 C in 100 ul HBSS containg 10% BSA and 25 ul specific monoclonal antibody at a saturating concentration. The cells were then washed three times with 1 ml ice-cold HBSS/BSA solution. Primary antibody was detected with 25 ul fluorescein-conjugated goat or rabbit antimouse immunoglobulin G (saturating). The cells were then washed as before, resuspended in 100 ul HBSS/BSA and fixed for 10 minutes in 1% paraformaldehyde. Aliquots of the fixed cells were then diluted 1:50 in saline solution and analyzed by flow cytometry Median channel fluorescence was used as a measure of relative antigen expression as it has been established that under saturating conditions median channel fluorescence is an accurate index of antigen density on the cell surface (8) In the FACS Analyzer system using log amplification, a 66 channel shift in median channel fluorescence represents a ten-fold increase in the number of molecules found on a given cell surface Each quantitation of antigen expression was based on flow cytometric analysis of 10 to 30 x 10^4 optical events.

RNA ISOLATION AND BLOTTING. For dot blot hybridization

analysis approximately 5 x 10^6 cells were collected at each time point and washed with ice-cold, sterile PBS prior to lysis with NP40. Nuclei were then pelleted and the supernatants retrieved. An equal volume of guanidine isothiocyanate solution was then added to each supernatant, the solution warmed to room temperature, and then extracted once with equal volume of hot phenol The resulting supernatant was then ethanol precipitated, washed once with ice-cold 70% ethanol and stored in 200 ul of 70% ethanol until use. Prior to transfer of RNA to Zeta probe membranes (Biorad, Richmond, CA. 94804), the pelleted RNA film was dried down under speed vacuum and resuspended in sterile TE (10mM Tris, 1 mM EDTA, pH 8.0). Equivalent aliquots of resuspended RNA were then removed from each sample and optical densities read to normalize the amounts of RNA loaded to each well of a dot-blot manifold. Samples were denatured with formaldehyde and combined with an equal volume of 20 x SSC, 1% SDS prior to loading. After transfer of the samples to the membrane, the membrane was baked for two hours under vacuum and prewashed in 2 x SSC, 1% SDS prior to prehybridization Prehybridization was carried out for a minimum of 12 hours in 5 x SSC, 50% formamide, 5 x Denhardt's solution, tRNA (300 ug/ml), polyadenylic acid (10 ug/ml), 0 1% SDS and salmon sperm DNA at 150 ug/ml. Hybridizations were carried out in fresh prehybridization solution Filters were washed extensively in 1 x SSC, 1% SDS and then autoradiographed overnight using

Kodak XK-1 film at room temperature with intensifying screens. Quantitation of the signals was carried out by densitometric scanning. For northern blot analysis, total RNA was purified by resuspension of cell pellets containing 1 x 10^7 cells in 10 ml of 5 mM HEPES pH 7.6 and 5% 2-mercaptoethanol followed by homogenization in a Brinkman polytron for two minutes. The homogenate was then filtered through a cheesecloth and centrifuged for 20 minutes at 12,062 x g. The supernatant was then collected, Sarkosyl added to 4% and the solution layered over previously prepared centrifuge tubes containing 5 7 M CsCl. After high speed centrifugation (35K, 16 hours), the pelleted RNA was rinsed with water and dissolved in 15 ml of 4 M urea, 50 mM HEPES pH 7 5, 10 mM EDTA. RNA was extracted twice with equilibrated phenol-chloroform (1:1), once with chloroform and three times with ether followed by ethanol precipitation RNA was resolved on 1.0% agarose gels after denaturation in glyoxal dimethyl sulfoxide and blotted onto Zeta-probe membranes Hybridization probes were prepared by nick translation of purified restriction fragments of murine MHC clones that were kindly donated by L. Hood, California Institute of Technology and R Germain, National Institutes of Health (9,10)

IN VITRO NUCLEAR TRANSCRIPTIONS. RIN5F cells were harvested at several time points after induction with 1 unit/ml recombinant rat gamma-interferon for run-on transcription

analysis. Nuclei from 5 x 10^6 cells were isolated following six strokes in a Dounce homogenizer and were incubated for 10 minutes at 30 C in the presence of 0.5 mM ATP, CTP and GTP with 2.5 uM alpha^{32}P UTP at a specific activity of 40 uCi/nM. Labeled nuclear RNA was extracted by hot phenol/SDS extraction and following ethanol precipitation, was purified by 2M LiCl precipitation (5 x 10^6 cells/ml) at 4 C Following mild alkali cleavage (0.1 M NaOH at 0 C for 10 minutes) and neutralization with 2.0 M HEPES, 10^7 cpm (RNA from approximately 2.5 x 10^6 cells) was used for each hybridization. The RNA hybridization to a DNA dot matrix was for 36 hours at 60·C. Filters were washed and digested with RNase prior to exposure to Kodak XK-1 film.

RESULTS

INDUCTION OF MHC GENES WITH SUPERNATANT FROM CONCANAVALIN A STIMULATED SPLENOCYTES. In order to determine the inducibility of MHC class I and II genes in RIN5F we first tested the ability of a crude lymphokine preparation (CLP) from conA stimulated splenocytes to elicit this response.

Addition of CLP to RIN5F cultures resulted in a five-fold enhancement in the amount of MHC class I heavy chain RNA as measured by densitometry of dot blot hybridizations (Fig. 1A). As was the case for all the experiments presented in this paper, hybridization with a beta-actin probe was used to normalize any variation in the amount of input RNA. In addition, cells incubated in medium containing conA alone served as a control and did not cause a change of mRNA levels over background (data not shown) Incubation of the cells with increasing amounts of CLP resulted in a dose-dependent increase in the amount of MHC class I heavy chain RNA (Figure 1B) Dot blot hybridizations with MHC class II I-A and I-E alpha chain probes indicated that both loci (whose rat analogs are termed RT1 B and RT1.D, respectively) are inducible in RIN5F cells with CLP. The densitometric signal for the two MHC class II alpha chains increased three-fold over background hybridization observed in untreated cells (Fig 2)

Subsequent northern blots and *in vitro* nuclear transcriptions have indicated that class II genes are either transcriptionally off or express an undetectable level of transcript in uninduced RIN5F cells. Enhancement in the abundance of MHC class I heavy chain RNA occurred between four and eight hours postinduction (Fig. 1) whereas induction of class II genes occurred between eight and twelve hours (Fig. 2).

We monitored the effect of CLP on the cell surface expression of MHC class I and II molecules by flow cytometric analysis using the monoclonal antibodies OX6 and OX18. These monoclonals are routinely used by us and others to detect MHC molecules on rat cells. Prior to incubation in CLP, RIN5F cells exhibited a class II negative, class I low expressor phenotype. We defined negativity as fluorescence less than or equal to that detected on cells incubated with the standard control negative monoclonal antibody P3 as the primary antibody. RIN5F cells exhibited a ten to fifteen fold enhancement in the levels of MHC class I cell surface antigen expression after incubation in 50% CLP for 48 hours while there was no detectable MHC class II antigen under identical conditions (Fig. 3).

INDUCTION OF MHC GENES WITH RECOMBINANT GAMMA INTERFERON

Having established that CLP could enhance MHC class I heavy chain RNA abundance and cell surface expression, and induce

MHC class II gene expression but not cell surface antigen expression, we set out to determine whether recombinant rat gamma interferon alone could elicit this phenotypic change.

Addition of recombinant gamma-interferon at a final concentration of 1 unit/ml to RIN5F cultures resulted in a ten-fold enhancement in the amount of MHC class I heavy chain RNA twenty hours after addition of the inducer as measured by densitometry of northern blots (Fig. 4A). I-E alpha chain transcripts were undetectable prior to induction, became evident at 20 hours postinduction and continued to rise as late as 48 hours postinduction (Figure 4B). Incubation of the cells with increasing amounts of recombinant interferon (from 1 unit/ml to 10 units/ml) resulted in a dose-dependent increase in the amount of MHC class I heavy chain RNA (Fig. 5).

Flow cytometric analysis of RIN5F cells treated with 1 unit/ml recombinant gamma-interferon revealed a fifteen-fold enhancement in the level of MHC class I surface antigen expression 48 hours postinduction (Fig. 6) The level of class I antigen expression was perceptively increased at 8 hours postinduction and continued to rise as late as 48 hours postinduction. On the other hand, class II antigen expression remained undetectable as late as 48 hours postinduction (Fig 6) Incubation in increasing amounts of recombinant gamma-interferon resulted in a dose-dependent increase in the level of class I antigen expression (Fig 7). We were unable to

of class I antigen expression (Fig. 7). We were unable to detect induction of class II antigen expression with 1, 5 or 10 units/ml doses of recombinant interferon 24 hours postinduction (Fig. 7).

INDUCTION OF MHC GENE EXPRESSION HAS A TRANSCRIPTIONAL BASIS

We performed in vitro nuclear transcriptions to determine the transcription rates of MHC class I and II genes prior to and 4 and 16 hours postinduction with 1 unit/ml recombinant gamma-interferon. Transcription rates were determined by densitometry of duplicate sets of autoradiographic signals resulting from the hybridization of labeled nascent RNA preparations to equivalent amounts of unlabeled class specific DNA immobilized to nitrocellulose. Variability in autoradiographic signals between experiments were normalized based on autoradiographic signals corresponding to the transcription rate of beta-actin. There was no change in the abundance of beta-actin transcript upon treatment with interferon. Prior to incubation in interferon, RINSF cells exhibited a low level of MHC class I heavy chain gene transcription (less than for beta-actin) and no detectable transcription of I-A or I-E alpha chain gene homologs (Fig. 8). Four hours postinduction, there was a five-fold increase in the rate of transcription of class I genes while transcription of class II genes remained undetectable. Sixteen hours postinduction the transcription rate of class I

genes had risen ten-fold over the basal rate and transcription of both alpha chain genes was detectable at a level approximately equivalent to the basal rate of class I transcription.

EFFECTS OF 5-AZACYTIDINE AND CYCLOSPORINE A ON INDUCIBILITY OF MHC CLASS I GENES. Preincubation of RIN5F cells for 24 hours in 2 uM of the hypomethylating agent 5-azacytidine resulted in higher levels of induction of class I gene expression by gamma-interferon (Fig. 9A). Forty-eight hours postinduction in 40% CLP, RIN5F cells pretreated with 5-azacytidine expressed approximately one-third more class I transcript than cells treated with CLP alone. Preincubation in 5-azacytidine also enhanced the ultimate level of class I antigen induction by CLP by three-fold (20 channel shift, Fig. 10). Addition of cyclosporine A to the standard induction reaction at a concentration of 1000 ng/ml (a concentration corresponding to levels found in the serum of patients treated with cyclosporine A) did not affect the transcriptional induction of class I genes with CLP (Fig. 9B). In addition, cyclosporine A did not block the induction of class I antigen by CLP, although CLP made in the presence of cyclosporine A (1000 ng/ml) was unable to induce class I antigen expression (Fig. 10).

DISCUSSION

RIN5F is a pancreatic beta-cell derived, continuous, clonal, insulin-producing cell line that was originally established by Gazdar and coworkers from a transplantable rat islet cell tumor. Due to the virtual impossibility of isolating a pure preparation of beta-cells, this cell line is an invaluable tool for the study of the immune-mediated destruction of the beta-cells that occurs in type I, juvenile or insulin-dependent diabetes mellitus and the mechanisms involved in evasion of immune surveillance by insulinoma cells. A central player in both of these phenomena are the MHC antigens. Both enhanced expression of class I and de novo expression of class II antigens on beta cells have been implicated in IDDM, and repressed levels of class I antigen has been implicated in tumor cell evasion of immune surveillance. These observations as well as similar findings in allograft rejection, graft versus host reactions, and a number of autoimmune disorders have stimulated the hypothesis that quantitative as well as qualitative variation in MHC gene expression might be a determinant in the immunogenicity of a cell.

In vivo, islet beta-cells normally exhibit a class II negative, class I low expressor phenotype Immune

histochemical analysis of pancreatic sections from newly diabetic or prediabetic human and rat biopsies reveals enhanced levels of class I antigen throughout the islet while the de novo appearance of class II antigens on islet beta cells remains a controversial finding. RIN5F cells are similar to endogenous islet beta-cells in that they exhibit a class II negative, class I low expresser phenotype. We do not know whether the level of class I antigen on RIN5F is lower than on normal untransformed beta-cells.

We have investigated the ability of CLP and recombinant gamma-interferon to modulate the expression of MHC class I and II genes in RIN5F. We demonstrate that incubation of RIN5F cells in various concentrations of CLP or gamma-interferon results in a dose-dependent enhancement of class I antigen expression eight to twelve hours postinduction. Class II antigen expression, on the other hand, is not inducible with these doses of inducer as late as 48 hours postinduction. The inability of CLP or gamma-interferon to induce class II antigen expression although inducing class II gene transcription, and the lag between transcription induction and RNA accumulation, demonstrate a role for posttranscriptional levels of control in gamma-interferon induced expression of MHC genes in RIN5F. Chen and coworkers report similar findings with the human leukemic cell line K562 (11). The class I antigen induction kinetics for RIN5F are similar to those described by Jones and coworkers for murine

myelomonocytic cells, Rosa and coworkers for human melanoma cells and by Chen and coworkers for K562 (11 13). Northern blots indicate that the level of class I heavy chain transcripts is enhanced at 8 hours postinduction while class II transcripts are not detectable until 20 hours postinduction. This striking difference in the induction kinetics of class I and II MHC genes supports the findings of Jones et al. that indicate that class I genes are early and class II genes late responders to gamma-interferon. Our findings using in vitro nuclear transcriptions clearly demonstrate a role for elevated rates of class I gene transcription in the increased abundance of class I transcripts and in the enhanced expression of class I antigen. They also show that the different kinetics of class I and II transcript accumulation are largely due to differences in the kinetics of transcriptional enhancement and activation of these genes. Friedman and Stark (using alpha-interferon) and Jones and coworkers (using gamma-interferon) report similar findings of distinct transcriptional kinetics for class I and II MHC genes (14,15) Both genes, however, exhibiting latency periods of hours, are late transcriptional responders to gamma-interferon relative to other interferon induced genes that respond within minutes (16) In summary, it appears that the mechanisms for the transcriptional induction of class I and II genes in RIN5F are complex in relation to other interferon induced genes and are likely distinct from each

other. RIN5F cells, then, represent a valuable tool for probing 1) the similarities and differences in the interferon mediated induction pathways for class I and II MHC genes and 2) posttranscriptional levels of control in class II gene expression

5-azacytidine is documented to have pleiotropic effects on cells and has been extensively used in studies of the role of DNA methylation in the regulation of gene expression (17). At a 2 uM concentration, 5-azacytidine is noncytotoxic but retains its ability to inhibit cytosine methytransferase Incubation in 2 uM 5-azacytidine, then, results in the hypomethylation of genomic DNA and has been documented to induce the expression of certain genes We demonstrate in this paper that preincubation of RIN5F cells in 2 uM 5-azacytidine results in an increased maximal level of CLP mediated induction of class I genes. These results suggest that 5-azacytidine and gamma-interferon additively induce class I gene expression (suggesting that 5-azacytidine and gamma interferon exert their effects via distinct mechanisms). Interestingly, incubation in 2 uM 5-azacytidine alone has little effect on the basal level of class I gene expression This suggests that 5-azacytidine might exert its effect by facilitating the interaction of (a) gamma-interferon induced second messenger(s) with regulatory regions of class I genes. Further investigations are necessary to understand this phenomenon

In vivo injection of cyclosporine A, a potent immunosuppressive drug, inhibits the induction of MHC antigens at sites of inflammation in allograft rejection and graft versus host disease (18). Cyclosporine A has a documented inhibitory effect on the expression of certain lymphokines (19). Indeed, we show in this paper that CLP made in the presence of cyclosporine A is unable to induce class I gene expression. We incubated RIN5F cells in the presence of cyclosporine A and CLP to assess whether cyclosporine A had direct inhibitory activity on the inducibility of class I MHC genes. The failure of cyclosporine A to inhibit the induction of class I genes with CLP suggests that in vivo cyclosporine A inhibits induction of class I genes via the suppression of lymphokine production and not due to a direct effect on class I gene expression.

The results presented in this current paper are in agreement with those of Campbell and coworkers who immunoprecipitated increased amounts of class I but no class II antigen from RIN5F cells induced with CLP or recombinant gamma-interferon (20). Separate immunohistochemical studies performed by our group on this and a different subclone of RIN5F cells confirm the class II interferon response negative phenotype of this clone but indicate that the second subclone harbors a small percentage of cells (5-10%) that can be induced to express low levels of class II antigen after prolonged incubation (48 hours) in high doses of gamma

interferon (in excess of 100 units/ml, data not shown) These results support the recently published findings of Thomas et al. that indicate heterogeneity of the cell surface phenotypes of subclones of the macrophage cell line WEHI-3 that have lost their Ia response but retained their class I response to gamma interferon (21) Clearly, however, in all subclones of RIN5F that we have analyzed, enhancement in MHC class I expression occurs 1) with a shorter latency period 2) at lower doses of inducer, and 3) on far greater percentage of cells (nearly 100%) than induction of class II antigen expression.

These in vitro findings parallel those of Campbell and coworkers who noted that incubation of murine pancreatic islets in gamma-interferon results in a ten-fold increase in H 2K antigen expression on beta cells while Ia antigen expression remains undetectable (22). On the other hand, Lacy's group has reported detecting class II antigen expression on beta cells in a similar experiment, as has Bottazzo's group using a combination of interferon and tumor necrosis factor (23,24). The in vivo results on de novo synthesis of class II antigen on beta cells prior to IDDM are also contradictory. The Cooke and Bottazzo laboratories report aberrant class II antigen expression on beta cells prior to IDDM in both man and the BB rat model of IDDM (25,26) In our laboratory we have never observed a class II positive beta-cell in prediabetic or diabetic BB rats (Issa-Chergui et al., manuscript submitted). A recent paper by

Chergui et al., manuscript submitted. In recent years, L. Pipeleers and coworkers might explain these discrepancies [17]. These investigators rigorously analyzed the insulin positive cells found within normal islets and found that these were mononuclear cells of nonendocrine origin that had phagocytosed fragments of damaged endocrine cells. The authors suggest that the insulin-positive, mononuclear cells in the pancreas of newly diagnosed diabetic individuals may be these mononuclear cells that have phagocytosed debris from ongoing beta-cell destruction. The, thus, suggest additional cell markers are necessary to test whether there are in fact class II expressing beta cells in the prediabetic or diabetic pancreas. As we and others have reported, however, one can consistently observe enhanced class I gene transcription and antigen expression throughout the islet prior to spontaneous IDDM. Enhanced class I antigen expression is another consistent finding in acutely diabetic BB rats where the syndrome is induced by the passive transfer of cytotoxic T lymphocytes from the spleens of acutely diabetic donors (Yale et al., manuscript submitted).

The *in vitro* and *in vivo* data from various laboratories suggest, then, that beta-cells in a prediabetic state are class II unresponsive while vigorously class I responsive to immunomodulators. One must question the clinical relevance of the ability to induce class II antigen expression on a small percentage of beta-cells *in vitro* using non-

these findings, we are in agreement with Campbell's hypothesis that enhanced class I gene expression on islet beta cells or surrounding cells (via a "bystander mechanism") might play an important role in the cascade of events leading to cytotoxic reactions in autoimmune IDDM. As it is not known whether gamma-interferon enhances the expression of only those class I genes constitutively expressed or activates normally silent class I genes, enhanced class I expression on target cells might result in the de novo recognition of the target cells by preexisting autoreactive T cells, or the activation of normally silent class I genes might present the target cell in an alloreactive context. Via either mechanism, we feel it is important to seriously consider a potential role for enhanced class I gene expression on beta-cells in IDDM.

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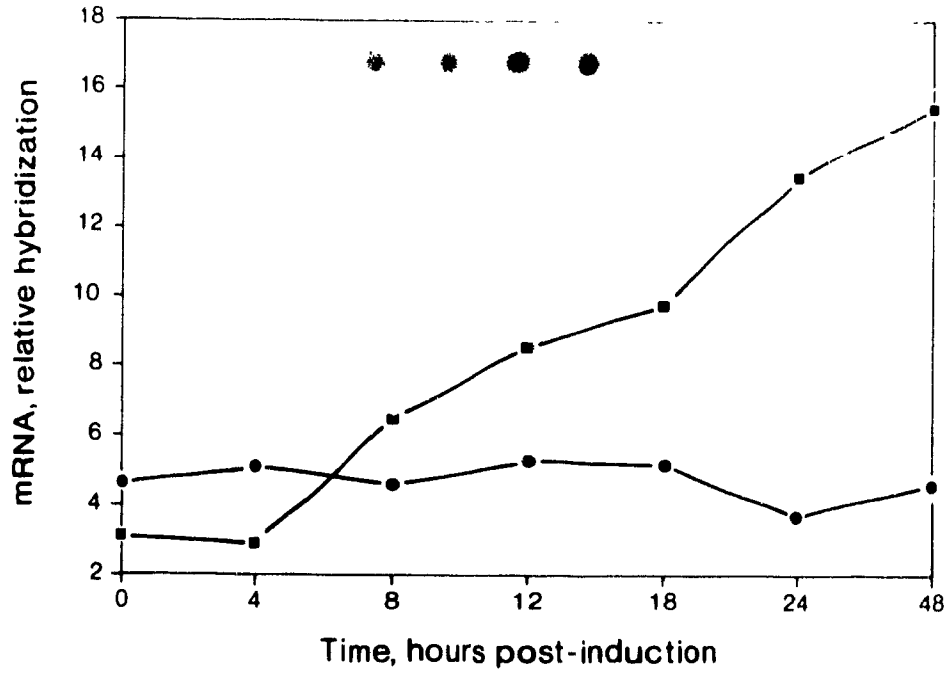
FIGURE 1. Induction of class I gene expression with supernatant from concanavalin A stimulated splenocytes Dot blot hybridization analysis of RNA isolated from 40% CLP treated RIN5F cells and densitometry of autoradiographic signals. A) (■,top panel of dots) representative results of hybridization with a ^{32}P-labeled 248 bp Pst I fragment from the class I cDNA clone ph2Ila; (●, bottom panel of dots) hybridization with the beta actin probe pA1. B) Dose dependency of induction of class I gene expression with CLP. The hybridization probe is the class I probe pH2Ila (▲,top panel of dots) cells treated with 60% CLP; (●,middle panel of dots) 40% CLP; (■,bottom panel of dots) 20% CLP, (◆) 40% CLP and annealed with the beta actin probe. Relative hybridization is in arbritrary units.

A


mRNA, relative hybridization
18
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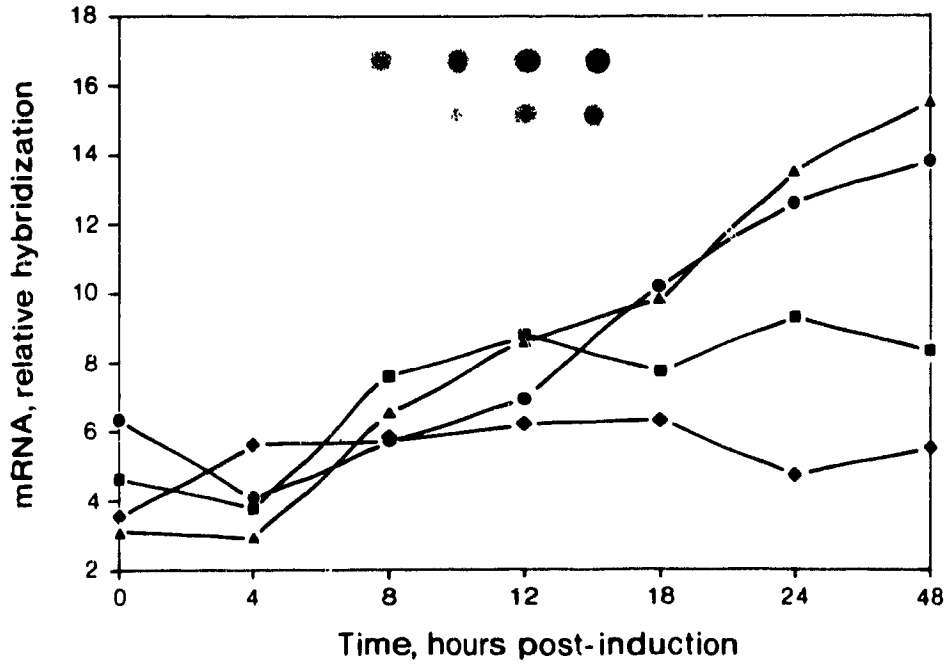
48
Time, hours post-induction

B


mRNA, relative hybridization
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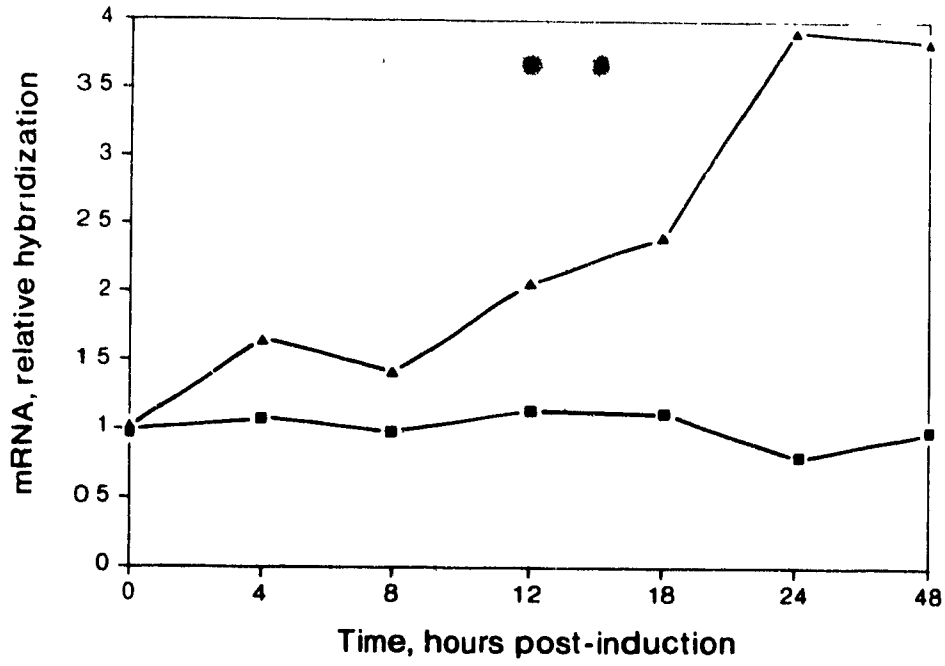
Time, hours post-induction

FIGURE 2. Induction of class II gene expression with CLP. A) Dot blot hybridization of RNA isolated from RIN5F cells treated with 40% CLP probed with the 4.7 kb BamHI/EcoRI I-A alpha probe from the BALB/c H-2^d clone 24.2 (▲, top panel of dots) and the beta actin probe (■, bottom panel of dots) B) cells induced with 40% CLP probed with the 700 bp Pst fragment from the I-E alpha cDNA clone pGem-IE^d alpha (▲, top panel of dots) and the beta-actin probe (■, bottom panel of dots).

A


mRNA, relative hybridization
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2 5
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1 5
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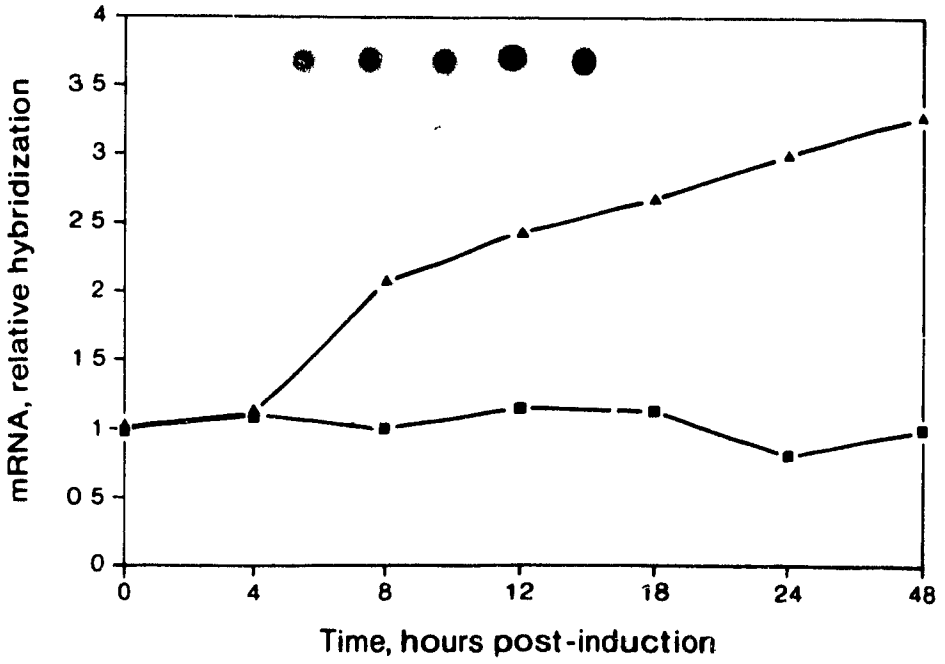
24
48
Time, hours post-induction

B


mRNA, relative hybridization
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12
18
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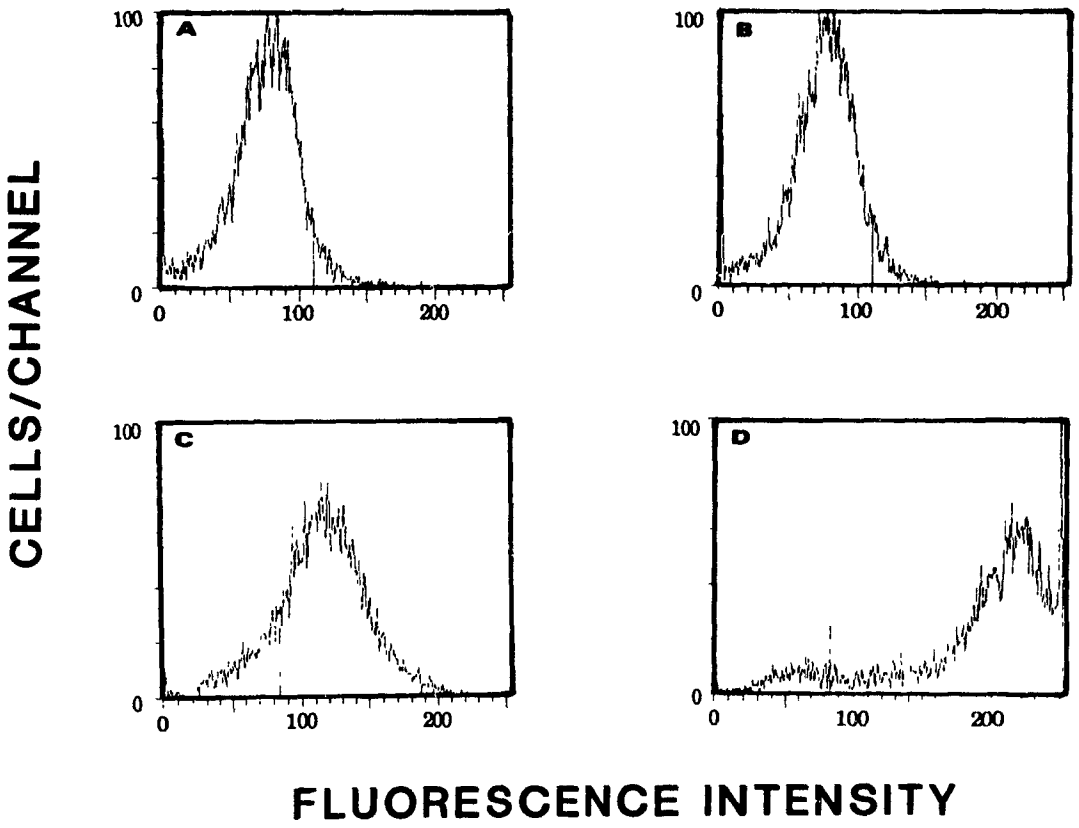
48
Time, hours post-induction

FIGURE 3. Inducibility of MHC antigens on RIN5F with 50% CLP 48 hours postinduction. FACS analysis using the class I directed monoclonal antibody OX18 and the class II directed monoclonal antibody OX6 followed by secondary incubation in FITC-conjugated goat or rabbit anti-mouse IgG2a. A) Uninduced RIN5F cells stained with FITC-labeled OX6. B) Induced RIN5F cells stained with FITC-labeled OX6. C) Uninduced RIN5F cells stained with FITC-labeled OX18. D) Induced RIN5F cells stained with FITC-labeled OX18.


CELLS/CHANNEL
A
B
C
D
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0
0
100
200
FLUORESCENCE INTENSITY

FIGURE 4. Kinetics of accumulation of MHC gene transcripts after incubation in 1 unit/ml recombinant rat gamma-interferon. A) Northern blot of RNA isolated from RIN5F cells at indicated time points postinduction with the class I probe and B) with the I-E alpha chain probe. RNA isolated from spleen was loaded in the lanes marked S. Equivalent amounts of RNA were loaded in each lane and was confirmed by hybridization with a beta-actin probe (data not shown).

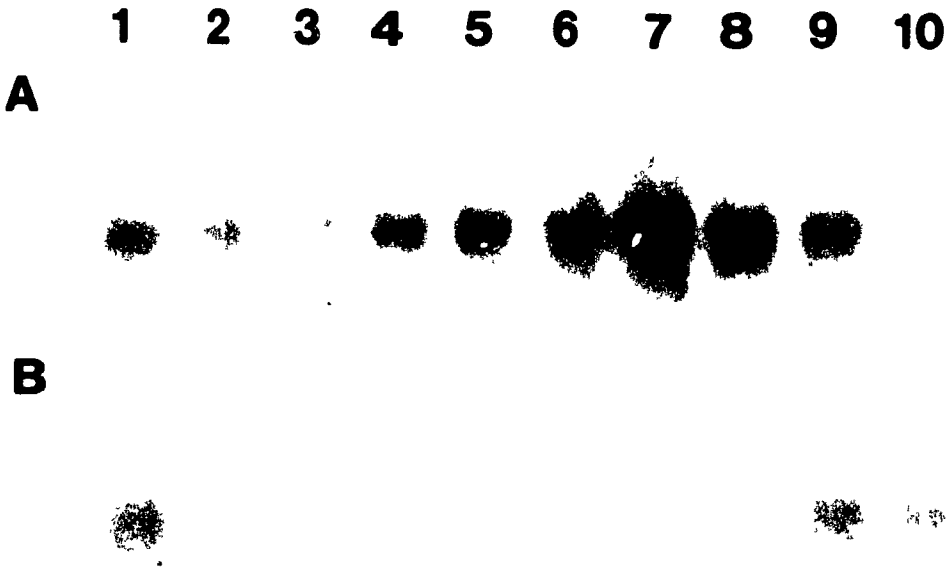

1 2 3 4 5 6 7 8 9 10
A
B

FIGURE 5. Dose-dependency of induction of class I gene expression after 20 hours of incubation of RIN5F cells in recombinant rat gamma-interferon. A) Northern blot of equivalent amounts of RNA isolated from cells induced with (lanes 1-5 in order). 0, 1, 5, 7, and 10 units/ml gamma interferon probed with the class I probe. B) Same northern blot annealed with the beta-actin probe after removal of the class I probe by boiling in dH_2O.

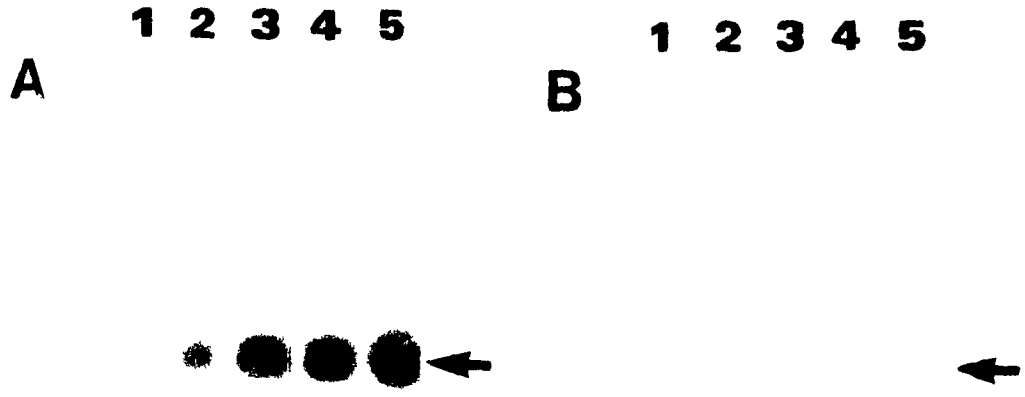

1 2 3 4 5
A
1 2 3 4 5
B

FIGURE 6. Kinetics of induction of MHC antigens after incubation in 1 unit/ml recombinant interferon. The bottom and middle rows of panels are data from the FACS analysis of RINSF cells induced with 1 unit/ml gamma-interferon and stained with FITC-labeled OX18. The individual panels in the middle row represent cell preparations removed after the indicated hours postinduction; the bottom row presents the same data sets as the middle row but in relation to forward light scatter. In the top row, the first two panels (from left to right) represent cells stained with the negative control monoclonal antibody P3 at t=0 and 48 hours postinduction, and the last two panels, cells at t=0 and t-48 stained with OX6. The middle panel in that row is a merged histogram of cells stained with OX18 at t=4, 12, and 48 hours to demonstrate the progressive induction of class I antigen expression.

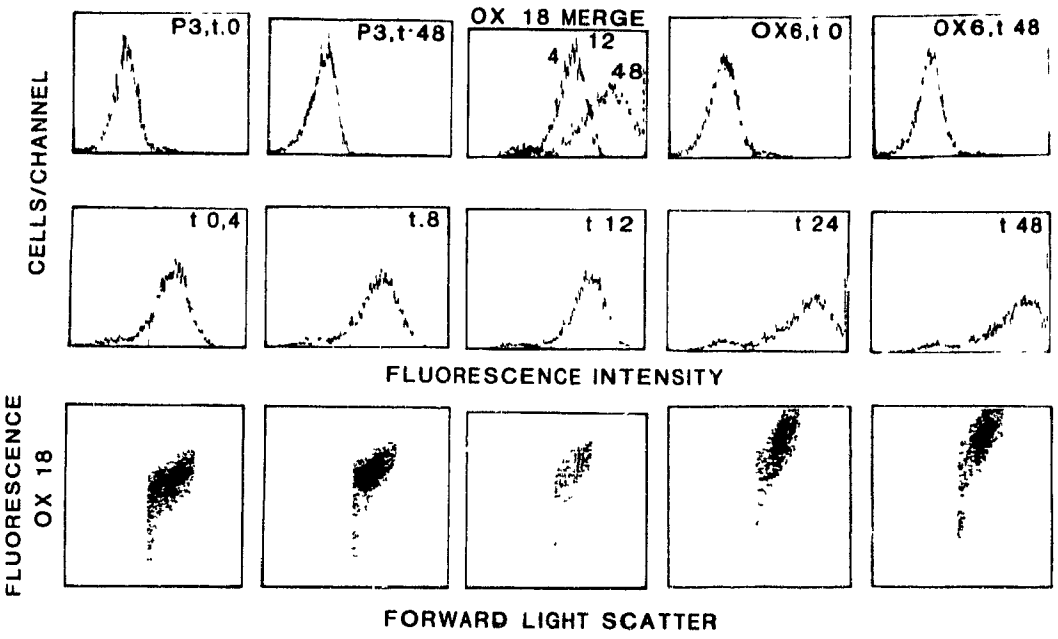
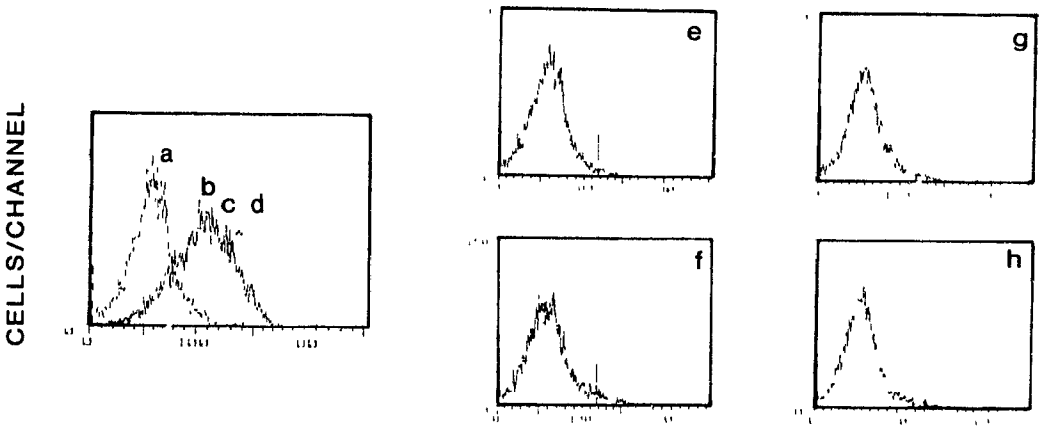

P3,t.0
P3,t·48
OX 18 MERGE
4
12
48
OX6,t 0
OX6,t 48
CELLS/CHANNEL
t 0,4
t.8
t 12
t 24
t 48
FLUORESCENCE INTENSITY
FLUORESCENCE OX 18
FORWARD LIGHT SCATTER

FIGURE 7. Dose-dependency of induction of MHC class I antigen after 24 hours of incubation in gamma-interferon. A) Unstained, untreated RIN5F cells, (b-d stained with FITC-labeled OX18) B) Untreated RIN5F cells C) 1 unit/ml interferon, D) 5 units/ml interferon; (e-h stained with FITC-labeled OX6) E) Untreated RIN5F cells, F) 1 unit/ml interferon for 48 hours, G) 5 units/ml interferon for 48 hours, H) 10 units/ml interferon for 48 hours.


CELLS/CHANNEL
a
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FLUORESCENCE INTENSITY

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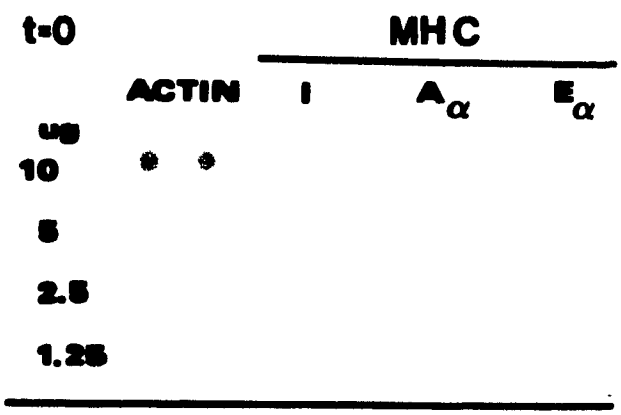
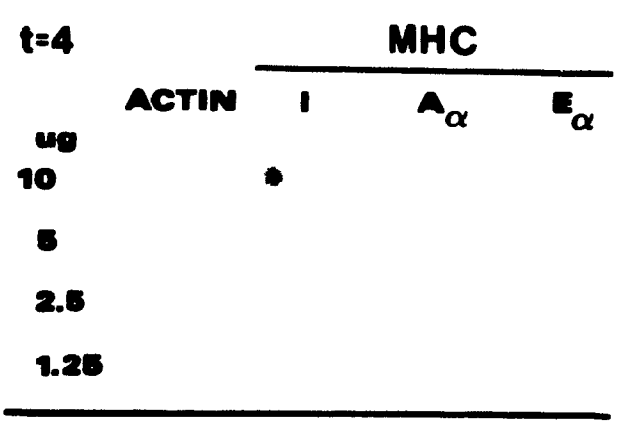
FIGURE 8. In vitro nuclear transcription of MHC genes in isolated nuclei from RIN5F cells induced with 1 unit/ml recombinant gamma-interferon. RIN5F cells were incubated for the indicated periods of time A) t=0, B) t=4, C) t=16 hours, nuclei were isolated, and labeled nuclear RNA extracted after nascent chain elongation was allowed to proceed in the presence of alpha^{32}P-UTP (28). Labeled RNA was annealed to dots of denatured DNA (corresponding to the locus specific fragments used as probes in the previous figures) at the indicated concentrations on nitrocellulose filters. Unpaired RNA was removed by digestion with RNase, and hybrids detected by autoradiography. Relative rates of transcription for a given gene at the different time points were compared by densitometry of the resulting autoradiographic signals (with the beta-actin signal serving as an internal control)

A


t=0
MHC
ACTIN
I
A_α
E_α
ug
10
5
2.5
1.25

B


t=4
MHC
ACTIN
I
A_α
E_α
ug
10
5
2.5
1.25

C

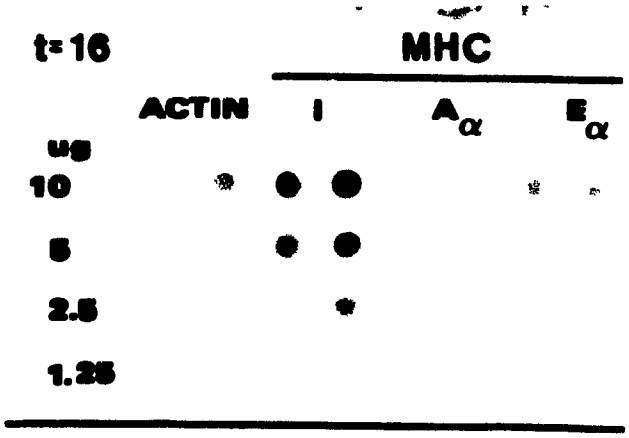
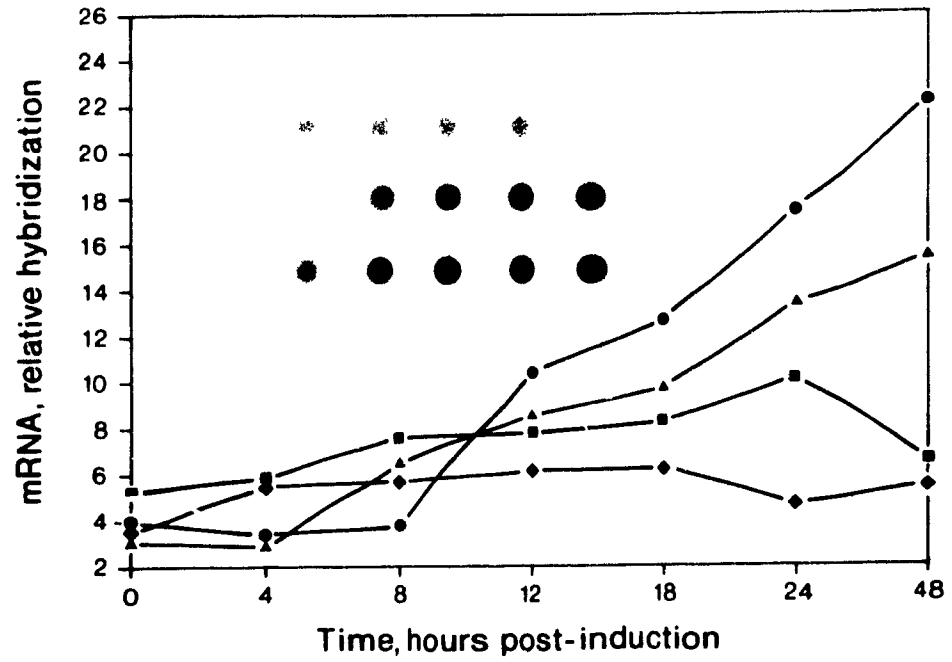

t=16
MHC
ACTIN
I
A_α
E_α
ug
10
5
2.5
1.25

FIGURE 9. Effects of 5-azacytidine preincubation or cyclosporine A coincubation on induction of class I gene expression with CLP. Dot blot hybridizations of RNA isolated from RIN5F cells at indicated time points postinduction in 40% CLP after preincubation for 24 hours in 2 uM 5-azacytidine (A) or coincubation in 1000 ng/ml cyclosporine A (B). The hybridization probe unless otherwise indicated is the class I cDNA probe. A) (◆, top panel of dots) cell treated with 40% CLP annealed with the beta-actin probe; (■) cells treated with 5-azacytidine alone; (▲, middle panel of dots) 40% CLP alone, (● ,bottom panel of dots) treatment with 40% CLP after 5-azacytidine preincubation. B) (◆) 40% CLP, annealed with the beta-actin probe, (■, top panel of dots) 40% CLP, (●) DMSO and 40% CLP; (▲ bottom panel of dots) 40% CLP and cyclosporine A.

A


mRNA, relative hybridization
26
24
22
20
18
16
14
12
10
8
6
4
2
0
4
8
12
18
24
48
Time, hours post-induction

B

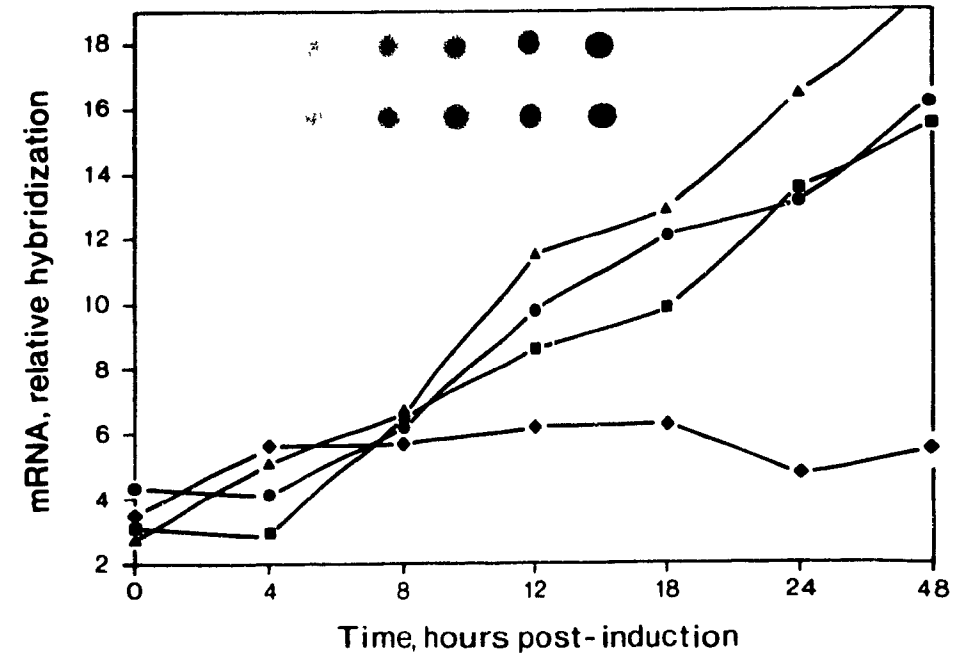
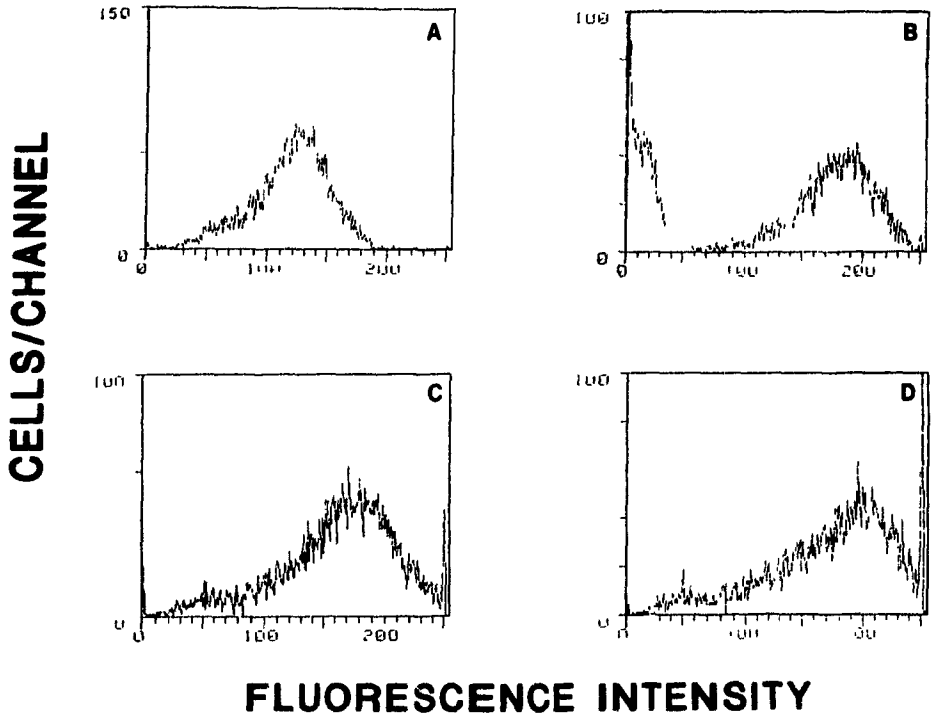

mRNA, relative hybridization
18
16
14
12
10
8
6
4
2
0
4
8
12
18
24
48
Time, hours post-induction

FIGURE 10. Effect of preincubation in 5-azacytidine or coincubation in cyclosporine A on class I antigen induction by CLP. FACS analysis of RIN5F cells induced with 40% CLP for 24 hours and stained with FITC-labeled OX18. A) cells treated with 40% CLP/cyclosporine A (this CLP was made using the standard conA stimulation protocol with the addition of cyclosporine A at 1000 ng/ml). B) cells treated with 40% CLP, C) cells treated with 40% CLP and cyclosporine A at 1000 ng/ml; D) cells preincubated in 2 uM 5-azacytidine for 24 hours prior to treatment with 40% CLP


CELLS/CHANNEL
A
B
C
D
FLUORESCENCE INTENSITY

CHAPTER SEVEN.

GENERAL DISCUSSION

Insulin-dependent diabetes mellitus results from the selective destruction of the insulin-producing islets of Langerhans of the pancreas. IDDM occurs spontaneously in mice, rats and humans, and has a multifactorial etiology. Development of IDDM depends in part on heritable susceptibility genes, of which at least one resides within the major histocompatibility complex (MHC). The aim of the research described in this dissertation was to investigate the nature of the MHC association of IDDM in the spontaneously diabetic BB rat.

ASSOCIATION OF INSULIN-DEPENDEMT DIABETES MELLITUS WITH GENES OF THE MAJOR HISTOCOMPATIBILITY COMPLEX

IDDM in mice, rats and man is associated with genes within the MHC Population and family studies indicate that at least one IDDM susceptibility gene resides within the HLA-D region of the human MHC. The genes which encode the alpha and beta chain polypeptides of the class II molecules of the MHC are located in this region. IDDM is most closely associated with the HLA DR and -DQ loci within this region. Of the two loci, restriction fragment length polymorphism analysis indicates that the strongest association is with the HLA-DQ locus. Two HLA-DR alleles are negatively associated with the

disease. Among Caucasian diabetics, the DR4 DQw3.2, DR3, DR1, DR2-AZH, and DRw8-Dw19 haplotypes are associated with susceptibility for IDDM, while DR2 Dw2, DR2-Dw12, DP5, and DR4-DQw3.1 are negatively associated with the disease.

In the nonobese diabetic (NOD) mouse IDDM is thought to involve at least three recessive susceptibility genes. At least one of these genes resides with the mouse MHC (H-2) located on chromosome 17. The NOD mouse does not express the I-E class II molecule, and the HLA DQ$_{beta}$ homolog, I-A$_{beta}$, is unique among all the sequenced I-A$_{beta}$ genes. It is assumed, but not rigorously proven that the I-A$_{beta}$ chain gene is one of the IDDM susceptibility genes in the NOD mouse.

IDDM in the BB rat absolutely requires at least one chromosome bearing the RT1^{u} haplotype at the MHC. The susceptibility determinant(s) encoded within the BB rat MHC are necessary but not sufficient for the development of IDDM. At least two other genes and unknown environmental stimuli are thought to contribute to the development of the disease.

PENETRANCE OF THE MHC SUSCEPTIBILITY DETERMINANT(S)

The mode of inheritance of human IDDM is complex. Several DQ alleles are found in patients with IDDM, so the MHC susceptibility component can act in a dominant fashion. Up to 10% of patients with IDDM carry one resistance allele at the MHC. The relative risk of individuals bearing two susceptibility alleles (although elevated) is not remarkably

different from individuals bearing a single susceptibility allele. In mice, the MHC susceptibility determinant is clearly recessive All non-NOD H 2 haplotypes confer dominant resistance to IDDM when placed on a NOD genetic background. In rats, although the frequency of IDDM is elevated in animals homozygous for the $RT1^u$ haplotype, diabetic heterozygotes are frequently observed. The penetrance of the MHC susceptibility determinant was investigated in Chapter II 18% of the F2 offspring from intercrosses between BB rats and normal Lewis and Buffalo rats were heterozygous for the $RT1^u$ haplotype as determined by serotyping at the class I RT1A locus and by restriction fragment length polymorphism analysis at the RT1B and D class II loci and class I genes flanking the immune response region. These data demonstrated that a single chromosome carrying the $RT1^u$ haplotype is sufficient for the development of IDDM in the BB rat and that the MHC associated susceptibility determinant(s) are dominant traits. The BB rat model, then, resembles human IDDM more closely than the NOD mouse model with regard to penetrance of the MHC susceptibility determinant(s).

MAPPING OF SUSCEPTIBILITY DETERMINANTS WITHIN THE ASSOCIATED MHC HAPLOTYPE

The association of IDDM with the HLA was first described fifteen years ago Subsequently, studies using serologic reagents mapped disease association to the HLA-D region.

Greater than 95% of patients with IDDM had the HLA DR3 and/or DR4 haplotypes compared to 45% of the normal population. Subsequent studies employing restriction fragment length polymorphism analysis indicated a stronger association with HLA-DQ than HLA-DR, but did not precisely map a susceptibility determinant or RFLP that correlated with IDDM in the three haplotypes that are permissive for IDDM (DR1, DR3 and DR4) It is still unclear how many susceptibility determinants reside within the HLA

The susceptibility determinant(s) in the NOD mouse MHC have not been formally mapped As the I-E molecule is not expressed in the NOD mouse, it is generally assumed that the I-A molecule is one of the susceptibility determinants It is also of interest that expression of the I-E molecule in transgenic NOD mice results in protection from IDDM The significance of this result will be discussed later in this chapter. Other susceptibility determinants could reside elsewhere in MHC.

Chapters 3 and 4 describe breeding studies that map the susceptibility determinants in the BB rat MHC between the RT1.A and RT1.C loci. Previous breeding studies had demonstrated that at least one chromosome bearing the u haplotype was necessary for the development of IDDM These studies also determined that other haplotypes (e.g. RT1°, were not permissive for the development of IDDM when placed in the BB rat genetic background. The breeding studies in Chapter 3

and 4 make use of the recombinant haplotypes .r4 and .r8. The .r4 haplotype results from a recombination between the RT1 D^u locus and the RT1.C^a locus. The .r8 haplotype results from a recombination between the RT1.A^a locus and the RT1.B^u locus. Southern blot analysis indicated that these haplotypes sustained only one recombinant event within the MHC. Animals homozygous for the recombinant haplotypes were identified by restriction fragment length polymorphism analysis. Both .r4 and .r8 homozygous animals developed IDDM. As these animals were respectively homozygous for a non-permissive allele at the RT1.C and RT1.A loci, these experiments mapped the IDDM susceptibility determinants in the BB rat MHC between RT1.A and RT1.C. It should be emphasized that these experiments are unique in that the mapping of susceptibility genes is not based upon statistical correlation (as is the case with human IDDM) or by inference (as is the case with the NOD mouse) but on functional assay in vivo

The rat immune response region, encoding the class II antigens RT1.B and RT1.D, resides between RT1.A and RT1.C. By inference, the mapping of IDDM susceptibility to this region would strongly suggest that one or both of the class II molecules are susceptibility determinants. Although there is no formal proof that this is the case, a report that IDDM could be prevented in BB rats by the injection of anti-class II monoclonal antibody provides circumstantial support for this hypothesis. Although it is likely that one of the class

II molecules is a susceptibility determinant, this does not exclude the possibility that other genes also found in the immune response region (or at least, mapping between RT1 A and RT1.C) participate in genetic predisposition for IDDM.

Although all of the MHC linked susceptibility determinants required for the development of IDDM map between RT1.A and RT1.C, it is of interest that the .u4 homozygous animals developed an IDDM with an altered pathology .u1 homozygous diabetic animals presented with sparse or absent pancreatic lymphocytic infiltration with true insulitis being a rare finding. This result indicates that a gene or genes closely linked to the RT1.C locus affects the magnitude of insulitis.

SEQUENCE ANALYSIS OF CLASS II BETA CHAIN GENES

Working on the assumption that the HLA D linked susceptibility determinant(s) for IDDM were the class II HLA genes, McDevitt and his coworkers sequenced genes coding the four class II molecules isolated from IDDM patients and healthy controls. They found that all sequences identified in patients were also present in the normal population and concluded that IDDM is not the result of mutant class II alleles. It was known, however, that IDDM was associated with the DQw3.2 allele of the DR4 haplotype, and negatively associated with the DQw3.1 allele. Sequence analysis of the DQ alpha and beta chain genes of the two alleles showed that

while the alpha chains were identical, the beta chains of the two DQ alleles differed at amino acid positions 13, 26, 45 and 57. Position 57 was the only polymorphic residue that correlated well with IDDM susceptibility. All DQ alleles that were negatively associated with IDDM had an aspartate at position 57, while those positively associated with disease had an alanine, valine or serine residue. Analysis of 39 IDDM patients indicated that 90% were homozygous for non-aspartate at position 57, while 10% were non-Asp/Asp heterozygotes. None of the patients were Asp homozygotes.

Sequence comparison of the I-A beta (the DQ beta chain homologue) chain genes of the NOD mouse and other I-A beta alleles indicate that the NOD mouse allele is unique among all sequenced I-A beta alleles. The NOD mouse I-A beta chain has a Ser at position 57, while all other I A beta chains have an Asp at that position. In contrast to human IDDM, however, homozygosity for the NOD I-A beta allele appears to be absolutely required for disease susceptibility.

The breeding studies presented in Chapters 3 and 4 indicated that susceptibility determinant(s) found between RT1.A and RT1.C of multiple u haplotypes could permit the development of IDDM when placed on a BB rat genetic background. These results strongly suggested that the susceptibility determinants encoded within this region were not unique to the BB rat u haplotype, and that resistance determinants within the Wistar Furth genome would not reside

within this region. In fact, sequence analysis of the beta chain genes of both the RT1.B and RT1.D class II molecules indicate that both beta chains from susceptible and non-susceptible BB rat strains have serine at position 57. Furthermore, serine at position 57 is found in several other RT1.B and RT1.D beta chain alleles that do not permit the development of IDDM on a BB rat genetic background. Thus, there is no correlation between position 57 and IDDM susceptibility in the BB rat, and the specific susceptibility determinant(s) within the rat MHC remain unidentified.

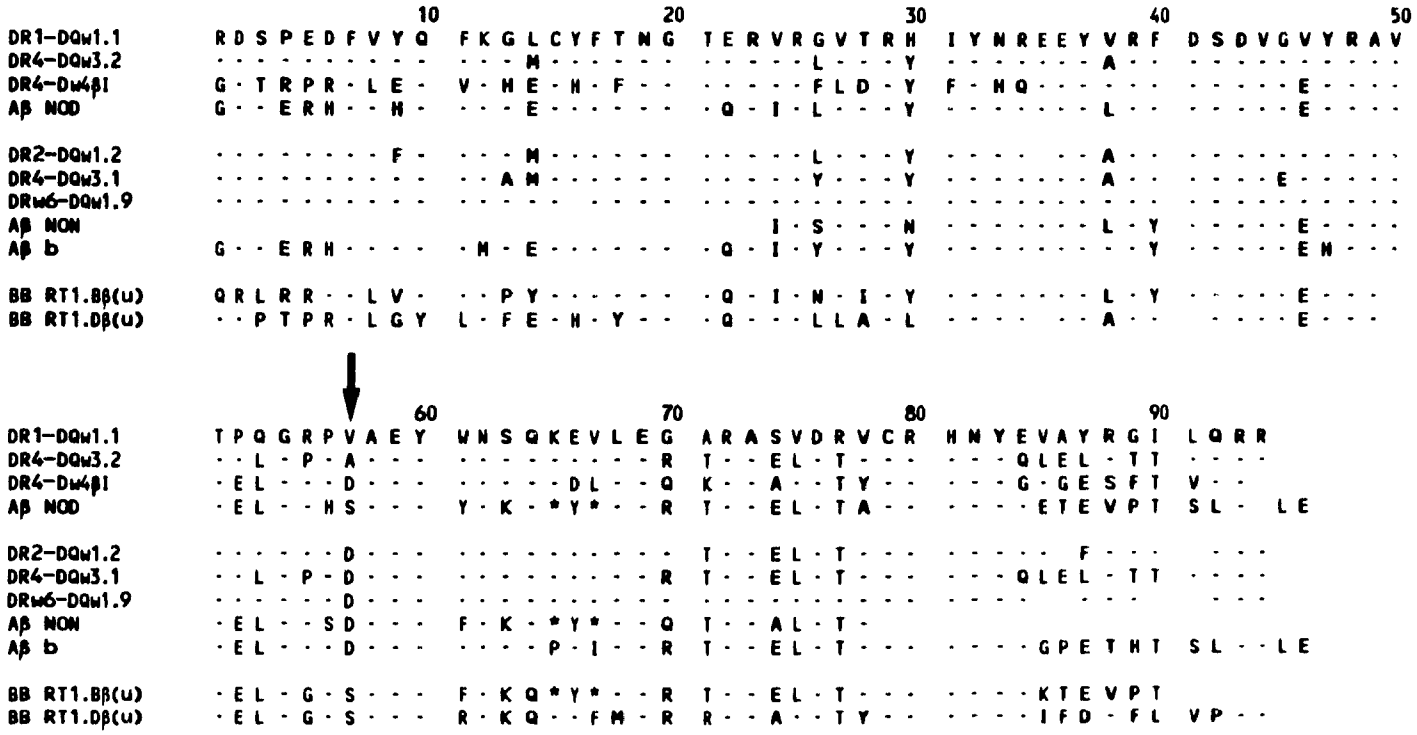


FIGURE 1. Sequence analysis of class II beta chains in diabetes prone and resistant humans, mice and rats (Todd et al., 1987).

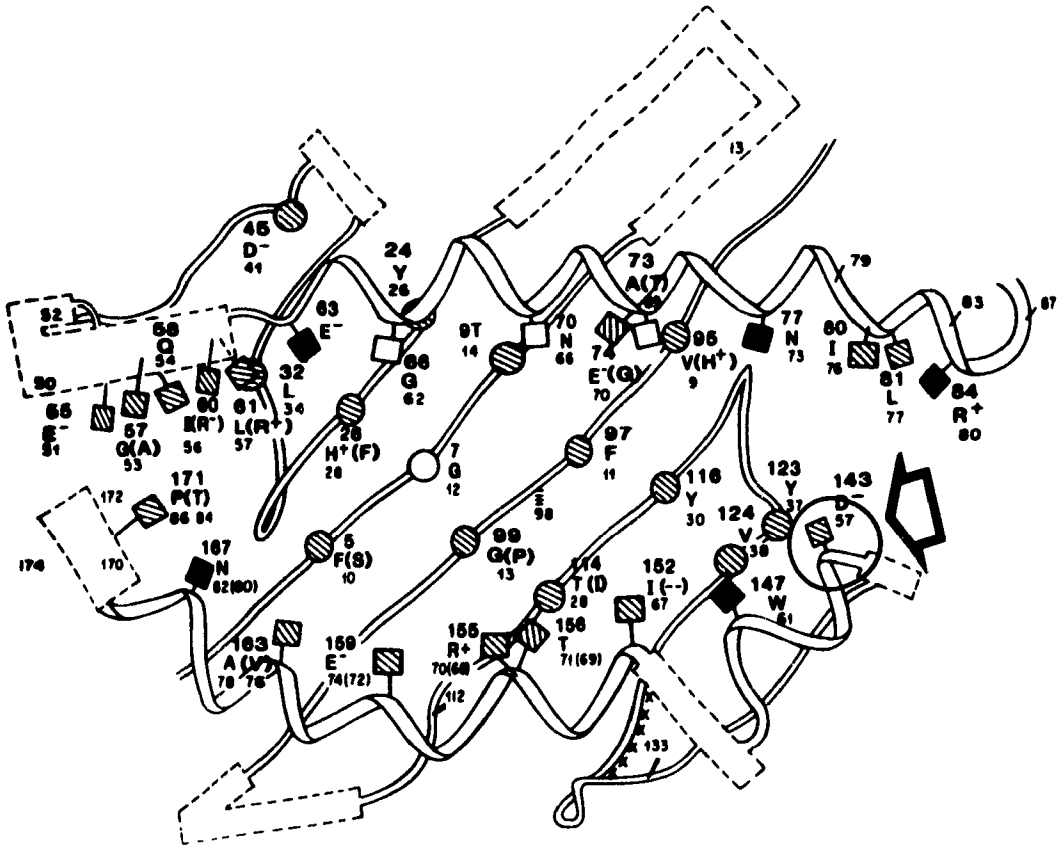


FIGURE 2. Predicted structure of a class II MHC molecule and location of amino acid position 57 (Paul, 1989).

ABERRANT EXPRESSION OF MHC ANTIGENS AND IDDM

Bottazzo and coworkers set forth the hypothesis that aberrant expression of class II molecules might explain the correlation of IDDM susceptibility with specific alleles of these molecules. Aberrant expression would present self antigen normally not seen by the immune system, and was hypothesized to be a potential primary event in the pathogenesis of IDDM. Circumstantial support for the hypothesis came from the observation of aberrant class II expression on the islets of pancreatic tissue taken from a child that died of acute IDDM. This observation stimulated several studies that investigated the expression of MHC antigens on pancreatic islets prior to and at the time of onset of IDDM. Additional studies were performed to investigate the inducibility of MHC antigens on pancreatic islets and on insulinoma cell lines upon exposure to crude lymphokine preparations and/or recombinant gamma-interferon. The results of these studies have been contradictory, and it remains unclear whether aberrant expression of class II antigens on beta cells actually occurs. It has been suggested that the Ia-positive, insulin-positive cells that were reported in Bottazzo's initial publication may have been mononuclear cells that had phagocytosed debris from ongoing beta-cell destruction. The studies described in this thesis indicate that aberrant class II expression does not occur on

the islets of prediabetic or newly diabetic BB rats. The results do indicate, however, that a very early event in the histopathology of the prediabetic pancreas is an enhancement in MHC class I expression. Subsequent studies have confirmed these observations, and the hypothesis that enhanced class I expression might play a role in the pathogenesis of IDDM is receiving growing support

ON THE ROLE OF THE MHC IN THE PATHOGENESIS OF INSULIN-DEPENDENT DIABETES MELLITUS.

Insulin-dependent diabetes mellitus is a disease that results from a multifactorial etiology. Several genes and unknown environmental factors are necessary for the development of the disorder. At least one IDDM susceptibility gene resides within the MHC. Several facts strongly suggest that one of the susceptibility genes is a class II MHC gene. 1) The breeding studies described in this thesis map the MHC associated susceptibility gene(s) in the BB rat to the immune response region. 2) There is a correlation betwen position 57 of the HLA-DQ beta chain in man, and the I-A beta chain in the NOD mouse, and susceptibility to IDDM. 3) Injection of anti-class II monoclonal antibody into prediabetic animals can abrogate the development of IDDM. Finally, association with a class II MHC gene is compatible with the proposed autoimmune pathogenesis of the disease.

Assuming that a class II MHC gene is a susceptibility

determinant, how might it operate? The correlation between IDDM susceptibility and position 57 of DQ beta and I-A beta serves as a good case. Position 57 is hypothesized to be located at one end of the peptide binding cleft of a class II MHC molecule. Based on the crystal structure of the class I HLA-A2 molecule, the amino acid at position 57 would point in to the peptide binding groove and could form a salt bridge with an arginine found on the other alpha-helix encoded by the alpha chain gene. Therefore, the residue at position 57 could profoundly affect the size and shape of the peptide binding groove. Several other residues could also affect the peptide binding groove and could explain association of IDDM with a particular class II molecule in the BB rat model

A specific conformation of a class II MHC molecule could affect the proper functioning of the immune system at two levels. Class II molecules expressed in the thymus directly influence the selection of the T cell repertoire. A specific class II molecule might fail to negatively select a T cell required for the pathogenesis of IDDM. Alternatively, the associated class II molecule might positively select an autoreactive T cell. The associated class II molecule could also operate in the periphery. A particular conformation of the peptide binding cleft might allow the binding of an autoantigen not normally seen by the immune system that shares an epitope with pancreatic beta cells. Exogenous antigen could also trigger autoimmunity A virus could harbor a beta

cell cross reactive peptide whose presentation by a class II molecule relies upon the conformation of the peptide binding cleft. Via any of these mechanisms, it is not difficult to envision a role for a particular class II MHC molecule in the pathogenesis of IDDM.

How might enhanced class I MHC expression on the pancreatic islets contribute to the development of IDDM? At first glance, the enhanced expression of class I molecules in the prediabetic pancreas is enigmatic due to the mapping of susceptibility determinants to the immune response region of the MHC. This dichotomy, however, could simply mean that the two phenomena operate via distinct mechanisms. Class II associated susceptibility determinants might operate as suggested previously, while enhanced class I expression might be controlled by allelic genes (found outside the MHC) encoding trans-acting factors. Class I antigens, then, would participate in the pathogenesis of IDDM by quantitative and not qualitative means. Therefore, unlike the class II case, any class I antigen, if overexpressed, could contribute to the development of IDDM. There is substantial evidence that the concentration of class I antigen on a target cell determines its immunogenicity. Pancreatic beta cells normally express very low levels of class I antigen. Overexpression of class I antigen on pancreatic islets might present beta cell antigen to the immune system de novo. Beta cells expressing high levels of class I antigen would also be more easily recognized

by cytotoxic T cells once autoimmunity had initiated. Finally, the transgenic experiments have indicated that overexpression of class I antigen on beta cells is directly cytocidal to the beta cell. Enhanced class I expression on beta cells could therefore contribute to the pathogenesis of IDDM at various levels.

THE PATHOGENESIS OF IDDM - A SCENARIO.

Multiple susceptibility genes are required for the development of IDDM. At least one of these genes is likely to encode a class II MHC antigen. Position 57 of the HLA-DQ beta chain and of the I-A beta chain correlates with disease susceptibility. This residue is positioned within the class II molecule where it can exert a profound effect on the conformation of the peptide binding cleft. As there is no correlation between position 57 and IDDM susceptibility in the BB rat, other residues likely define disease susceptibility in the rat system. These residues are not unique to the BB rat MHC, but are associated with the u haplotype. A particular conformation of the associated class II molecule probably exerts a primary effect at the level of thymic education. The importance of thymic education in influencing the development of IDDM is best illustrated in the I-E transgenic NOD mouse that is no longer prone to insulitis and IDDM. Heritable predisposition for IDDM, however, only sets the stage for an unknown initial trigger. It is unlikely that this trigger is

the aberrant expression of class II antigens. In the BB rat, class II positive beta cells are not observed, and in man their existence is controversial. Hyperexpression of class I MHC antigens throughout the pancreatic islets of prediabetic animals is a consistent finding. In our studies, we were unable to determine conclusively whether enhanced class I expression preceded or was a consequence of early insulitis. Subsequently, Cooke and coworkers have reported that enhanced class I expression is the earliest phenomenon observed in the pancreas of prediabetic BB rats. They report that enhanced class I expression precedes insulitis and the accumulation of ED1 macrophages. The cause of enhanced class I expression on pancreatic islets is unknown. It has been suggested that viral infection might induce enhanced class I expression. Alternatively, a non-MHC encoded susceptibility determinant might encode a transactivator of class I genes. If enhanced class I expression results from viral infection, this would involve endogenous viruses in BB rats where IDDM develops in rats raised in gnotobiotic conditions. Whatever the cause, enhanced class I expression could play either a direct or indirect role in the pathogenesis of IDDM. High levels of class I expression on cells that normally express very low levels of class I antigen might result in the de novo presentation of beta cell antigen to the immune system. In this scenario, enhanced class I expression would play a direct role in disease pathogenesis. Alternatively, enhanced class I

antigen could play a secondary role by making beta cells easier targets for cytotoxic T cells once autoimmunity has already initiated. Finally, enhanced class I expression could be directly cytocidal to beta cells

Clearly, many questions remain to be answered with respect to the causes of IDDM. The research described in this thesis and in the literature suggest that both class I and class II MHC molecules may play a role in the development of IDDM. In the case of class II molecules, researchers must focus their efforts on how particular class II molecules predispose the individual for IDDM Other research should probe what role enhanced class I expression might play in disease pathogenesis. These experiments should make use of transgenic mice and should focus on methods to selectively down regulate class I expression in pancreatic islets Finally, an intensive effort should be made to locate other IDDM susceptibility genes. The existence of well defined and characterized animal models of the disease will facilitate these efforts.

CLAIMS TO ORIGINALITY

The following results presented in this thesis are original:

1) A single dose of the u haplotype of the rat major histocompatibility complex (RT1) is shown to be sufficient to permit the development of insulin-dependent diabetes mellitus in the BB rat. Diabetic animals heterozygous for the u and b haplotypes at the RT1 complex were identified by restriction fragment length polymorphism analysis (RFLP) using multiple class I and class II MHC probes.

2) Diabetic animals homozygous for the .r8 recombinant haplotype were identified by RFLP analysis. The development of IDDM in these animals demonstrates that the RT1.A locus is not involved in disease susceptibility.

3) Diabetic animals homozygous for the .r4 recombinant haplotype were identified by RFLP analysis. The development of IDDM in these animals demonstrates that the RT1.C locus is not involved in disease susceptibility. Together with claim 2, IDDM susceptibility determinants map between RT1.A and RT1.C, i.e. to the RT1 immune response region.

4) Susceptibility determinants located with the BB rat u haplotype are likely not to be unique to the BB rat The demonstration that rats homozygous for the _rt1.1_, _rt8_ and Wistar Furth u haplotypes develop diabetes strongly suggests that any u haplotype can permit the development of IDDM when placed on a BB rat genetic background.

5) Expression of MHC class I heavy chain gene(s) is enhanced in the pancreatic islets of prediabetic and newly diabetic BB rats. Northern blotting of RNA isolated from islets of age-matched BB and Wistar Furth rats indicates that enhanced class I expression is a very early event that precedes the onset of overt IDDM.

6) Incubation of RIN5F cells with crude lymphokine preparations or recombinant gamma-interferon resulted in the induction of both class I and II MHC genes Induction of the two classes of genes followed very different kinetics, however. In vitro nuclear transcription assays indicated that induction of the genes had a transcriptional basis Although class II RNA was induced by treatment with gamma interferon, class II antigen was not detected on the cell surface as assayed by flow cytometric analysis. Preincubation of RIN5F cells in the hypomethylating agent 5-azacytidine enhanced the induction of class I RNA, while the addition of cyclosporine A did not inhibit the induction of class I gene(s)