Insulin-Dependent Diabetes Mellitus Induced in Transgenic Mice by Ectopic Expression of Class II MHC and Interferon-Gamma

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Summary

We have produced transgenic mouse strains harboring class II major histocompatibility complex or interferon-gamma genes linked to the human insulin promoter. These experiments were designed to investigate the consequences of the expression of immunological effector molecules by nonimmunological cells. In both of these studies we observed the disappearance from the pancreas of the insulin-producing β cells coinciding with the development of insulin-dependent diabetes mellitus. Transgenic mice expressing both chains of the I-A gene showed progressive atrophy of the islets of Langerhans, whereas mice expressing interferon-gamma suffered an inflammatory destruction of the islets.

Introduction

Insulin-dependent (Type I) diabetes mellitus (IDDM) is a metabolic disorder resulting from the lack of endogenous insulin secretion. This is due to a disease process caused by the disappearance of the insulin-producing β cells from the pancreatic islets of Langerhans (reviewed by Gepts and LeCompte, 1985). Human IDDM and laboratory animal experimental diabetes mellitus are thought to be the result of both genetic and epigenetic components. The genetic component consists of at least one gene in the major histocompatability complex (MHC) that contributes to susceptibility to the disease. For the disease to develop, some epigenetic triggering event must also occur. Experimentally, this triggering event can be mimicked by either pharmacologic or infectious agents, including several viruses. The pharmacologic agents are thought to be toxic to the β cells, whereas the pathway of disease induction by the viral agents is unknown. Evidence has been mounting that IDDM has an autoimmune pathogenesis (reviewed by Rossini et al., 1985; Eisenbarth, 1986).

MHC class II molecules, normally expressed on the surface of B lymphocytes, macrophages, and dendritic cells, play a central role in the immune response by presenting antigen to T helper (T_H) cells. The presence of the class II molecules may be sufficient for antigen presentation in vitro; fibroblasts transfected with the appropriate class II genes and lipid bilayers incorporating purified class II molecules can present antigen and activate appropriate T cell responses (Malissen et al., 1984; Norcross et al.,

1984; Watts et al., 1984; Walden et al., 1985). Whether the class II molecules are sufficient for T_H activation in vivo has not been resolved (Unanue, 1984). The class II genes lie within the I-A and I-E subregions of the H-2I region in mice and within the HLA-D region in man and encode heterodimeric molecules composed of a 34 kd α chain and a 28 kd β chain.

The ectopic expression of class II MHC molecules on nonimmune cell types has been documented in a variety of autoimmune states (reviewed by Pujol-Borrell et al., 1986b). Class II MHC molecules have been found on pancreatic β cells taken from newly diagnosed patients with IDDM (Bottazzo et al., 1985; Foulis and Farquharson, 1986; Foulis et al., 1987). While the significance of this is unknown, it has been speculated that the class II expression could cause presentation of self antigens, resulting in immune mediated destruction of the tissue. Although the cause of the aberrant class II expression is not known, it is noteworthy that the lymphokine interferon gamma (IFN-γ) can induce class II expression on a wide variety of cell types in vitro (reviewed by Rosa and Fellous, 1984). However, it is unclear whether IFN-γ alone can induce class II expression on β cells of cultured islets (Campbell et al., 1985; Campbell et al., 1986; Pujol-Borrell et al., 1986a; Wright et al., 1986; Pujol-Borrell et al., 1987). IFN-γ is produced by the host in response to viral and bacterial infections. As opposed to the other interferons, IFN-γ is apparently a product only of T lymphocytes and natural killer (NK) cells, possesses unique immunomodulatory properties, and can also affect nonlymphoid tissues.

An intriguing potential pathway for autoimmune endocrine disorders has recently been proposed (Bottazzo et al., 1983). Briefly, local viral infection or other environmental factors would lead the activation of T cells and subsequent local production of IFN-γ. The IFN-γ may then induce the expression of class II MHC molecules not only on normal antigen-presenting cells, but also on local epithelial cells, such as the pancreatic β cells. Such expression could cause the β cell to act as an antigen-presenting cell, which may then present autoantigens to the host immune system and initiate an autoimmune response.

We sought to examine the relevance of these concepts by determining whether the expression of IFN-γ or class II MHC molecules by the pancreatic β cells in a laboratory animal would lead to IDDM. To investigate these questions, we derived novel transgenic mouse lines designed to direct pancreatic β cells to express class II MHC molecules or IFN-γ. In both cases, a clinical diabetic state was produced.

Results

Generation of Transgenic Animals

In order to derive transgenic mice expressing class II MHC molecules or IFN-γ in the pancreatic β cells, we constructed recombinant DNA plasmids that joined the potential controlling sequences of the human insulin gene to
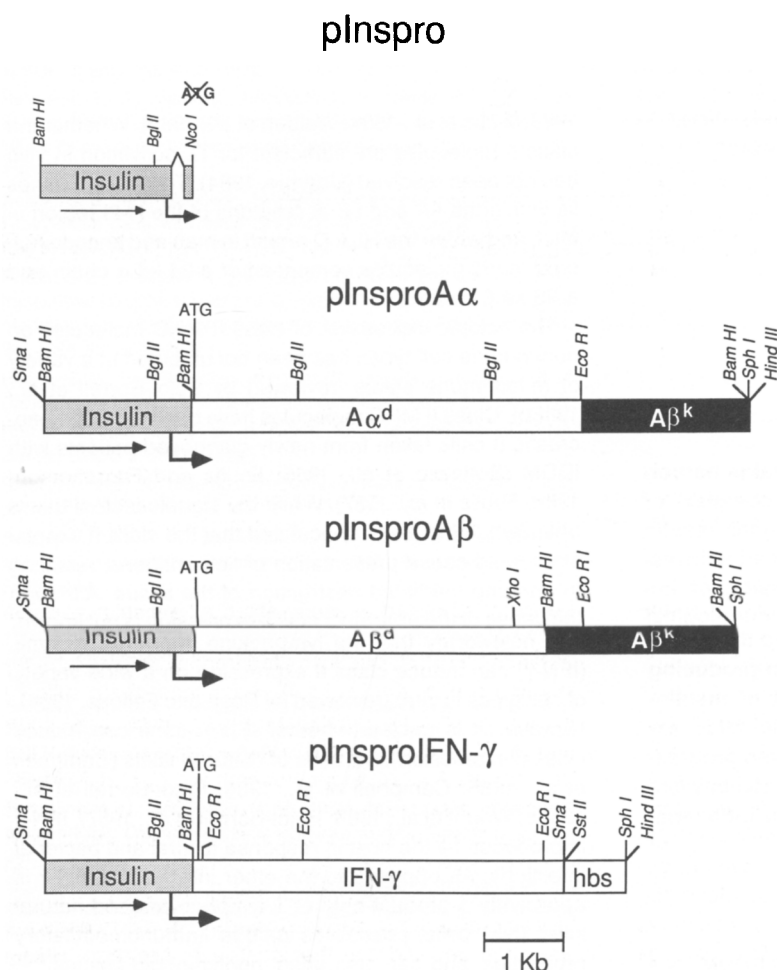
Figure 1. Structure of Recombinant Insulin/A_α, A_β, and IFN-γ Genes

The plasmids consist of coding region from the A_α^d, A_β^d, or IFN-γ genes fused with the 5' flanking region of the human insulin gene. The sequences from the human insulin gene include the entire first noncoding exon, followed by the first intron, and 15 bp of the second exon extending to one base before the initiator ATG codon of the insulin gene (see pInspro). In pInsproAα, the terminator portion of the construct is contributed by the 3' untranslated region (noncoding) from the A_β^k gene. In the pInsproAβ construct, the introcytoplasmic and 3' untranslated region are again contributed by fusion with the A_β^k sequences. This substitution does not affect the A_β^d amino acid sequence since the two haplotypes are identical in this region. The pInsproIFN-γ construct includes terminator sequences from the hepatitis virus B gene.

the mouse class II MHC A_α and A_β genes, and to the mouse IFN-γ gene (Figure 1). To maximize the potential for tissue-specific expression, a relatively large upstream portion (1867 bp of 5' sequences) of the human insulin gene promoter was used. It has previously been shown in transgenic mice that 660 bp of 5' sequences of the rat insulin II gene was sufficient to direct tissue-specific expression of a transgene (Hanahan, 1985). The complete human insulin gene similarly leads to tissue-specific expression in transgenic mice (Bucchini et al., 1986; Selden et al., 1986). The human insulin transcription start site and the complete untranslated first exon and part of the second exon of the human insulin gene were included in the construction. This promoter cassette ends immediately prior to the insulin translation start site, and was fused to the mouse genes in regions after their normal transcription start sites but before translation initiation. Since T cells must recognize foreign antigen in association with self class II molecules, the class II genes used to make the fusion genes were from the same mouse strain as the zygotes used to make the transgenic mice; thus the class II genes were A_α^d and A_β^d, and the zygotes were BALB/c (H-2^d).

For the microinjection to create transgenic animals, linearized DNA molecules, separated from plasmid sequences, were injected into mouse zygotes. The insulin-A_α and insulin-A_β chimeric genes were injected separately into inbred BALB/c zygotes. In the insulin-A_α experiment, 528 zygotes were implanted into pseudopregnant females. Of the 50 mice born, 4 proved to be transgenic when analyzed by Southern hybridization, and the transgene copy numbers were estimated by comparison to the endogenous gene: 443-11 (3-5 copies per haploid complement), 449-2 (3-5 copies), 451-1 (3 copies), and 443-5 (10 copies). In the insulin-A_β experiment, implantation of 409 zygotes yielded 63 mice, 2 of which proved to be transgenic (474-13 [8-10 copies] and 466-5 [20 copies]). For the insulin-IFN-γ experiment, a total of 254 zygotes of varying genotype were implanted into pseudopregnant females. Sixty-three mice were born and their DNA analyzed by Southern hybridization. Three mice proved to be transgenic, two of which were CD-1 × BALB/c F1 hybrids: (461-2 (3-5 copies) and 462-4 (3-5 copies), the other was derived from a CD-1 × CD-1 cross: 454-4 (10 copies). All three have been backcrossed to inbred BALB/c mice, and have subsequently segregated their transgene to a fraction of their progeny.

Analysis of the Insulin Class II Transgenic Animals

None of the founder transgenic insulin-A_α or insulin-A_β mice exhibited obvious phenotypic differences from controls. However, when founder A_α (443-11) and A_β (474-13)
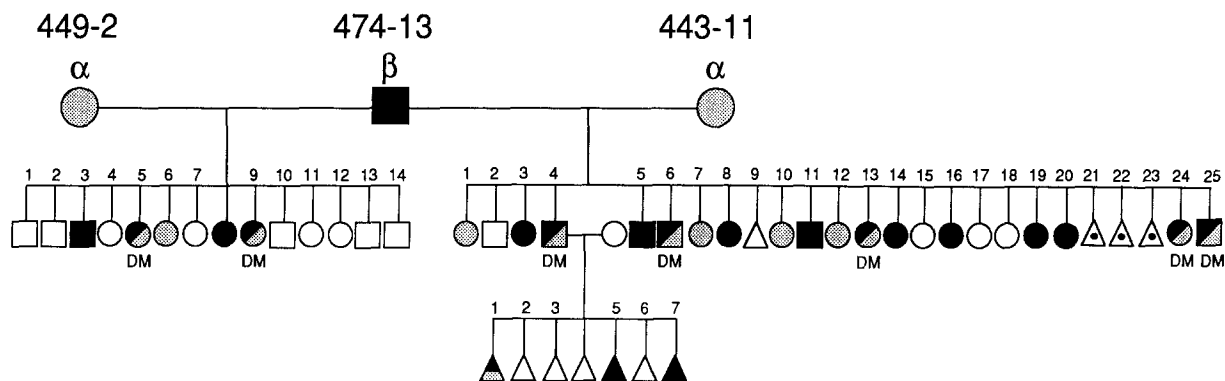
449-2 **474-13** **443-11**

Figure 2. A Partial Pedigree of Insulin-A$_\alpha$ and Insulin-A$_\beta$ Transgenic Mice Is Presented to Examine the Heritability of the Phenotype of Diabetes Mellitus (DM)

Females are indicated by circles, males by squares, and animals where sex was not determined by triangles. Solid black shading denotes the animals that carry the A$_\beta$ gene. Gray shading denotes animals that carry the A$_\alpha$ gene. A dot indicates animals that were not analyzed. The second generation animals (1–7) were sacrificed 16 days after birth for pancreatic RNA analysis and not analyzed by blood or urine glucose for diabetes mellitus.

mice were crossed, some of the offspring (at approximately 8 weeks of age) appeared inactive, had a "huddled" posture, distended abdomens, polydipsea, and polyuria. Dipstick (Chemstrip, Boehringer Mannheim) testing of urine from these mice revealed glycosuria (2%–5% glucose) and hyperglycemia (greater than 400 mg/dl, usually 600–1000 mg/dl). A subcutaneous insulin regimen (0–4 units/day ultra-lente insulin, Lilly) was begun to control the clinically apparent diabetes mellitus, based on minimizing the glycosuria, and the animals' health improved in approximately 2 weeks. Prior to insulin treatment, blood glucose levels of the diabetic mice remained above 400 mg/dl (up to 1000 mg/dl); with insulin treatment, blood glucose levels were approximately 150–350 mg/dl. Similar results have been obtained mating founder A$_\alpha$ (449-2) with A$_\beta$ (474-13) mice. This phenotype segregated only with the in-

heritance of both the insulin-A$_\alpha$ and the insulin-A$_\beta$ transgenes (Figure 2).

Pancreatic RNA was extracted from mice carrying neither transgene, the insulin-A$_\alpha$ transgene, the insulin-A$_\beta$ transgene, or both transgenes. When these RNAs were analyzed by Northern blotting with a probe specific for the transgenes, it was evident that both the A$_\alpha$ and A$_\beta$ transgenes were expressed in a tissue-specific manner in the transgenic animals. In animals with both transgenes, both transcripts were seen (Figure 3). In all other tissues analyzed, no expression could be detected (data not shown). To demonstrate that mRNA derived from the transgenes was translated and to further localize the tissue specificity of expression, cryostat sections of young (1 month) diabetic and control pancreata were examined after treatment with a monoclonal antibody that reacts with a mouse
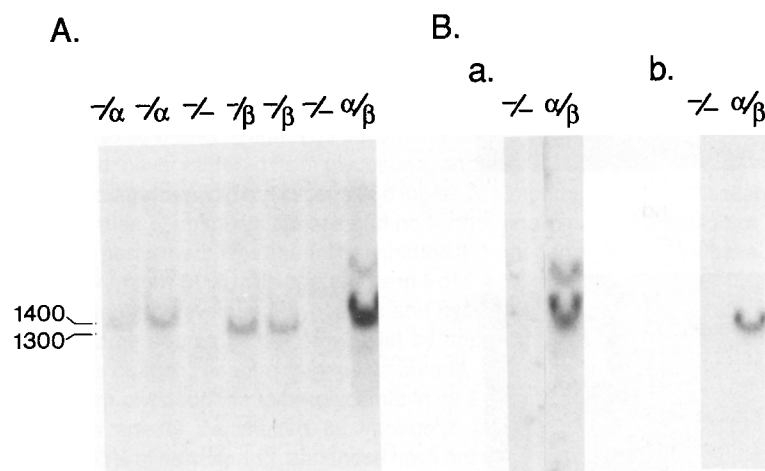
A. **B.**

Figure 3. Detection of Transgene Specific Transcripts in the Pancreases of Transgenic Mice

(A) Expression of the exogenous gene was assessed by hybridization with a radiolabeled oligonucleotide that is complementary to the 5' untranslated region of the human insulin mRNA and included in our constructs upstream to the coding regions. Total cellular pancreatic RNA samples are from the offspring of 443-11 insulin-A$_\alpha$ and 474-13 insulin-A$_\beta$ as follows: lane 1, 443-11-1; lane 2, 443-11-7; lane 3; 443-11-15; lane 4, 443-11-3; lane 5, 443-11-8; lane 6, 443-11-2; lane 7, 443-11-6 (diabetic). Genotypes of these animals are given in Figure 2. The animal numbers are designated by the insulin-A$_\alpha$ founder animal number (443-11) followed by the progeny number. The indicated sizes of the transcripts were calculated from the positions of the 18S and 28S ribosomal RNA transcripts visualized in the ethidium-bromide-stained formaldehyde gel.

The larger transcript seen in lane 7 (and in B, lane 2) is of unknown origin. It probably represents an unprocessed precursor or a 3' terminator read-through of the A$_\alpha^d$ transgene since it is detected with the 5' oligonucleotide probe as well as the A$_\alpha^d$ gene probe. A similar transcript was noted in transfection studies utilizing the same A$_\alpha^d$:A$_\beta^k$ fusion gene (Norcross et al., 1984).

(B) Expression of both the insulin-A$_\alpha$ and the insulin-A$_\beta$ transgenes in a diabetic mouse. (a) Expression of the insulin-A$_\alpha$ transgene was assessed by hybridization of total cellular pancreatic RNA to the radiolabeled A$_\alpha^d$ gene. Lane 1, BALB/c control; lane 2, 443-11-6. (b) Expression of the insulin-A$_\beta$ transgene was assessed by hybridization of total cellular pancreatic RNA to the radiolabeled A$_\beta^k$ cDNA. Lane 1, BALB/c control; lane 2, 443-11-6.
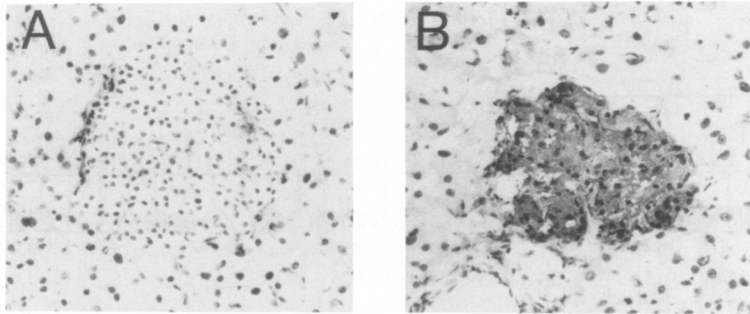
Figure 4. Class II (I-A) Antigen Expression in a Normal BALB/c Mouse and in a Young Diabetic Mouse from the A_α/A_β Transgenic Line Was Assessed Using the Avidin-Biotin Immunoperoxidase Method with DAB as a Chromogen

(a) Nonstaining islet of Langerhans from a BALB/c mouse. (b) Darkly staining islet of a 1 month old diabetic mouse.

I-A framework region (Bhattacharya et al., 1981; Davignon et al., 1981). The islets from the diabetic mouse showed intense I-A staining whereas the control mouse had only occasional positive nonislet cells stained (Figure 4). The diabetic pancreata were not stained when the first anti-body was omitted (data not shown). Thus, the transgenes exhibit tissue-specific expression, and the inheritance and expression of both transgenes are linked to the diabetes.
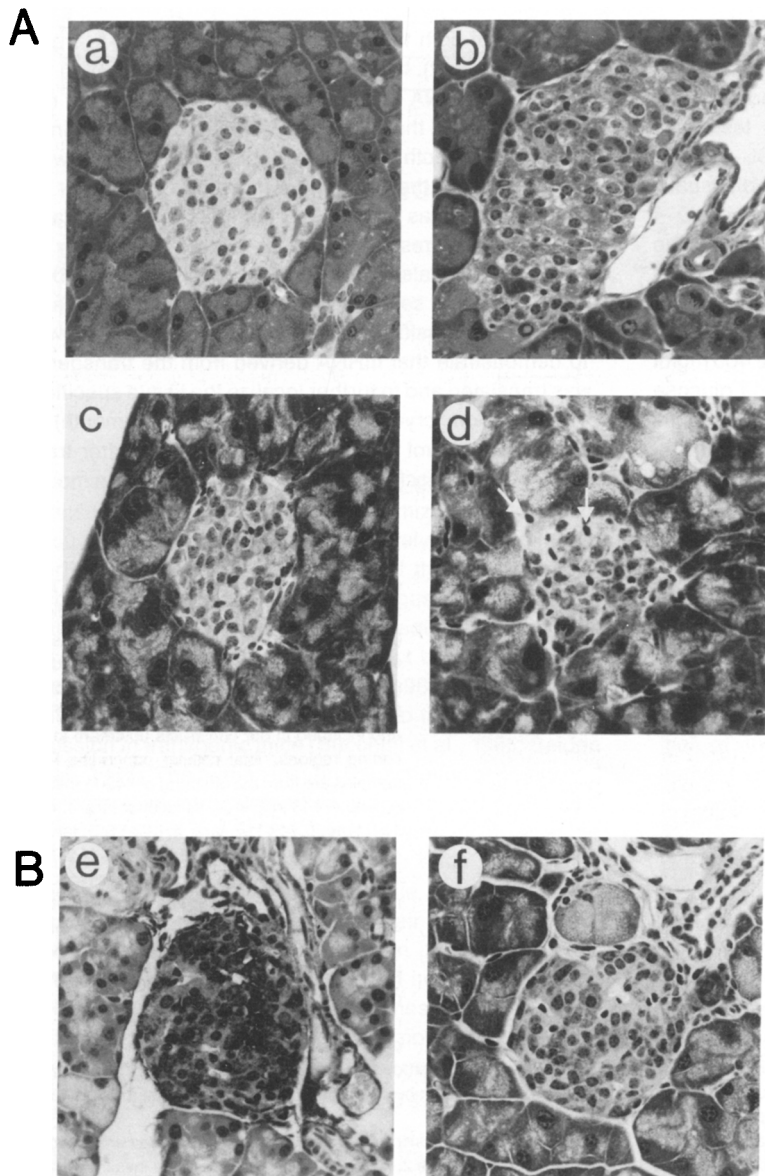
Histological examination of islets of Langerhans from

Figure 5.
(A) Sections of pancreas containing islets of Langerhans from a normal BALB/c and several insulin-A_α/A_β transgenic diabetic mice (haematoxylin and eosin, X240). (a) Normal islet. (b) Islet from a 4 week old insulin-A_α/A_β diabetic mouse. (c) Islet from a 6 week old insulin-A_α/A_β diabetic mouse. (d) Islet from an 8 week old insulin-A_α/A_β diabetic mouse. When compared to a typical normal islet (a), cells within islets shown in (b), (c), and (d) are less uniform in size, type, number, and distribution. Islets from older diabetic mice are subjectively smaller. Mononuclear inflammatory cells, such as those in (d) (arrows) are occasionally present.
(B) Insulin antigen expression in a normal BALB/c mouse and in an insulin-A_α/A_β transgenic diabetic mouse was assessed using the avidin-biotin immunoperoxidase method with DAB as a chromogen. (e) Darkly staining islet of Langerhans from a normal BALB/c mouse. (f) Faintly staining islet from an insulin-A_α/A_β, 6 week old diabetic mouse.

the diabetic mice revealed them to be composed of a morphologically heterogeneous group of cells clearly contrasting with the homogeneous cells forming islets of age-matched controls (see Figures 5a–5d). Cells in islets from 6 week or 2 month old transgenic mice were typically degranulated, had pleomorphic nuclei that stained with variable intensity, and were unevenly distributed (Figures 5c and 5d). Changes were less pronounced in younger mice (Figure 5b). There was generally an increased number of nuclei within affected islets, some of which may represent nuclei of collapsed β cells or infiltrating inflammatory cells. Necrotic debris and mononuclear inflammatory cells were infrequently present in islets from 6 and 8 week old transgenic mice (Figures 5c and 5d). Immunoperoxidase studies using antibodies to human insulin demonstrated reduced staining of the islet cells in diabetic transgenic mice. In 2 month old diabetic mice, the intensity of staining was dramatically reduced in comparison with controls, and the percentage of faintly staining cells within various islets ranges from less than 10% to approximately 50% compared with islets from age-matched controls in which greater than 80% of the islet cells stain intensely for insulin antigen (Figures 5e and 5f). Similar results were obtained when aldehyde fuchsin stain was used to demonstrate β cells. Quantitative morphometrics were not performed in this study; however, examination of step-sectioned pancreases from 1 or 2 month old diabetic mice revealed islets clearly smaller and less numerous than those in controls.

Analysis of Insulin-IFN-γ Transgenic Animals

One of the three founder insulin IFN-γ transgenic animals, 461-2, appeared inactive at 10 weeks of age and abdominal distention, polydipsea, and polyuria were noted. The mouse had glycosuria (2%–5%) and the serum was lipemic. A subcutaneous insulin regimen, similar to that used for the diabetic transgenic insulin-A_α, A_β transgenic mice, was begun and the animal's health improved. This animal transmitted its transgene to approximately 25% (20/76) of its offspring; all of the transgenic offspring developed glycosuria (2%–5%) and hyperglycemia (greater than 400 mg/dl, up to 1000 mg/dl) beginning at approximately 6 to 10 weeks of age. The second transgenic founder, 462-4, never suffered from glycosuria, and blood glucose measurement did not exceed 260 mg/dl. At 5 months of age it died of unknown causes and no further analysis could be performed. This animal transmitted its transgene to approximately 30% of its offspring and 5 of the 14 transgenic offspring developed glycosuria and hyperglycemia (to the same degree as the 461-2 line) by approximately 20 weeks of age. The third transgenic animal, 454-4, has not shown glycosuria or hyperglycemia in its 8 months of life. This animal transmitted its transgene to approximately 50% of its offspring, and these have not developed either glycosuria or hyperglycemia. All of the diabetic offspring have been treated with insulin regimens with resultant improved health.

Pancreata from diabetic transgenic mice harboring the IFN-γ gene were characterized histologically by variable accumulations of inflammatory cells within the parenchyma. These cells were predominantly lymphocytes and macrophages, although plasma cells and neutrophils were occasionally recognized. Pancreatic lesions tended to be less severe in younger mice and progress with age. Lesions in mildly affected, generally neonatal mice, consisted of interstitial edema accompanied by small clusters of lymphocytes between acini, surrounding islets, and small vessels (Figure 6a). In more severely affected mice there is substantial obliteration of islet as well as acinar structures by lymphocytic and histiocytic accumulations (Figures 6b and 6c). Primary insulitis (inflammation confined to the islets) was not an obvious feature in these mice; however, interstitial inflammatory cells commonly surrounded and infiltrated islets. Pancreata from older diabetic mice sustained on exogenous insulin had focally extensive areas of fibrosis interspersed with clusters of inflammatory cells (Figure 6d). Immunoperoxidase studies of inflamed pancreata demonstrated a marked reduction in numbers and staining intensity of islet cells labeled with antibody to human insulin (Figures 6e and 6f). Immunoperoxidase studies utilizing a monoclonal antibody against I-A antigens (Bhattacharya et al., 1981; Davignon et al., 1981) revealed diffuse light to moderate staining of acinar tissue, islets, and epithelial cells lining the ducts (data not shown).

Discussion

We studied transgenic mice harboring fusion genes between the human insulin 5′ regulatory region and the IFN-γ gene and class II MHC genes. The two diseases produced in the transgenic mice are pathologically distinct.

In the doubly transgenic mice expressing both Aα and Aβ class II sequences, the pancreatic β cells largely disappear from the islets of Langerhans by the time the animals are 2 months old. This loss of cells is specific to the islets and does not notably affect the acinar tissue of the pancreas. In older transgenic diabetic mice, the islets resemble the pseudoatrophic islets seen in human IDDM. Overt diabetes in these mice (at least up to 3 months of age) only occurs in those transgenic mice that have inherited a copy of each of the Ins-A_α and Ins-A_β genes. The conclusion is that the diabetes is caused by and is dependent on the expression of both these genes.

We have shown that the pancreata of the Ins-A_α and Ins-A_β transgenic mice contain mRNA molecules derived from the transgenes. We have also directly demonstrated the presence of class II molecules within the islets of Langerhans of the doubly transgenic mice. It is possible that just the expression of the class II molecules in the doubly heterozygous class II mice could be deleterious to the β cells, causing their eventual demise. Injury of cells due to expression of class II molecules has never been reported in transfection experiments (reviewed by Germain and Malissen 1986) or in the induction of class II expression in β cells in vitro (Wright et al., 1986; Pujol-Borell et al., 1987). Transgenic mice in which the pancreatic β
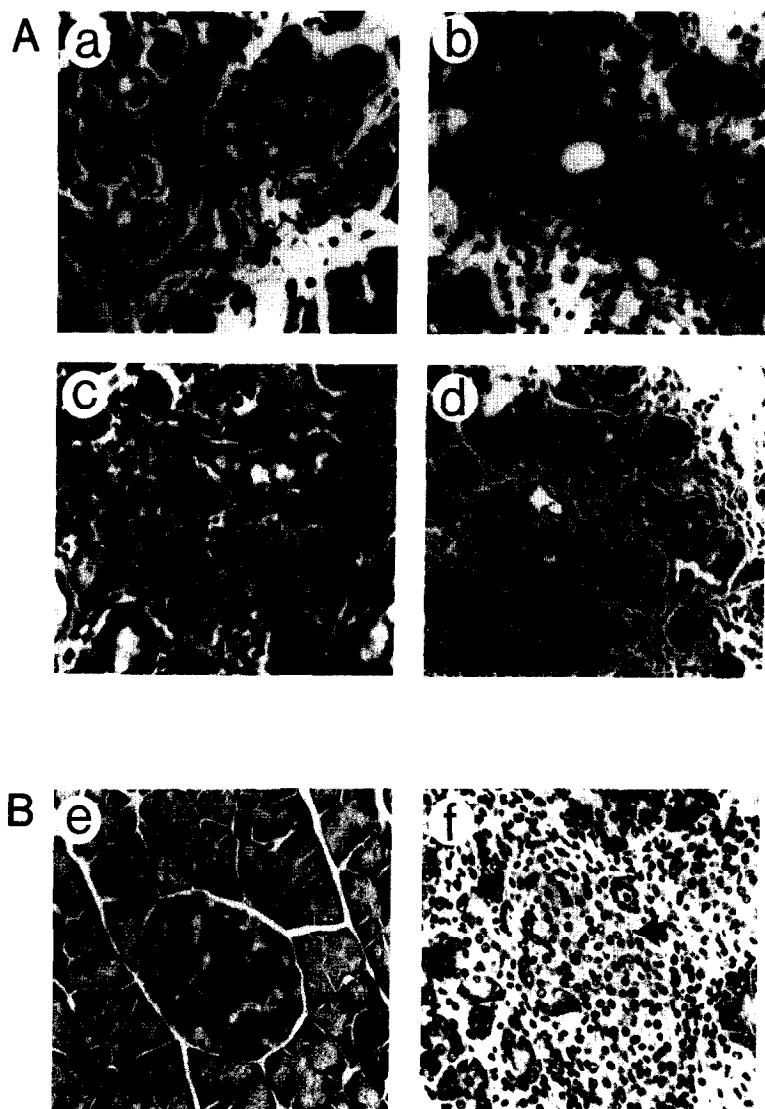
Figure 6.

(A) Sections of pancreas containing islets of Langerhans from prediabetic and diabetic insulin-IFN-γ transgenic mice (haematoxylin and eosin). (a) Mildly affected pancreas from a 1 week old mouse. Mononuclear inflammatory cells are present in the interstitium within the vicinity of an islet (X180). (b) Moderately affected pancreas from a 3 week old mouse. Numerous inflammatory cells are present in the interstitium and are beginning to obscure the centrally located islet. (c) A higher magnification of a severely affected pancreas from a 6 week old mouse. Islets (arrow) are nearly totally obscured by accumulations of mononuclear inflammatory cells (X240). A few faintly staining (using peroxidase) insulin antigen positive cells were shown to be present in a duplicate section (data not shown). (d) Pancreas from an 8 week old mouse that had been maintained on exogenous insulin. Faint outlines of islets are surrounded by a maturing inflammatory cellular component which includes fibroblasts (X100).

(B) Insulin antigen expression in a normal mouse and in an insulin-IFN-γ diabetic mouse was assessed using the avidin-biotin immunoperoxidase method using DAB as a chromogen. (e) Dark staining islet from a normal BALB/c mouse. (f) Faintly staining islets from an insulin-IFN-γ transgenic mouse (arrows). Islets are surrounded and nearly obscured by mononuclear inflammatory cells.

cells express the SV40 early region can develop antibodies directed against SV40 T antigen (Adams et al., 1987). This "intolerance" phenomenon does not in this case lead to diabetes. Several other exogenous genes have been expressed in the pancreatic beta cells of transgenic mice without causing IDDM: human insulin (Bucchini et al., 1986; Selden et al., 1986), polyoma middle T antigen (V. Bautch, personal communication), and human placental lactogen (S. Alpert, personal communication). However, we have noticed an intermittent, mild hyperglycemia in some of the A_α transgenic (singly heterozygous) mice from the 443-11 line, with blood glucose values averaging approximately 180–200 mg/dl in comparison with A_β transgenic mice or control mice, which were 120–150 mg/dl (data not shown). We cannot account for this at present. It may be pertinent that in a line of transgenic mice carrying a class II MHC E_α transgene, abnormal constitutive expression of the endogenous E_β gene was observed (Yamamura et al., 1985). A similar phenomenon might apply here, and further investigation of this line should eluci-

date the cause of the hyperglycemia. It is also possible that the expression of class II molecules affects the insulin producing function of the β cells without killing them, resulting in diabetes. Loss of differentiation in β cells without cell death has been observed in virally initiated diabetes (Tishon and Oldstone, 1987).

Our results also indicate the possibility that the β cells could present antigen to T_H cells, thus initiating an autoimmune reaction. New immunological responsiveness has been demonstrated in transgenic mice through the introduction of an I-E gene (Le Meur et al., 1985; Pinkert et al., 1985; Yamamura et al., 1985). Activation of an autoimmune response by presentation of presumably self antigens by the β cells implies a failure in self recognition (tolerance). Nonresponsiveness to self antigens is thought to involve the deletion or suppression of clones of T cells that recognize self antigens. It is possible that T cell clones reactive to components of nonimmune cells are not suppressed or deleted since these molecules are not normally seen by the host immune system. Thus, in non-

immune cells the expression of class II molecules may lead to the presentation of self antigens to which the host immune system is naive, resulting in the activation of an autoimmune response. Alternatively, the extreme overexpression of class II molecules in the β cells of these mice might lead to the bypass of tolerance by adventitious antigen presentation, since in transfected fibroblasts the level of class II expression is correlated with antigen presenting capacity (Lechler et al., 1985).

The data presented in this communication do not directly address the question of whether the β cell destruction in the Ins-IA transgenic mice is a consequence of an autoimmune response. Experiments in progress examining the response to pancreatic grafts in these Ins-IA mice and characterizing mice with MHC Class I and an "irrelevant" protein inserted into the β cell membrane will begin to address this question.

The possibility also exists that antigen being presented by β cells is a foreign antigen derived from a virus (see Suzumura et al., 1986). An extensive search for serological evidence by viral infection in diabetic and nondiabetic mice did not reveal the presence of antibodies against any virus known to be associated with diabetes, although antibodies against mouse hepatitis virus were detected in both diabetic and nondiabetic mice.

Relevant to this work are studies involving patients with autoimmune thyroid disorders. Thyroid follicular cells from such patients have been shown to aberrantly express class II MHC molecules (Hanafusa et al., 1983). These cells have the capacity to present foreign antigen to T cells in vitro (Londei et al., 1984). In addition, infiltrating T cells autoreactive to host thyroid cells have been isolated and cloned from diseased thyroid glands (Londei et al., 1985). The diabetes in the A_α/A_β transgenic mice could occur as a result of analogous processes. However, the apparent paucity of inflammatory cells in the islets is curious. In human IDDM, although insulitis is thought likely to be present frequently during the initial stages of the disease, it is often not detected, perhaps because of its transient and nonuniform nature (Gepts and LeCompte, 1985). Examination of younger, prediabetic mice, specific cell labeling studies, and electron microscopic analysis will help define the mechanism of β cell destruction. The few scattered inflammatory cells observed within the islets of these mice could be producing factors that are directly or indirectly toxic to β cells. Recent studies have suggested exquisite sensitivity of β cells to oxygen free radicals that are induced by lymphokines (Nomikos et al., 1986; Tsumimoto et al., 1986; Prowse et al., 1986). The sparse amount of islet inflammation present in the current study may be sufficient to kill the β cells in these islets in this manner.

In the IFN-γ model, the β cells also disappear from the transgenic pancreas. However, in this case it is concomitant with a progressive inflammatory process that appears to be focused on the islets but involves the entire pancreas. The active inflammatory lesion in younger mice consists primarily of chronic inflammatory cells of the type seen in response to viral infections and in autoimmune diseases. Interestingly, the pathology is also reminiscent of that seen in transplant graft rejection of the acute cellu-

lar type (Robbins et al., 1984). The mechanism by which the inflammation is initiated, however, is unknown.

In diabetic mice, the IFN-γ could be directly inducing the expression of class II molecules that would present self antigens to the host immune system as described above for the A_α/A_β transgenic mice. If IFN-γ were acting solely by inducing the expression of class II molecules, then we might expect the histology of the IFN-γ transgenic diabetic mice to resemble that of the A_α/A_β transgenic diabetic mice. As this is clearly not the case, the IFN-γ must be participating in other cellular processes as well. Interferon-γ is known to induce increased pancreatic class I MHC molecule expression in vitro (Campbell et al., 1985; Campbell et al., 1986; Pujol-Borrell et al., 1986a). Increase class I MHC expression is seen in the islets of newly diagnosed diabetics (Bottazzo et al., 1985; Foulis et al., 1987). One component of β cell destruction may involve cytotoxic T lymphocytes that recognize class I MHC molecules in conjunction with antigen. Additionally, because of the large inflammatory infiltrate, other lymphokines may be produced and contribute to the pathology. Leukocytes secrete tumor necrosis factor, which acts as a chemoattractant for polymorphonuclear neutrophils (Figari et al., 1987). Interleukin-1 has been implicated in vitro as a causative factor in β cell damage (Bendtzen et al., 1986), and the related proteins, tumor necrosis factor and lymphotoxin, have been shown to synergize with IFN-γ in vitro to cause class II expression by β cells (Pujol-Borrell et al., 1987). The IFN-γ transgenic mice demonstrate the deleterious cascade of effects that are initiated by local production of IFN-γ in vivo, and may therefore provide an animal model for study of virus-caused, interferon-mediated inflammation.

The incomplete penetrance of the diabetic phenotype in the 462-4 derived transgenic progeny is not readily understandable. Since these progeny are the second backcross of the CD1 (outbred) line to BALB/c, there will be much genetic nonequivalence between these progeny. It is possible that the variability in genetic background contributes to the penetrance of the transgene.

It has recently been reported that in both the nonobese diabetic (NOD) mouse model and in human IDDM, an amino acid residue change (specifically, the absence of aspartic acid at residue 57) in the mouse I-A_β or its human correlate HLA-DQβ chain is highly correlated with the propensity to develop IDDM (Acha-Orbea and McDevitt, 1987; Todd et al., 1987). The introduced A_β chain and the recipient BALB/c mice in the present experiment are of the same haplotype (H-2^d) and contain aspartic acid at residue 57 of A_β (Choi et al., 1983; Malissen et al., 1983). Thus the allelic amino acid difference, while clearly significant, may not be necessary for the development of diabetes.

Human IDDM represents a heterogeneous group of disease syndromes with a common final pathologic expression. Diverse mechanisms likely play a role in the development of IDDM. These experiments were designed to bypass the initiating insult in addition to the genetic predisposition to the disease, thereby separating and isolating several individual steps in a proposed pathway

for autoimmune reaction generation. To do so, identifiable genetic perturbations were made which led to diabetes. Localized class II MHC expression causes a depletion of β cells; whereas IFN-γ, which may lead to aberrant class II expression, causes cell depletion associated with an inflammatory disease process. Neither created a pathological phenocopy of what is thought to be the general progression of disease in human IDDM, although each recapitulated parts of the damage noted.

Studies with transgenic mice may lead to a deciphering of the mechanisms of autoimmune disease. The respective roles of adventitious class II MHC expression, self-antigen presentation, lack of tolerance to self-antigens, and local production of lymphokines can now be assessed in vivo.

Experimental Procedures

DNA Constructs

A recombinant plasmid containing an insulin promoter cassette was made by isolating in two steps a 1.9 kb BamHI–NcoI fragment from the human insulin B allele gene (Ullrich et al., 1980; Ullrich et al., 1982), blunt-ending the NcoI site with S1 nuclease, and inserting it into a BamHI–HincII cleaved PucI18 vector to yield pInspro. In the remaining constructions, all restriction enzyme cleavage sites utilized for cloning were made blunt-ended with the Klenow fragment of DNA polymerase I except where noted. To derive pInspro Aα, pInspro was cleaved at its unique SphI site and a 7 kb BamHI fragment containing the A_α^d coding sequence fused to the 3' untranslated region to the A_β^k 3' sequences (kindly provided by Dr. E. Choi, University of Cincinnati, unpublished data) was inserted at this site. pInsproAβ was derived by cleaving a recombinant plasmid (kindly provided by Dr. E. Choi, unpublished data) containing the A_β^d 5' untranslated and coding region fused in the intracytoplasmic domain with the A_β^k 3' sequences with EcoRI and isolated a 5.4 kb restriction fragment containing the majority of the gene. The amino acid sequence of the A_β^d and the A_β^k proteins are identical in the cytoplasmic region, thus the presence of these sequences in our construct does not alter the syngeneic nature of this experiment. In order to remove 5' ATG potential start codons, this restriction fragment was treated for varying amounts of time with Bal31 nuclease and inserted into a HincII cleaved pUC118 plasmid (without Klenow treatment). Restriction and sequence analysis was performed on recombinant clones, and a clone containing 48 bp of sequence 5' to the ATG translation initiator of the Aβ protein sequence was chosen. This plasmid was cleaved at its unique XbaI site and the BamHI–SphI insulin promoter fragment inserted. This was cleaved with XhoI and SphI and the large restriction fragment, containing the plasmid and 5' sequences, was ligated (without Klenow treatment) to the 2.9 kb XhoI–SphI restriction fragment containing 3' sequences from the original Aβ plasmid. To derive pInsproIFN-γ, a recombinant plasmid containing the genomic murine IFN-γ gene (Gray and Goeddel, 1983) fused with the hepatitis B 3' untranslated sequence (Kpn–SstII) was cleaved with PvuII to remove 5' sequences, and the BamHI–SphI insulin promoter fragment from pInspro was inserted in their place.

Production of Transgenic Mice

Restriction fragments of the above plasmids, free of plasmid sequences, were isolated from low melting temperature agarose gels by NaI-glass bead extraction (Geneclean, BIO101). DNA was further purified free of contaminants by ultracentrifugation.

Four week old BALB/c or CD-1 (outbred, closed stock) female mice were superovulated by injecting intraperitoneally (i.p.) 5 U of gonadotropin from pregnant mare serum 48 hr prior to injecting 5 U of human chorionic gonadotropin. Female mice were then mated with BALB/c or CD-1 proven breeder male mice.

The next day, one-cell stage embryos were flushed from the oviducts of the mated female mice with M3 medium (Quinn et al., 1982). The eggs were freed of cumulus cells and zona pellucida by hyaluronidase treatment. The male pronuclei of the fertilized eggs were microinjected with approximately 2 picoliters of DNA at a concentration of 1 ng/μl. The injected, fertilized eggs were then transferred into the ampullae of half-day pseudopregnant recipient CD-1 female mice to complete their development.

When the litters were 4 weeks old, 1–2 cm of their tails were cut. DNA was extracted from the tail segments for Southern blot analysis.

DNA and RNA Blots

Five to ten micrograms of genomic DNA from the tails were restricted with NcoI (IFN-γ), BglII (A_α), or HincII (A_β). The DNA was electrophoresed on 0.8% agarose gels and transferred to nylon membranes (Zetaprobe). Hybridization with radiolabeled random-primed probes was performed with restriction fragments of the IFN-γ cDNA (Gray and Goeddel, 1983), the genomic A_α^d gene, or the A_β^k cDNA (Estess et al., 1986).

Northern analysis was of RNA isolated from the pancreata and other tissues of nontransgenic and transgenic mice. RNA was extracted from the tissues by the guanidine HCl/ethanol procedure (Chirgwin et al., 1979), electrophoresed through a 1% agarose, 6% formaldehyde gel, transferred to a nylon membrane, and hybridized to either a 50 base oligonucleotide complementary to the first untranslated exon of the human insulin gene, the A_α gene, or the A_β cDNA. Following three 20 min washes in 0.1% SDS, 0.1 × SSC at 65°C (42°C with the 50 base oligonucleotide probe), the filters were exposed to a Kodak XAR film with an intensifying screen at −70°C for 5 days.

Glucose Determination

Urine glucose percentages were determined by dipstick analysis (Chemstick, Boehringer Manheim) of spontaneously expressed urine. Random blood glucose determinations were made on whole blood using the Accucheck II (Boehringer Manheim) or on serum or plasma by standard enzymatic methods. Mice were considered diabetic when urine glucose was 2%–5% and the nonfasting blood glucose was greater than 400 mg/dl for 3 consecutive days. Normal (nontransgenic) mice never showed glycosuria, and had blood glucose concentrations of approximately 100–200 mg/dl.

Histological Analysis

Tissues and fetuses were fixed in 10% neutral buffered formalin for 24 hr, dehydrated in S-29 Dehydrant (Technicon Corp.), cleared in toluene, and infiltrated with paraffin. Three micron sections were cut on a Reichert-Jung 2040 microtome, picked up on poly-L-lysine coated slides, and dried in a 37°C oven overnight. Sections were deparaffinized in three changes of toluene, and rehydrated through graded alcohols to distilled water. The sections were then blocked for endogenous peroxidase activity with hydrogen peroxide-methanol, endogenous biotin with avidin-biotin blocking kit (Vector Laboratories) and endogenous immunoglobulin binding, and then stained for insulin with antibody to human insulin using the avidin-biotin peroxidase method (Vector Laboratories) with DAB as the chromagen. Negative controls were no primary antibody, nonimmune serum, and control tissue. Positive controls of both human and mouse pancreas were also used.

Sections were also cut at 3 microns and stained with a haematoxylin and eosin stain for viewing morphogenic evaluation. Selected sections were stained with aldehyde-fuchsin stain to detect β cells. Approximately three to four sections were initially evaluated from each pancreas. Several pancreata were also chosen for complete step-sectioning (3 sections every 50 microns). Additional sections were evaluated when islets were not found on initial sections.

For analysis of I-A antigen expression, 10 micron cryostat sections were fixed in acetone for 5 min at −20°C, blocked for endogenous peroxidase activity, and stained for I-A antigens with monoclonal antibody TIB120 (gift from Drs. Robertson Parkman and Maxwell Cooper) using the avidin-biotin peroxidase method (Vector Laboratories).

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