Expression of a Tumor Necrosis Factor α Transgene in Murine Pancreatic β Cells Results in Severe and Permanent Insulitis without Evolution towards Diabetes

By Y. Higuchi,*[1] P. Herrera,‡ P. Muniesa,‡ J. Huarte,‡ D. Belin,* P. Ohashi,§ P. Aichele,§ L. Orci,‡ J.-D. Vassalli,‡ and P. Vassalli*

*From the *Department of Pathology and the ‡Institute of Histology and Embryology, Centre Médical Universitaire, University of Geneva, CH 1211 Geneva 4; and the §Department of Experimental Pathology, Institute of Pathology, University of Zurich, CH 8091 Zurich, Switzerland*

Summary

Mice bearing a tumor necrosis factor (TNF) α transgene controlled by an insulin promoter developed an increasingly severe lymphocytic insulitis, apparently resulting from the induction of endothelial changes with features similar to those observed in other places of intense lymphocytic traffic. This was accompanied by dissociation of the endocrine tissue (without marked decrease in its total mass), islet fibrosis, and the development of intraislet ductules containing, by places, β cells in their walls, suggesting a regenerative capacity. Islet disorganization and fibrosis did not result from lymphocytic infiltration, since they were also observed in SCID mice bearing the transgene. Diabetes never developed, even though a number of potentially inducing conditions were used, including the prolonged perfusion of interferon γ and the permanent expression of a nontolerogenic viral protein on β cells (obtained by using mice bearing two transgenes). It is concluded that (*a*) a slow process of TNF release in pancreatic islets induces insulitis, and may be instrumental in the insulitis resulting from local cell-mediated immune reactions, but (*b*) that insulitis per se is not diabetogenic, lymphocyte stimulation by cells other than β cells being necessary to trigger extensive β cell damage. This provides an explanation for the discrepancy between the occurrence of insulitis and that of clinical disease in autoimmune diabetes.

Insulin-dependent diabetes is thought to be of autoimmune origin. In animal models of the human disease, the nonobese diabetic (NOD)[2] mouse (for review see reference 1) and the Bio Breeding (BB) rat (2), it begins with a lymphocytic insulitis, which can progress towards eventual destruction of the insulin-secreting β cells. Although the antigenic target(s) of the autoimmune reaction taking place in the pancreatic islets may be different in human diabetes and animal models, destruction of β cells probably proceeds through similar pathogenetic mechanisms, based on T cell–mediated tissue damage.

Among these pathogenetic mechanisms, direct cytotoxicity of T lymphocytes to antigen-bearing β cells and release of cytokines harmful to β cells have been incriminated. Cells containing mRNA for perforin, a protein involved in cytotoxicity, and for TNF-α, a broad mediator of inflammatory reaction, have been detected in the insulitis of NOD mice by in situ hybridization (3). Based on in vitro alterations of islet cells in the presence of TNF or IL-1, another broad mediator of inflammation with many effects resembling those of TNF, and the potentialization of these effects by IFN-γ, a product of stimulated lymphocytes, it has been proposed that local release of these various cytokines plays a role in the appearance of diabetes (4–8). Mice bearing an IFN-γ transgene placed under the control of the insulin promoter, and thus releasing INF-γ in the pancreatic islets through β cells, develop diabetes (9). Since TNF is a possible mediator of autoimmune β cell destruction, we decided to explore the effects of β cell expression of a TNF transgene. A severe insulitis developed, but, in spite of its persistence, β cell damage extensive enough to result in diabetes was not observed, even

[1] The contributions of Y. Higuchi and P. Herrera should be considered equal.

[2] *Abbreviations used in this paper:* BB, Bio Breeding; LCMV gp, lymphochoriomeningitis virus glycoprotein; NOD, nonobese diabetic; RIP, rat insulin promoter.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/92/12/1719/13 $2.00
Volume 176 December 1992 1719–1731

after more than 1 yr of observation. Attempts at eliciting T lymphocytes within the insulitis to develop a diabetogenic immune reaction against β cells were made by perfusing other cytokines (e.g., IFN-γ), by mildly damaging β cells, or/and by expressing a viral antigen on β cells (using mice bearing an additional transgene) (10). Failure to elicit diabetes in all these conditions leads to the conclusion that two successive, pathogenically distinct phases may be required for the appearance of autoimmune diabetes, and that insulitis, the first phase, may be necessary but not sufficient to create diabetes. This probably explains why a large proportion of NOD mice, which all develop insulitis of comparable severity, does not progress toward diabetes.

Materials and Methods

Mice. A NarI-SalI fragment containing all the coding and 3′ untranslated regions of the TNF-α gene was obtained from a λ EMBL 3 genomic clone containing most of the mouse strain C57Bl/6 TNF locus (11) and inserted behind a 660-bp fragment of the rat insulin promoter (RIP) II (12). Transgenic mice were produced by microinjection of the purified DNA fragment into the male pronucleus of B6D2F$_1$ × B6D2F$_1$ zygotes. Among 41 mice born, three contained integrated copies of the transgene, as judged by tail genomic DNA PCR analysis, using a 5′ primer corresponding to the RIP II promoter (5′-TAAGGCTAAGTAGAGG-TGT-3′) and a 3′ primer corresponding to the TNF coding region (5′-GAGAAGAGGCTGAGACATAG-3′), and 30 cycles of amplification (30 s at 94°, 30 s at 54°C, and 2 min at 72°C). To determine transgene copy numbers, Southern analysis of EcoRI-digested DNA was performed, yielding 2.8 and 2.4 kb fragments for the endogenous genes and the transgene, respectively, using hybridization to a TNF probe. The founders were crossed to NMRI mice, and transgenic progeny being detected by PCR analysis of the tail DNA. To obtain SCID mice bearing the TNF transgene, SCID mice (Iffa Credo, L'Arbresle, France) were crossed with TNF transgenic mice. The progeny was analyzed by PCR for presence of the transgene and by lymphopenia on blood smears for SCID homozygosity after backcrossing to SCID mice. NOD mice, 5–6-mo-old, were obtained from the colony of Hans Acha-Orbea (Ludwig Institute, Lausanne Branch, Switzerland). TNF activity in the blood was explored by a bioassay (13) using WEHI 164 cells as targets.

When indicated, transgenic or control mice received the following treatments: cyclophosphamide (Bristol-Myers Squibb, Princeton, NJ), two 300-mg/Kg i.p. injections 2 wk apart; rat mAb anti-mouse CD3 (14), five daily 20-μg i.v. injections, accompanied when mentioned by intraperitoneal injections of human rIL-2 (Glaxo IMB, Geneva, Switzerland); intraperitoneal perfusions in osmotic minipumps (Alzet 2002; Alza, Corp., Palo Alto, CA) of mouse rIL-1β (gift of J. Y. Bonnefoy, Glaxo IMB), rIFN-γ (gifts of Drs. N. Sarvetnick, La Jolla, CA, and G. Adolf, Behringwerke, Vienna, Austria) or PBS (as a control); bromo-deoxyuridine (BrdU; Sigma Chemical Co., St. Louis, MO), either by three daily 15-mg i.p. injections for 3 d, or by intraperitoneal perfusion with osmotic pumps for 4 d, total amount, 2 mg; streptozotocin (Upjohn, Kalamazoo, MI), either 40- or 100-mg/kg i.p. daily by injection for 5 d; $^{35}SO_4$ (Amersham International, Burlinghamshire, UK) given as a single 5–10-mC i.p. injection, followed by killing after 4 h.

Histochemistry, Immunocytochemistry, and Electron Microscopy. Tissues were frozen in liquid nitrogen or fixed either in Bouin's or 4% glutaraldehyde and embedded in paraffin or epon 812, respectively. The following antibodies were used: rat mAb anti-mouse CD4 (GK1.5 [15]), CD8 (H35-17.2 [16]); μ Ig chains (gift of H. Bazin, Brussels), F4/80 (17), Ia (TIB 120; American Type Culture Collection, Rockville, MD), invariant chain (gift of N. Koch, Heidelberg, Germany), ICAM-1 (18), type II cytokeratin Endo A (TROMA 1; gift of R. Kemler, Freiburg, Germany), anti-BrdU (Becton Dickinson & Co., San José, CA); and guinea pig or rabbit IgG against porcine insulin, porcine glucagon, bovine pancreatic polypeptide, and synthetic stomatostatin (gifts respectively of P. Wright, Indianapolis, IN; R. H. Unger, Dallas, TX; R. E. Chance, Indianapolis, IN, and R. Guillemin, La Jolla, CA). FITC- or TRITC-conjugated goat, sheep, and rabbit IgG antibodies were obtained from Biosys (Compiègne, France) and Nordic (Tilburg, The Netherlands). Autoradiographs were performed with Ilford L4 emulsion. Aldehyde-fuchsin staining was done as described (18a). The volume density of total endocrine tissue and of β cells, was studied separately by the point-counting method of morphometric analysis (19) on paraffin sections, stained either with hemaluneosin or aldehyde-fuchsin, respectively, of the pancreata from three transgenic and three nontransgenic mice. A minimum of 10,000 points were counted per pancreas. Statistical significance was determined using the unpaired Student-Fisher t test (20). Islet fibrosis was studied on paraffin sections stained with Gomori's silver impregnation method. Ultrastructural studies were performed with an electron microscope (model EM 300; Philips). For explorations of the pancreatic islets' vascular tree, mice under general anesthesia (0.5-ml i.p. injection of 2.5% tribromoethanol in saline) were perfused through the abdominal aorta with heparin and 1% glutaraldehyde as fixative. The pancreata were postfixed with OsO_4 before embedding in epon 812.

Results

Development of Insulitis in TNF Transgenic Mice, and Comparison to the Insulitis Observed in Autoimmune NOD Mice

Three transgenic founder mice were obtained, bearing respectively, as judged by Southern blot analysis, 1, 2, and 8–10 copies of the insulin promoter-TNF transgene (data not shown). The transgenic progeny of these mice showed a very comparable evolution of lesions at various ages, although the line with the highest copy number tended to have a more severe insulitis at earlier ages. All the experiments reported in this work used the two lines with low copy number of the transgene. Their nontransgenic littermates were used as controls. As determined by histologic examination of various tissues, the lesions observed in the transgenic mice were restricted to the pancreatic islets. Blood samples taken at various ages did not show detectable TNF levels, as judged by a bioassay. TNF mRNA was detectable in the pancreas of transgenic but not control mice. At 3 wk of age, lymphocytes began to accumulate in the islets, usually in a central localization. This was followed by a progressively increasing lymphoid infiltration involving all islets (Fig. 1, *A–C*). In mice killed between 2 and 16 mo-old, the islets were, because of the extent of the infiltration, of progressively increasing size. The largest diameter, considering as islets the endocrine cells and the associated lymphocytic infiltration,

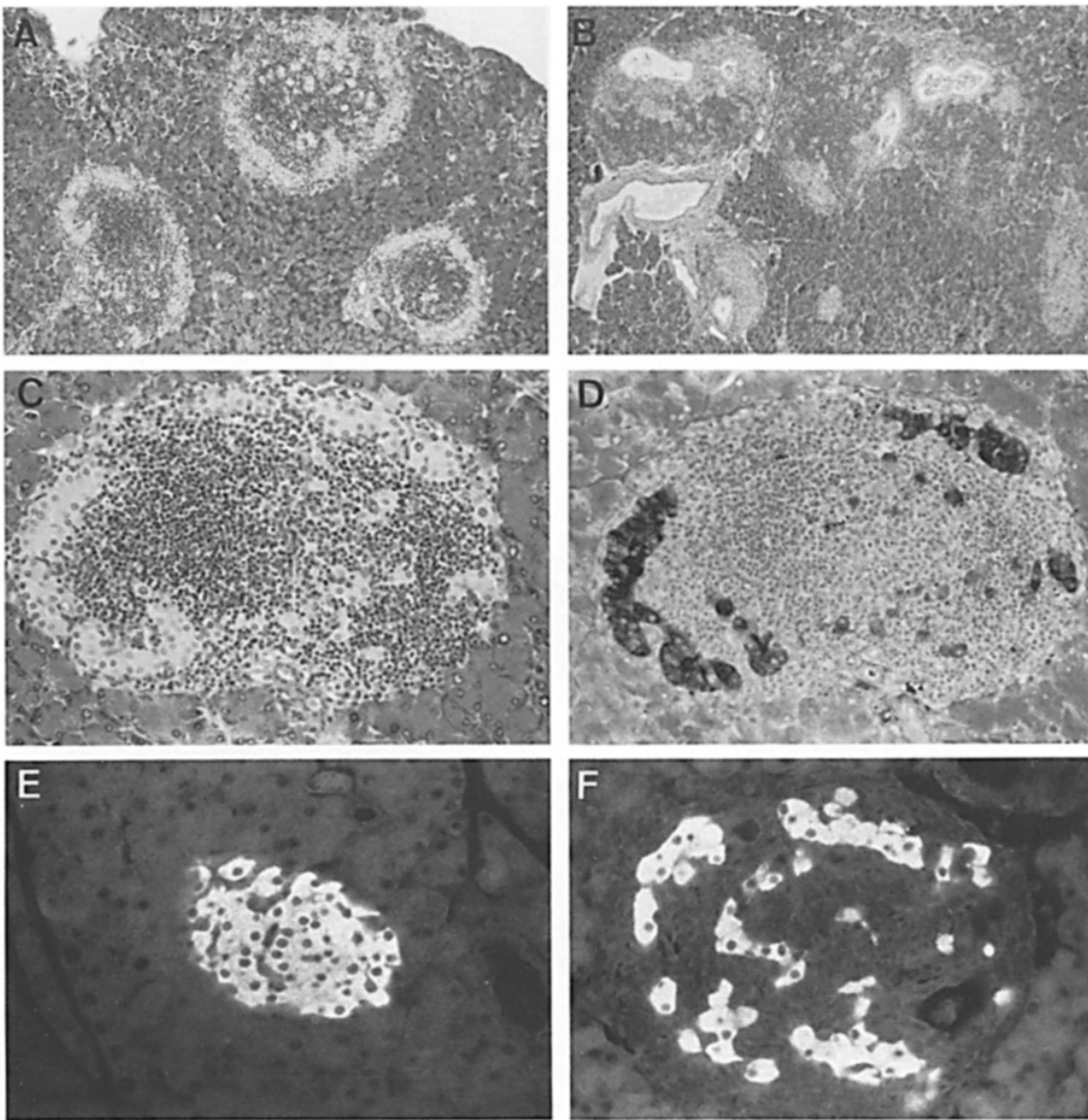

Figure 1. 5 μm-thick paraffin sections of pancreas of transgenic or control mice (4–6-mo-old), showing islets of Langerhans. (*A*) Three infiltrated islets from a TNF transgenic mouse (×62). (*B*). Two infiltrated islets of a transgenic mouse showing large ductules (×58). (*C* and *D*) Two serial sections of an infiltrated islet shown in *A*; the dispersion of β cells stained by aldehyde-fuchsin can be appreciated in *D* (×155); (*E* and *F*) Immunofluorescent staining with an antiinsulin antibody of islets of control (*E*) and transgenic (*F*) mice. (×250).

of 31 islets of seven TNF transgenic mice ranging from 5 to 12-mo-old was 347 ± 22 μm, and that of 25 islets of five control mice of comparable ages, 181 ± 10 μm (P <0.001). The largest islets observed had between 700 and 800 μm in diameter. Immediately adjacent to some of these large islets were extraislet accumulations of lymphoid cells that appeared to correspond to dilated lymphatic channels, probably indicative of an enormously increased lymphocytic traffic. At all times during the evolution of these lesions, the fasting glycemias of transgenic mice were not significantly different from those of control littermates (average of 141 ± 5 mg/dl, n = 20). Using a variety of techniques, the following detailed observations were made.

The Lymphoid infiltrate Consisted Mostly of T and B Lymphocytes, and also Included Macrophages. Immunohistochemistry showed that most infiltrating cells were either Thy1$^+$ (CD4$^+$ or CD8$^+$) T, or sIg$^+$ B lymphocytes with occasionally, a few nests of plasma cells. Staining with the mAb F4/80, which recognizes macrophages, showed F4/80$^+$ cells in a strikingly peripheral distribution. Few of the lymphocytes appeared to be activated, as judged by: (*a*) the scarcity of cells stained with anti-IL-2R antibody; and (*b*) the relative scar-

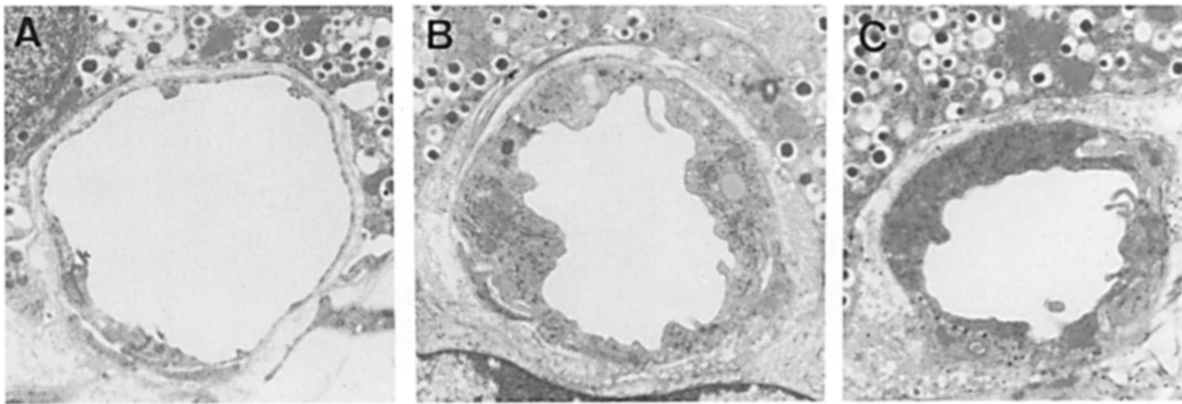

Figure 2. Ultrathin sections of intraislet capillaries (fixation by in vivo perfusion). (*A*) Control mouse. Capillary with a thin rim of endothelial cytoplasm, taking by place a fenestrated appearance. (*B*) TNF transgenic mouse. The endothelium cell lining is typically much higher. (*C*) NOD mouse. The aspect of the endothelium is similar to that shown in *B*. ×16,900.

city of dividing lymphoid cells detected by BrdU labeling (three daily injections of BrdU on 3 consecutive d, or 4 d of continuous perfusion through an osmotic pump) (data not shown).

Disorganization of the Islet Architecture. Staining by aldehyde-fuchsin (Fig. 1 *D*) or by antiinsulin antibody (Fig. 1, *E* and *F*) showed that β cells either formed a peripheral irregular rim in the islets where lymphocytic infiltration was predominantly central, or appeared as scattered microcolonies of cells in the massively infiltrated islets. Morphometric analysis showed that the volume density of the total endocrine tissue and of β cells were not statistically different between normal and transgenic mice, studied between 3 and 14 mo of age. Presence of non-β cells (glucagon, somatostatin, and pancreatic polypeptide cells) in the inflamed islets was verified by incubating consecutive paraffin or epon semithin sections with the corresponding antihormone serum. It should be noted that no clear immunofluorescence staining for MHC class II antigens was detected on the endocrine cells (not shown).

Alteration of the Islet Endothelial Cells. A striking ultrastructural feature was the thickening of the endothelial cells' cytoplasm with a paucity of capillaries presenting a fenestrated endothelium. 82% of islet capillaries presented a clearly altered morphology, compared with the islet capillaries of control mice (Fig. 2, *A* and *B*). A property of the high endothelial cells of the lymph node postcapillary venules is that they rapidly incorporate $^{35}SO_4$ in a sulfated glycoprotein, resulting in characteristic radioautographic labeling (21). After $^{35}SO_4$ injection and autoradiography, the islets of control mice showed no labeling (Fig. 3 *A*), while a marked labeling following the vascular outline was observed in the islets of transgenic mice (Fig. 3, *B* and *C*). The presence of adhesion molecules on these endothelial cells was explored using an anti-mouse ICAM mAb on frozen sections. Since lymphocytes were stained, a diffuse staining was observed, and it was not possible to detect a distinct staining of the capillaries (which were not opened, as is the case with fixation under perfusion).

Presence of an Intraislet Fibrotic Reaction. After 2 mo of age, spindle-shaped cells with elongated nuclei were detectable in islets of transgenic mice. They were more clearly seen in mice in which the lymphocytic infiltration was decreased as the result of anti-CD3 antibody injection (see below). By electron microscopy, they appeared to be fibroblasts, frequently surrounded by bundles of collagen fibrils. The extent of this fibrosis was most clearly revealed on histologic sections by silver staining (Fig. 4, *A* and *B*).

Development of Epithelial Ductules in the Islets. The presence of intraislet ductules was commonly observed with increased islet alterations (Fig. 5). These ductules were stained by antikeratin antibodies (Fig. 5 *D*), and often contained lymphocytes in their lumens (Fig. 5 *B*). In serial sections, the ductules were seen to be connected with extraislet exocrine ducts (Fig. 5 *A*), showing that they did originate from these ducts. A striking observation was the presence in their walls of isolated or sometimes contiguous β cells, identified by histochemistry, antiinsulin antibodies, or electron microscopy (Fig. 5, *B* and *C*). On ultrathin serial sections, these β cells were always separated from the ductular lumen by a layer, sometimes very thin, of epithelial cell cytoplasm (Fig. 6). No direct evidence for an origin of these β cells from precursors present among the ductular epithelial cells, under the form of cells with transitional appearance, was found. However, in some serial sections, a topographical continuity was observed between β cells located in the wall of a ductule and β cell aggregates apparently independent of the ductules. This suggested that these ductules might represent a regenerating process for the appearance of new endocrine cells. After BrdU injections or perfusions as described above, about one third of the ductular epithelial cells were labeled. However, labeling of ductular β cells was only very rarely observed, and was not detected in other β cells.

All these features were studied in parallel in the pancreas of 5–6-mo-old NOD mice, displaying a marked insulitis most often without overt diabetes. Comparable observations were made, with minor differences: lymphocytic infiltration tended to start more often at the periphery rather than in the center

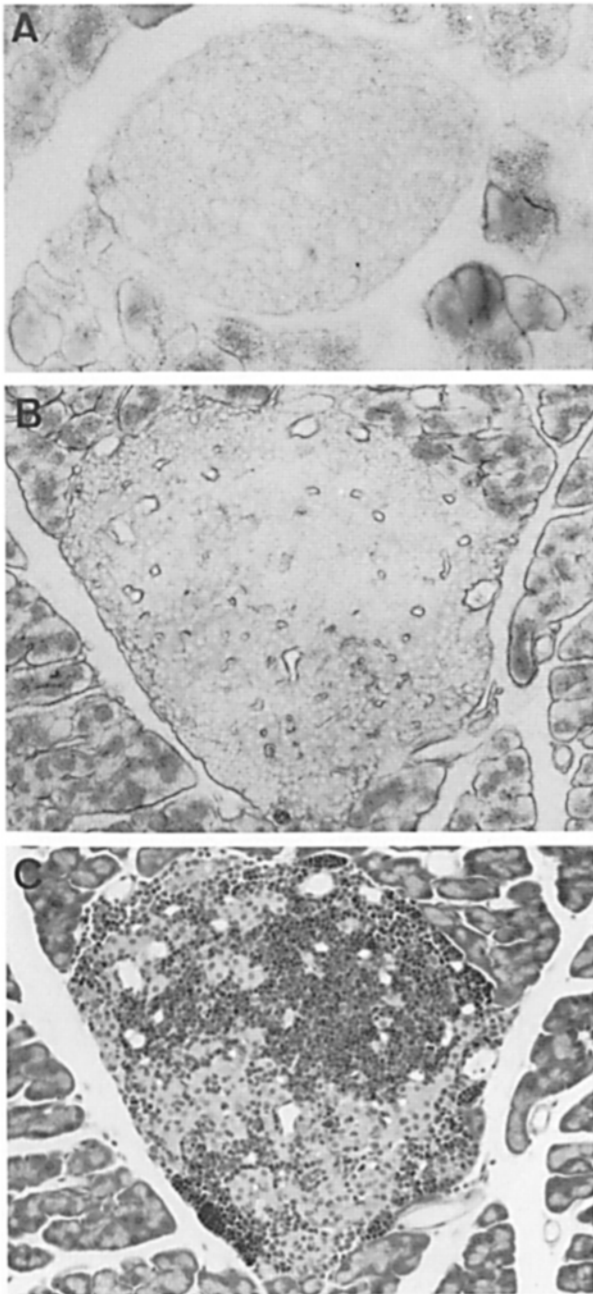

Figure 3. (*A* and *B*) Radioautographs, after $^{35}SO_4$ injection, of islets of a control mouse (*A*) (grains are present only on exocrine pancreas) and of a transgenic mouse (*B*), showing grains on the vascular walls within the islets. The vessels appear dilated and empty because of the perfusion. To better visualize the localization of the silver grains, the sections are unstained, the exposure time limited to 2 wk only. (*C*) Same section as *B*, stained to show the lymphocytic infiltration. (*A*) ×360; (*B* and *C*) ×180.

of islets, and the level of fibrosis, as judged by silver staining (Fig. 4 *C*), was less marked. The infiltrated islets did not reach the size ($P < 0.001$) of those seen in the transgenic mice (average of largest diameter of 17 islets from four mice: 217 ± 12 μm). Presence of F4/80$^+$ macrophages as a peripheral rim, thickening of endothelial cell cytoplasm (Fig. 2*C*), $^{35}SO_4$ incorporation, and immunofluorescence patterns with all antibodies mentioned above, were closely comparable. Development of intraislet ductules, however, was not observed, although ductules have been seen by others in the insulitis of NOD mice (see Fig. 1 of Signore et al. [22]).

Development of Lesions in Islets of TNF Transgenic SCID Mice

To explore which of the features observed in the islets of TNF transgenic mice were secondary to the lymphocytic infiltration, TNF transgenic mice were crossed with mice homozygous for the SCID mutation, which have neither T nor B lymphocytes (23). Transgenic SCID mice ∼3-mo-old did not show detectable lymphocytic infiltration, but a marked disorganization of the normal islet architecture was nevertheless obvious (Fig. 4, *D* and *E*). Infiltration by F4/80$^+$ cells was present in a peripheral pattern (Fig. 4 *F*), and appeared more marked than in non-SCID transgenic mice. Electron microscopy and silver staining for collagen fibrils showed that the altered β cell distribution was due to a fibrotic reaction (Fig. 4 *D*) as severe as that observed in TNF transgenic non-SCID mice, the only consistent difference with non-SCID mice being that the β cells were usually arranged in clusters of a few cells (Fig. 4 *E*). This probably simply reflects the lack of lymphocytic infiltration further separating the β cells. Intraislet ductules were rarely observed. On the other hand, ducts immediately adjacent to islets were common, but they did not contain β cells. All the cells within the islets were strongly stained with anti-ICAM antibody, except β cells, which were not stained (that islet cells can be induced in vitro to express ICAM is known [24]). It was thus not possible to specifically explore the endothelial cells. By electron microscopy, endothelial changes were similar to those described above; $^{35}SO_4$ incorporation was not studied. During the period of observation, the glycemia of these mice did not differ from those of non-SCID transgenic or control mice.

Attempts at Inducing Diabetes in TNF Transgenic Mice

These experiments, which are summarized in Table 1, were performed with transgenic mice that were at least 3-mo-old, and that displayed a severe insulitis.

Injections of Cyclophosphamide. In NOD mice that are not yet diabetic or that do not develop diabetes, one or two injections of cyclophosphamide are known to induce diabetes in a high percentage of cases (25). Four transgenic mice treated in this way had not developed a significant increase in glycemia 3 mo later.

Injections of Anti-CD3 Antibody. In vivo injections of anti-CD3 are known to stimulate T lymphocytes and to induce cytokine release (14). 12 transgenic mice received various schedules of anti-CD3 mAb injections, for the most part 20 μg daily for four consecutive days. Some of these mice had transient and mild hypoglycemia, rather than hyperglycemia. After killing on day 5, the lymphocyte infiltration was usually somewhat decreased, making the fibroblasts easier to observe. In two mice, this treatment was followed by injections of rIL-2 (5 μg, twice daily for 5 d). Lymphocytic infiltration

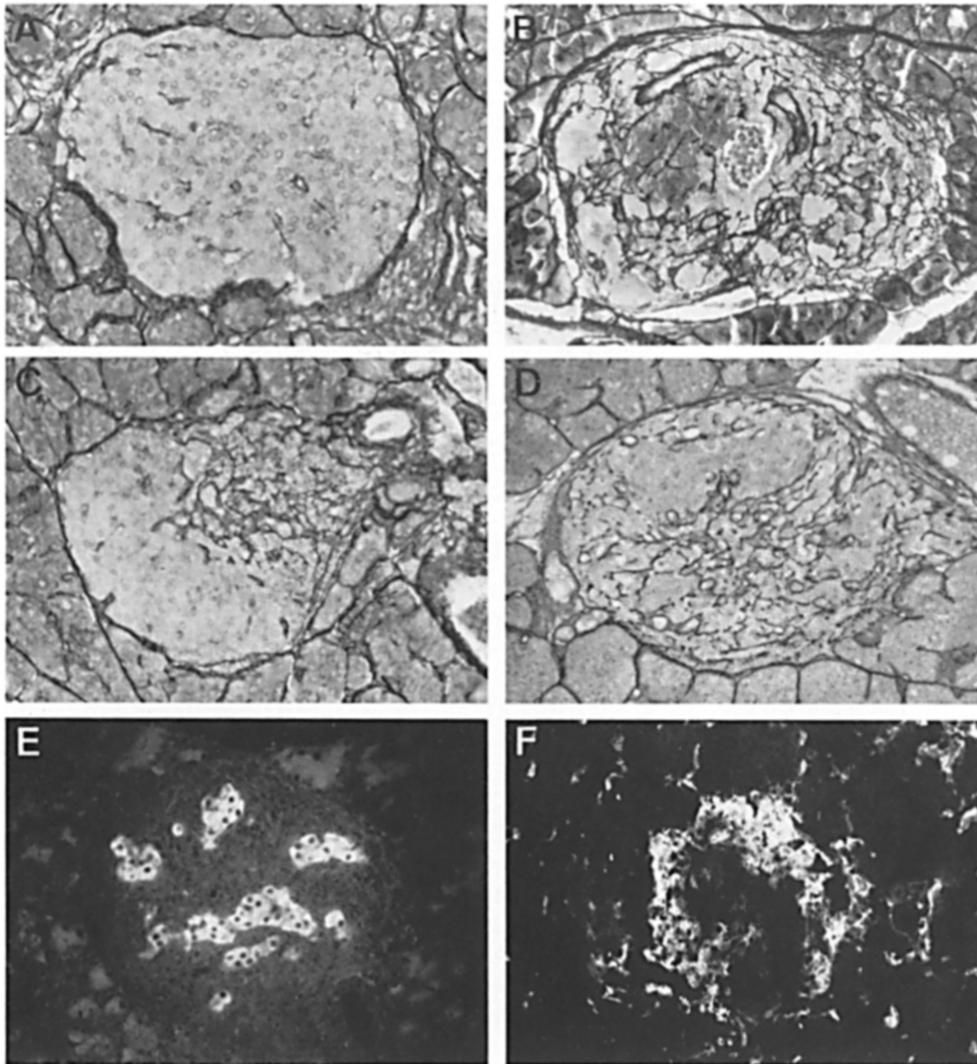

Figure 4. Histologic and immunofluorescent staining of paraffin (*A–E*) or cryostat (*F*) sections of pancreatic islets from normal or TNF transgenic mice. (*A*) Silver stain, control mouse. Staining limited to perivascular spaces. (*B*) Silver stain, TNF transgenic mouse. Heavy deposition of stained fibrils. (*C*) Silver stain, NOD mouse. Stained fibrils are found only in the part of the islet infiltrated by lymphoid cells. (*D*) Silver stain, TNF transgenic SCID mouse. Heavy depositions of stained fibrils. (*E*) Antiinsulin immunofluorescent staining of an islet from a TNF transgenic SCID mouse. In spite of the lack of lymphocyte infiltration, β cells appear as clusters. (*F*) Immunofluorescent staining with F4-80 mAb of an islet from a TNF transgenic SCID mouse. Positive macrophages are abundant, and are localized at the periphery of the islet. (*A*, *C*, and *D*) ×188. (*B*, *E*, and *F*) ×141.

was very marked after killing on day 11, without obvious signs of β cell damage. Glycemia did not vary significantly.

Perfusions of Cytokines. In vitro, TNF, in association with IL-1 or IFN-γ, has been observed to induce islet cell damage and/or increased expression of MHC class II antigens by β cells (4–8, 26). Three transgenic mice received 6 μg of rIL-1β in 3 d through osmotic pumps. They were killed since they appeared to be very sick or dying. Glycemia was low (50–60 mg/dl). Extensive areas of tissue necrosis were seen in the liver and the exocrine pancreas. The spleen red pulp was markedly hematopoietic. The islets of these mice were similar to those of untreated TNF transgenic mice, and antiinsulin immunofluorescence showed well-labeled β cells. Seven transgenic mice and one control mouse received 200 μg of mouse rIFN-γ as a constant perfusion over 2 wk, and two transgenic mice received the solvent only. The IFN-γ–treated transgenic mice (but not the normal mouse, nor the transgenic mice receiving the solvent only) had changes in their liver (hematopoietic clusters, small foci of necrosis with occasional neutrophils) and exocrine pancreas (focal areas of fibrosis and necrosis with occasional neutrophils). Since these lesions were not seen in the single normal mouse treated with IFN-γ, it appears probable that some TNF diffuses out of the islets in transgenic mice, and creates damage in synergy with IFN-γ, in particular in the exocrine pancreas. The important point, however, was that the islets of these mice were not different from those of control TNF transgenic mice in their structure and β cell content. Glycemias were comparable with those of control mice.

Injections of Streptozotocin. Five consecutive daily injections of subdiabetogenic doses of streptozotocin induce diabetes within a few days, often with insulitis. This has led to the suggestion that this is an autoimmune reaction to β cell damage (27), although this diabetes does not seem to be T cell mediated (28). To explore whether small doses of streptozotocin may induce diabetes, possibly by autoimmune mechanisms, more efficiently when an insulitis is already present, several groups of 3–10 TNF transgenic mice and their con-

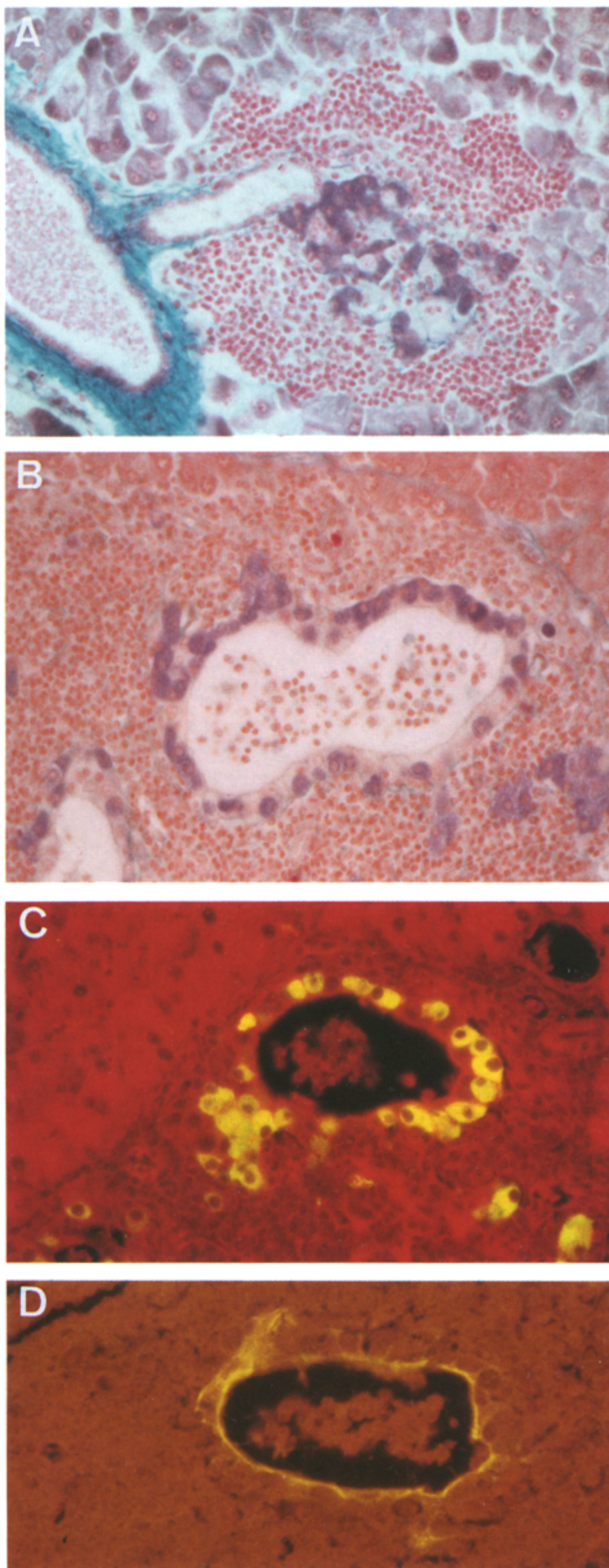

Figure 5. Intraislet ducts in transgenic mice. (*A*) Aldehyde-fuchsin staining of the pancreas from a TNF transgenic mouse. A branch from an interlobular duct is entering an infiltrated islet. (*B*) Aldehyde-fuchsin staining. Positive β cells embedded among the epithelial cells of ductule are especially striking. Around the ductule, a lymphocyte infiltrate replaced most β cells, and numerous lymphocytes are also present in the ductule's lumen. (*C*) Immunofluorescence with antiinsulin shows staining of the numerous β cells in the wall of an intraislet ductule. (*D*) Immunofluorescence with anticytokeratin antibody to stain ductule epithelial cells (see Fig. 6). (*A*) ×240. (*B*) ×250. (*C*) ×280. (*D*) ×312.

trols were subjected to serial injections of streptozotocin (40 or 100 mg/kg per injection). No diabetes was induced by low doses of this drug, whereas with high doses, 6 of 13 (46%) transgenic and 10 of 13 (77%) control mice became diabetic. Thus, mice with insulitis are not more susceptible to developing diabetes after this treatment than are control mice.

Presence of an Antigenic Viral Antigen on β Cells. TNF transgenic mice were crossed with mice bearing a transgene that causes the expression of the lymphochoriomeningitis virus glycoprotein (LCMV gp) on their islet β cell membranes (10). These mice do not respond spontaneously to this antigen, but they are not tolerant since, when infected with the live LCMV virus, they develop insulitis and diabetes within a few days (10). Mice carrying the two TNF and LCMV gp transgenes did not become spontaneously diabetic over a period of 6 mo of observation. They were not tolerant to the antigen, since when injected with the live virus, they became diabetic within a few days, much as their control littermates bearing only the LCMV gp transgene. Four mice carrying the two transgenes also received perfusions of IFN-γ (15 d), using the same protocols as described above for the TNF transgenic mice, with comparable results.

Discussion

The permanent production of TNF-α by pancreatic islet β cells results in a damage restricted to the islets of Langerhans, the major features of which are: lymphocytic insulitis, endothelial cell alterations, fibrotic reaction, disorganization of the arrangement of the endocrine cells, and development of epithelial ductules containing, by places, β cells in their walls. The lack of lesions in the exocrine regions of the pancreas and the good health conditions observed in the three lines of transgenic mice studied suggests that the release of TNF-α remained low. This may be due to the presence of the entire 3′ untranslated region of TNF in the transgene. This region indeed exerts a negative effect, not only on TNF mRNA stability, but also on its translation (29).

The lesions observed in TNF transgenic mice can be analyzed from two perspectives: the chronic inflammatory reaction elicited by continuous local TNF release, and the pathogenesis of autoimmune diabetes, the first stage of which presents, in NOD mice, comparable lesions.

The insulitis consisted of a progressively growing lymphoid infiltration made of $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes and, to a lesser extent, macrophages. At no time was a polymorphonuclear infiltration, even limited, observed. With age, the lymphoid cell infiltration became so large that the average islet diameter was doubled. This appears to be associated with an intense local cell traffic, as suggested by the dilated lymphatic channels full of packed lymphocytes

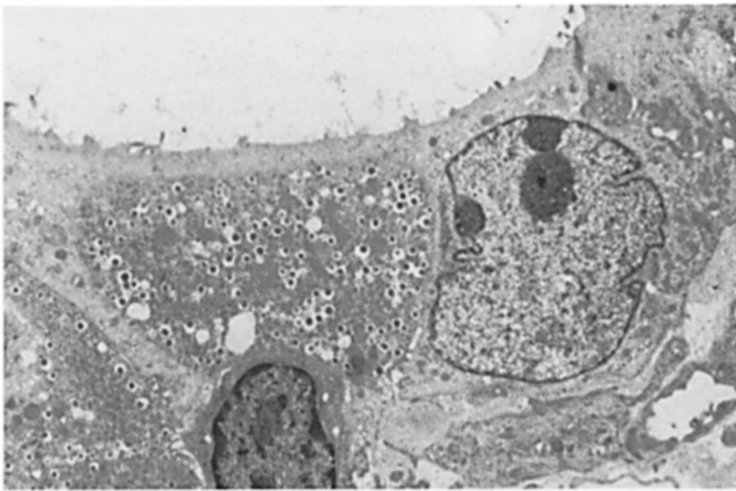

Figure 6. Electron microscopic appearance of intraislet ductules of TNF transgenic mice containing β cells in their wall. β cells are not in contact with the ductule lumen, but are covered by a thin sheet of epithelial cell cytoplasm. This explains the staining with anticytokeratin antibody shown in Fig. 5 *D.* ×13,500.

often seen adjacent to the infiltrated islets. The main cause of this increased local traffic is likely to be endothelial changes resulting from TNF action.

Two striking alterations of the islet capillary walls were indeed seen: a general cytoplasmic endothelial thickening with loss of the fenestrated structure of the normal islet capillaries, and the ability to incorporate $^{35}SO_4$ in capillary walls after in vivo injection. Sulfate incorporation is a characteristic feature of the peculiar high endothelial cells of the lymph nodes' postcapillary venules (21), also called high endothelial venules, which are a site of massive emigration of circulating lymphocytes from the blood. In these cells, this sulfate group is present on the carbohydrate moiety of a mucin-like glycoprotein (30) which functions as a "vascular adressin" (31). It is a ligand to a L-selectin (32) of lymphocytes, an adhesion protein with a lectin domain which functions as a lymph node "homing receptor" (33–35). Expression of this selectin ligand is induced by TNF on endothelial cells in culture (36), as is that of other adhesion proteins, namely ICAM-1 (37, 38), endothelial leukocyte adhesion molecule (ELAM-1) (39–41), and vascular cell adhesion molecule (VCAM-1) (42). Presence of these molecules on the transgenic islet endothelial cells could not be explored, either because many other islet cells were stained, as was the case for ICAM-1, or because

Table 1. *Attempts at Eliciting Diabetes in TNF Transgenic Mice*

Protocol used	Glycemia* >250 mg/dl and/or histologic β cell damage in recipients	Known effects‡
1. Cyclophosphamide (repeated injections)	No	Triggers diabetes in NOD mice with insulitis and no diabetes
2. Anti-CD3 antibody ± IL-2 (5 d)	No	Triggers cytokine release by T cells; activates T cells
3. IL-1β perfusion (3 d)	No	Damages islet cells in vitro
4. IFN-γ perfusion (15 d)	No	In vitro, increases MHC class II antigen expression on β cells and synergizes TNF action, including islet cell damage
5. Streptozotocin:		Subdiabetogenic damage to β cells;
40 mg/kg/d, 5 d	No	reported to trigger cell-mediated
100 mg/kg/d, 5 d	In a fraction of mice	autoimmune response to β cells
6. β cells bearing a viral Ag	No	No immune response in mice without viral infection
7. As 6, plus viral infection	Yes	Viral infection triggers diabetes
8. As 6, plus IFN-γ perfusion (15 d)	No	Combines nos. 6 and 4

* Nonfasting values

‡ See text for details and references.

of the lack of relevant antibodies. The endothelial changes observed in the islets of TNF transgenic mice are likely to be instrumental in the development of the insulitis, inducing a lymphocytic traffic resting on mechanisms comparable with those operative at the level of lymph nodes' high endothelial venules.

Together with lymphoid infiltration, the other major alteration leading to disorganization of endocrine cells within the islets of the TNF transgenic mice was fibrosis. This was not secondary to the lymphocytic infiltration, since fibrosis was also prominent in TNF transgenic SCID mice, which have no lymphocytic infiltration. Fibrosis may be related to macrophage infiltration, which was particularly conspicuous in TNF transgenic SCID mice. Previously, TNF has been found to be involved in fibrotic reactions (43), and in particular, as judged by the preventive effect of anti-TNF antibody treatment, on some fibrosing pneumopathies (44, 45). Whether TNF acts indirectly, by enhancing the release and processing of fibrogenic cytokines, such as TGF-β, has not been determined. The extent of the fibrotic reaction at the site of prolonged TNF release may vary depending upon the nature of the cellular infiltrate and the composition of the surrounding tissue. Islet fibrosis has also been observed in human insulin-dependent diabetes (46).

The last prominent alteration in the pancreatic islets was the appearance of epithelial ductules containing, by places, β cells in their walls, sometimes arranged in a more or less continuous row. Serial sections showed the connection of these ductules with extraislet ducts. Is this a process of regeneration, starting from exocrine ducts and leading to the neoformation of endocrine cells? Although strongly suggested by the topographical organization of these β cell–containing ductules, this hypothesis could not be ascertained, since no cells displaying intermediate features compatible with progressive differentiation into β cells of precursors located in the ductular walls were detected. What could be the inducing mechanism(s) of ductular development? A primary role of β cell damage or disorganization does not seem likely, since these structures have not been described after direct β cell damage occurring after streptozotocin treatment (47). Local release of cytokines is an attractive possibility. Although TNF-α itself is not known to act directly upon epithelial cell growth, it does trigger cytokine release from a variety of cell types. Macrophages, for instance, which are present in the insulitis, may release, among many other cytokines, TGF-α, an epithelial cell growth factor (48). Can a putative process of β cell regeneration play a significant role in the lack of progression towards diabetes of the TNF-mediated insulitis? After injections or perfusion of BrdU, the extent of labeling of the ductular epithelial cells did not indicate very rapid proliferation, and only very few β cells were found to be labeled. However, it seems plausible that a slow but constant process of β cell regeneration might play some protective role against the occurrence of diabetes in conditions where β cell injury does not occur at a very rapid rate. Very extensive proliferation of intraislet ductules is observed in the form of diabetes developed by db/db mice, although the pathogenic mechanisms of this disease are still obscure (49, 50). It is interesting to note that in human insulin-dependent diabetes, comparable intraislet, lymphocyte-containing ductules have also been observed (46).

When the TNF-mediated insulitis was compared with the autoimmune insulitis of NOD mice, two salient observations emerged. First, the TNF-mediated lesions bear, in almost all details discussed above except the development of ductules, a marked resemblance to those found in NOD mice analyzed in parallel in the present study. Second, in spite of the increasing extent with age of the insulitis of TNF transgenic mice, overt diabetes never developed, at least during the long period of observation (up to 16 mo). Similarly, in the first phase of the autoimmune disease in NOD mice, there is a marked insulitis without obvious decrease in β cells and without clinical diabetes. $CD4^+$ T cell clones have been obtained from the lymphocytes infiltrating the islets of NOD mice at this stage of the disease. These clones proliferate in vitro in the presence of islet cells of NOD mice only, and, after in vivo injection to young NOD mice, lead to the rapid development of insulitis without diabetes (51). TNF may be a mediator of the NOD insulitis. TNF mRNA has indeed been detected by in situ hybridization in the NOD insulitis (3). This would not exclude a contribution of TNF-β, which shares the same receptors, and of IL-1, which has comparable effects on endothelial cells (52). The puzzling observation that several weekly injections of TNF for several months in NOD mice retard insulitis and subsequent diabetes (53, 54) may seem to argue against this hypothesis. However, there are examples of desensitization to TNF effects by systemic injections of TNF which could explain this apparent paradox (55). In any event, the point that must be stressed most forcefully in the present context is that, in a large proportion of NOD mice (i.e., 20–30% of females and 80–90% of males), which all have initial insulitis of comparable severity, a second, diabetic phase of the disease never develops. To develop the β cell injury resulting in diabetes, which occurs around the sixth month, secondary events are necessary, leading to the appearance of new islet T cell subpopulations, perhaps as the result of recognition of new antigens. $CD4^+$ T cell clones have indeed been obtained from NOD mice in the diabetic phase which destroy NOD islet cells in vitro (56), and induce the rapid appearance of diabetes after injection into mice presenting insulitis only (57). Activated islet $CD8^+$ T cells are also capable of cytotoxicity against islet cells in vitro (58). In any event, the respective roles of $CD4^+$ and $CD8^+$ T lymphocytes in the development of initial insulitis and of subsequent diabetes are still a matter of controversy (59, 60). Some BB rats, although diabetes-prone, fail to develop diabetes. In these rats too, an insulitis has been observed (61).

Thus, in spite of the severity of their insulitis, the TNF transgenic mice may lack secondary events required to precipitate the β cell damage. Alternatively, or in addition, the lymphocytes present within the transgene-induced insulitis may lack antigenic stimulation, since the transgenic mice do not have the unique genetic background alterations, in particular within the MHC, which is the basis of the susceptibility of NOD mice to diabetes. A variety of protocols, listed in Table

1, was used, to try to mimic secondary events capable of inducing diabetes, or to induce an antigenic stimulation within the islets, or a combination of both. This included cyclophosphamide treatment (known to rapidly induce the appearance of diabetes and to considerably increase its incidence in NOD mice [25]), T lymphocyte stimulation, and constant perfusion of cytokines reported to strongly synergize with TNF in vitro to induce islet cell damage (4–8) and/or to increase β cell expression of MHC class II antigens and thus the presentation of antigenic peptides (26, 62). None of these protocols induced overt diabetes or immunohistochemical signs of β cell damage. The lack of effect of cyclophosphamide indicated that the transgenic insulitis differs from the NOD insulitis, perhaps because of the lack of a proper antigenic stimulation in the absence of a NOD genetic background. It is intriguing that the constant presence of high doses of circulating IFN-γ for 2 wk did not have, in mice releasing TNF in their islets and already having a severe insulitis, a diabetogenic effect, since mice bearing an IFN-γ transgene under the control of the insulin promoter develop diabetes with some degree of islet lymphocytic infiltration (9). Evidence has been presented for the existence in these mice of circulating lymphocytes specifically cytotoxic to syngeneic normal islet cells, i.e., of a tissue-specific cell-mediated autoimmunity (63). In NOD mice, anti-IFN-γ antibody treatment decreases the incidence of diabetes triggered by cyclophosphamide injection (64). The possibility that an intraislet simultaneous release of these two cytokines (by breeding double transgenic mice) indeed has a synergistic effect on diabetes occurrence could unfortunately not be explored.

Two lines of evidence argue against the interpretation that the lack of progression of the TNF-mediated insulitis towards diabetes might simply reflect the lack of antigenic determinants borne by β cells. First, repeated injections of various amounts of subdiabetogenic doses of streptozotocin, which repeatedly damages β cells (27), did not result in an incidence of diabetes in TNF transgenic mice higher than that observed in control littermates. Second, and more decisively, mice with a strong insulitis and expressing a viral antigen on islet β cell membranes were obtained by crossing TNF transgenic mice with mice bearing a LCMV-gp transgene under the control of an insulin promoter (10, 65). These last transgenic mice do not spontaneously respond to the LCMV-gp antigen (10). They have normal glycemias and intact islets, but when exposed to a strong antigenic stimulus (infection with the live virus), they develop an acute diabetes after 9–11 d, with an insulitis in which specific cytotoxic $CD8^+$ cells can be found (10). Mice bearing the two LCMV-gp and TNF transgenes did not develop diabetes spontaneously, at least not over a 6-mo period of observation. Perfusion with IFN-γ during 2 wk did not lead to a rapid increase in glycemia, which, however, was observed in these mice 1 wk after infection with live virus (indicating that they had not been rendered tolerant). Taken together, these results indicate that antigen presentation on β cells is by itself not able to efficiently stimulate specific lymphocytes among the large amounts of lymphocytes that circulate through islets as a result of the insulitis, even in conditions considered to enhance antigen presentation such as exposure to IFN-γ. Recently, mice bearing an IL-2 transgene under the control of the rat insulin promoter were described. Some of these mice developed an insulitis, without evidence of autoimmunity to islet antigens, in particular diabetes, nor of recognition of a foreign MHC class I antigen (expressed on β cells by the use of double transgenic mice) (66).

The conclusion of these last observations is that, to obtain β cell destruction leading to diabetes, severe insulitis, release of cytokines, and presence of antigenic determinants on β cells are not sufficient. Antigen-bearing β cells may be adequate targets only for T lymphocytes already stimulated by other cells presenting these antigenic determinants. The increased lymphocytic traffic in the islets resulting from insulitis may increase the changes of contact between specifically stimulated T lymphocytes and antigen-bearing β cells, and thus enhance the risk of diabetes or accelerate its emergence. The concept that lymphocytic insulitis may be by itself only potentially diabetogenic, i.e., may represent but an increased risk factor for specific and extensive β cell damage, provides an explanation for the existing discrepancy between the occurrence of insulitis and that of diabetes in autoimmune NOD mice. In human autoimmune diabetes too, there is evidence for a long period of antiislet autoimmunity before the abrupt clinical occurrence of diabetes (67).

In summary, the study of TNF transgenic mice has shown that: (*a*) a slow local release of TNF leads to a massive lymphoid cell infiltrate, probably resulting mainly from endothelial cell alterations, accompanied by local fibrosis and ductular proliferation, which may represent a process of endocrine regenerations; and (*b*) a lymphoid insulitis, whatever its severity, is not by itself sufficient to create diabetes. It appears that lymphocyte stimulation by APCs other than β cells is necessary for diabetes to develop.

This work is dedicated to the memory of Albert E. Renold. We want to thank Ms. J. Ntah for secretarial work; Ms. Danielle Ben Nasr, Ms. Monique Eissler, Ms. Ileana Condacci, Mr. P. Henchoz, and Mr. Max Bauman for skillful technical assistance; and Mr. Gérard Negro and Mr. Beat Favri for photographic work.

This work was supported by grants from the Swiss National Foundation (31-28866.90 and 31-34088.92). P. Ohashi is supported by the Medical Research Council of Canada.

Address correspondence to Dr. Pierre Vassalli, Département de Pathologie, Université de Genève, Centre Medical Universitaire, 1, rue Michel-Servet, 1211 Geneva 4, Switzerland. Y. Higuchi is currently at Depart-

ment of Pathology, Medical College of Oita, Oita 879-56, Japan; P. Muniesa is currently at Catedra de Anatomia y Embriologia, Facultad de Veterinaria, Universidad de Zaragoza, Zagaroza, Spain; P. Ohashi is currently at the Department of Medical Biophysics, Ontario Cancer Institute, University of Toronto, 500 Sherbourne Street, Toronto, Canada M4K 1K9.

Received for publication 25 June 1992 and in revised form 25 September 1992.

References

1. Shafrir, E., and A.E. Renold, editors. 1988. Frontiers in Diabetes Research. Lessons from Animal Diabetes II. John Libbey & Co. Ltd., London. 99–166.
2. Marliss, E., A.F. Nakhooda, P. Poussier, and A.A.F. Sima. 1982. The diabetic syndrome of the "BB" Wistar rat: possible relevance to Type I (insulin-dependent) diabetes in man. *Diabetologia.* 22:225.
3. Held, W., H.R. Macdonald, I.L. Weissman, M.W. Hess, and C. Mueller. 1990. Genes encoding tumor necrosis factor α and granzyme A are expressed during development of autoimmune diabetes. *Proc. Natl. Acad. Sci. USA.* 87:2239.
4. Bendtzen, K., T. Mandrup-Poulsen, J. Nerup, J.H. Nielsen, C.A. Dinarello, and M. Svenson. 1986. Cytotoxicity of human pI 7 interleukin-1 for pancreatic islets of Langerhans. *Science (Wash. DC).* 232:1545.
5. Mandrup-Poulsen, T., K. Bendtzen, C.A. Dinarello, and J. Nerup. 1987. Human tumor necrosis factor potentiates human interleukin 1-mediated rat pancreatic β-cell cytotoxicity. *J. Immunol.* 139:4077.
6. Mandrup-Poulsen, T., G.A. Spinas, S.J. Prowse, B.S. Hansen, D.W. Jorgensen, K. Bendtzen, J.H. Nielsen, and J. Nerup. 1987. Islet cytotoxicity of interleukin 1. Influence of culture conditions and islet donor characteristics. *Diabetes.* 36:641.
7. Pukel, C., H. Baquerizo, and A. Rabinovitch. 1988. Destruction of rat islet cell monolayers by cytokines. Synergistic interactions of interferon-γ, tumor necrosis factor, lymphotoxin, and interleukin 1. *Diabetes.* 37:133.
8. Campbell, I.L., A. Iscaro, and L.C. Harrison. 1988. IFN-γ and tumor necrosis factor-α. Cytotoxicity to murine islets of Langerhans. *J. Immunol.* 141:2325.
9. Sarvetnick, N., D. Liggitt, S.L. Pitts, S.E. Hansen, and T.A. Stewart. 1988. Insulin-dependent diabetes mellitus induced in transgenic mice by ectopic expression of class II MHC and interferon-gamma. *Cell.* 52:773.
10. Ohashi, P.S., S. Oehen, K. Buerki, H. Pircher, C.T. Ohashi, B. Odermatt, B. Malissen, R.M. Zinkernagel, and H. Hengartner. 1991. Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. *Cell.* 65:305.
11. Semon, D., E. Kawashima, C.V. Jongeneel, A.N. Shakhov, and S.A. Nedospasov. 1987. Nucleotide sequence of the murine TNF locus, including the TNF-α (tumor necrosis factor) and TNF-β (lymphotoxin) genes. *Nucleic Acids Res.* 15:9083.
12. Walter, M.D., T. Edlund, A.M. Boulet, and W.J. Rutter. 1983. Cell-specific expression controlled by the 5'-flanking region of insulin and chymotrypsin genes. *Nature (Lond.).* 306:557.
13. Ruff, M.R., and G.E. Gifford. 1980. Purification and physicochemical characterization of rabbit tumor necrosis factor. *J. Immunol.* 125:1671.
14. Ferran, C., K. Sheehan, M. Dy, R. Schreiber, S. Merite, P. Landais, L. H. Noel, G. Grau, J. Bluestone, J.F. Bach, and L. Chatenoud. 1990. Cytokine-related syndrome following injection of anti-CD3 monoclonal antibody: further evidence for transient in vivo T cell activation. *Eur. J. Immunol.* 20:509.
15. Dialynas, D.P., Z.S. Quan, K.A. Wall, A.Pierres, J. Quintans, M.R. Loken, M. Pierres, and F.W. Fitch. 1983. Characterization of the murine T cell surface molecule, designated L3T4, identified by monoclonal antibody GK1.5: similarity of L3T4 to the human Leu-3/T4 molecule. *J. Immunol.* 131:2445.
16. Pierres, M., C. Goridis, and P. Golstein. 1982. Inhibition of murine T cell-mediated cytolysis and T cell proliferation by a rat monoclonal antibody immunoprecipitating two lymphoid cell surface polypeptides of 94000 and 180000 molecular weight. *Eur. J. Immunol.* 12:60.
17. Austyn, J.M., and S. Gordon. 1981. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur. J. Immunol.* 11:805.
18. Horley, K.J., C. Carpenito, B. Baker, and F. Takei. 1989. Molecular cloning of murine intercellular adhesion molecule (ICAM-1). *EMBO (Eur. Mol. Biol. Organ.) J.* 8:2889.

18a. Warren, S., P.N. Lecompte, and M.A. Legg. 1966. the Pathology of Diabetes Mellitus. 4th ed. Lea & Febiger, Philadelphia. pg. 504.

19. Weibel, E.R. 1969. Stereological principles for morphometry in electron microscopy. *Int. Rev. Cytol.* 26:235.
20. Snedecor, G.W., and W.G. Cochran. 1967. Statistical Methods. Iowa State University Press, Ames. 120–122.
21. Andrews, P., D.W. Milsom, and W.L. Ford. 1982. Migration of lymphocytes across specialized vascular endothelium. V. Production of sulphated macromolecule by high endothelial cells in lymph nodes. *J. Cell Sci.* 57:277.
22. Signore, A., Pozzili, E.A.M. Gale, D. Andreani, and P.C.L. Beverley. 1989. The natural history of lymphocyte subsets infiltrating the pancreas of NOD mice. *Diabetologia.* 32:282.
23. Bosma, G.C., R.P. Custer, and M.J. Bosma. 1983. A severe combined immunodeficiency mutation in the mouse. *Nature (Lond.).* 301:527.
24. Campbell, I.L., A. Cutri, D. Wilkinson, A.W. Boyd, and L.C. Harrison. 1989. Intercellular adhesion molecule 1 is induced on isolated endocrine islet cells by cytokines but not by reovirus infection. *Proc. Natl. Acad. Sci. USA.* 86:4282.
25. Charlton, B., A. Bacelj, R.M. Slattery, and T.E. Mandel. 1989. Cyclophosphamide-induced diabetes in NOD/WEHI mice. Evidence for suppression in spontaneous autoimmune diabetes mellitus. *Diabetes.* 38:441.
26. Campbell, I.L., L. Oxbrow, J. West, and L.C. Harrison. 1982. Regulation of MHC protein expression in pancreatic β-cells by interferon-γ and tumor necrosis factor-α. *Mol. Endocrinol.* 2:101.
27. Like, A.A., M.C. Appel, R.M. Williams, and A.A. Rossini. 1978. Streptozotocin-induced pancreatic insulitis in mice. Morphologic and physiologic studies. *Lab. Invest.* 38:470.
28. Dayer-Métroz, M.D., M. Kimoto, S. Izui, P. Vassalli, and A.E. Renold. 1988. Effect of helper and/or cytotoxic T-lymphocyte

depletion on low-dose streptozocin-induced diabetes in C57BL/6J mice. *Diabetes.* 8:1082.
29. Kruys, V., K. Kemmer, A. Shakhov, V. Jongeneel, and B. Beutler. 1992. Constitutive activity of the tumor necrosis factor promoter is canceled by the 3' untranslated region in nonmacrophage cell lines; a trans-dominant factor overcomes this suppressive effect. *Proc. Natl. Acad. Sci. USA.* 89:673.
30. Lasky, L.A., M.S. Singer, D. Dowbenko, Y. Imai, W.J. Henzel, C. Grimley, C. Fennie, N. Gillett, S.R. Watson, and S.D. Rosen. 1992. An endothelial ligand for L-selectin is a novel mucin-like molecule. *Cell.* 69:927.
31. Streeter, P.R., B.T.N. Rouse, and E.C. Butcher. 1988. Immunohistologic and functional characterization of a vascular addressin involved in lymphocyte homing into peripheral lymph nodes. *J. Cell Biol.* 107:1853.
32. Springer, T.A. 1990. Adhesion receptors of the immune system. *Nature (Lond.).* 346:425.
33. Stoolman, L.M. 1989. Adhesion molecules controlling lymphocyte migration. *Cell.* 56:907.
34. Lasky, L.A., M.S. Singer, T.A. Yednock, D. Dowbenko, C. Fennie, H. Rodriguez, T. Nguyen, S. Stachel, and S.D. Rosen. 1989. Cloning of a lymphocyte homing receptor reveals a lectin domain. *Cell.* 56:1045.
35. Siegelman, M.H., I.C. Cheng, I.L. Weissman, and E.K. Wakeland. 1990. The mouse lymph node homing receptor is identical with the lymphocyte cell surface marker Ly-22: role of the EGF domain in endothelial binding. *Cell.* 61:611.
36. Spertini, O., F.W. Luscinskas, G.S. Kansas, J.M. Munro, J.D. Griffin, M.A. Gimbrone, Jr., and T.F. Tedder. 1991. Leukocyte adhesion molecule-1 (LAM-1, L-selectin) interacts with an inducible endothelial cell ligand to support leukocyte adhesion. *J. Immunol.* 147:2565.
37. Marlin, S.D., and T.A. Springer. 1987. Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). *Cell.* 51:813.
38. Simmons, D., M.W. Makgoba, and B. Seed. 1988. ICAM, an adhesion ligand of LFA-1, is homologous to the neural cell adhesion molecule NCAM. *Nature (Lond.).* 331:624.
39. Bevilacqua, M.P., S. Stengelin, M.A. Gimbrone, Jr., and B. Seed. 1989. Endothelial-leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. *Science (Wash. DC).* 243:1160.
40. Picker, L.J., T.K. Kishimoto, C.W. Smith, R.A. Warnock, and E.C. Butcher. 1991. ELAM-1 is an adhesion molecule for skin-homing T cells. *Nature (Lond.).* 349:796.
41. Shimizu, Y., S. Shaw, N. Graber, T.V. Gopal, K.J. Horgan, G.A. Van Seventer, and W. Newman. 1991. Activation-independent binding of human memory T cells to adhesion molecule ELAM-1. *Nature (Lond.).* 349:799.
42. Osborn, L., C. Hession, R. Tizard, C. Vassallo, S. Luhowskyj, G. Chi Rosso, and R. Lobb. 1989. Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. *Cell.* 59:1203.
43. Piguet, P.F., G.E. Grau, and P. Vassalli. 1990. Subcutaneous perfusion of tumor necrosis factor induced local proliferation of fibroblasts, capillaries, and epidermal cells, or massive tissue necrosis. *Am. J. Pathol.* 136:103.
44. Piguet, P.F., M.A. Collart, G.E. Grau, Y. Kapanci, and P. Vassalli. 1989. Tumor necrosis factor/cachectin plays a key role in bleomycin-induced pneumopathy and fibrosis. *J. Exp. Med.* 170:655.
45. Piguet, P.F., M.A. Collart, G.E. Grau, A.P. Sappino, and P. Vassalli. 1990. Requirement of tumor necrosis factor for development of silica-induced pulmonary fibrosis. *Nature (Lond.).* 344:245.
46. Gepts, W. 1965. Pathologic anatomy of the pancreas in juvenile diabetes mellitus. *Diabetes.* 14:619.
47. Steiner, H., O. Oelz, G. Zahnd, and E.R. Froesch. 1970. Studied on islet cell regeneration, hyperplasia and intrainsular cellular interrelations in long lasting streptozotocin diabetes in rats. *Diabetologia.* 6:558.
48. Rappolee, D.A., D. Mark, M.J. Banda, and Z. Werb. 1988. Wound macrophages express TGF-α and other growth factors in vivo: analysis by mRNA phenotyping. *Science (Wash. DC).* 241:708.
49. Coleman, D.L., and K.P. Hummel. 1967. Studies with the mutation, diabetes, in the mouse. *Diabetologia.* 3:238.
50. Like, A.A., and W.L. Chick. 1970. Studies in the diabetic mutant mouse: I. Light microscopy and radioautography of pancreatic islets. *Diabetologia.* 6:207.
51. Reich, E.P., R.S. Sherwin, O. Kanagawa, and C.A. Janeway, Jr., 1989. An explanation for the protective effect of the MHC class II I-E molecule in murine diabetes. *Nature (Lond.).* 341:326.
52. Pober, J.S., and R.S. Cotran. 1990. Cytokines and endothelial cell biology. *Physiological Rev.* 70:427.
53. Jacob, C.O., S. Aiso, S.A. Michie, H.O. McDevitt, and H. Acha-Orbea. 1990. Prevention of diabetes in nonobese diabetic mice by tumor necrosis factor (TNF): similarities between TNF-α and interleukin 1. *Proc. Natl. Acad. Sci. USA.* 87:968.
54. Satoh, J., H. Seino, T. Abo, S.I. Tanaka, S. Shintani, S. Ohta, K. Tamura, T. Sawai, T. Nobunaga, T. Oteki et al. 1989. Recombinant human tumor necrosis factor α suppresses autoimmune diabetes in nonobese diabetic mice. *J. Clin. Invest.* 84:1345.
55. Vassalli, P. 1992. The pathophysiology of tumor necrosis factors. *Annu. Rev. Immunol.* 10:411.
56. Haskins, K., M. Portas, B. Bergman, K. Lafferty, and B. Bradley. 1989. Pancreatic islet-specific T-cell clones from nonobese diabetic mice. *Proc. Natl. Acad. Sci. USA.* 86:8000.
57. Pankewycz, O., T.B. Strom, and V.E. Rubin-Kelley. 1991. Islet-infiltrating T cell clones from non-obese diabetic mice that promote or prevent accelerated onset diabetes. *Eur. J. Immunol.* 21:873.
58. Nagata, M., K. Yokono, M. Hayakawa, Y. Kawase, N. Hatamori, W. Ogawa, K. Yonezawa, K. Shii, and S. Baba. 1989. Destruction of pancreatic islet cells by cytotoxic T lymphocytes in nonobese diabetic mice. *J. Immunol.* 143:1155.
59. Thivolet, C., A. Bendelac, P. Bedossa, J.F. Bach, and C. Carnaud. 1991. $CD8^+$ T cell homing to the pancreas in the nonobese diabetic mouse is $CD4^+$ T cell-dependent. *J. Immunol.* 146:85.
60. Wang, Y., O. Pontesilli, R.G. Gill, F.G. La Rosa, and K.J. Lafferty. 1991. The role of $CD4^+$ and $CD8^+$ T cells in the destruction of islet grafts by spontaneously diabetic mice. *Proc. Natl. Acad. Sci. USA.* 88:527.
61. Komiya, I., D. Baetens, L. Inman, A. Perrelet, L. Orci, and R.H. Unger. 1989. Morphometric and functional studies of islets in diabetes-prone BB/W rats that are discordant for overt diabetes. *Diabetes, Nutrition and Metabolism (Milan).* 2:263.
62. Pujol-Borrell, R., I. Todd, M. Doshi, G.F. Bottazzo, R. Sutton, D. Gray, G.R. Adolf, and M. Feldmann. 1987. HLA class II induction in human islet cells by interferon-γ plus tumour necrosis factor or lymphotoxin. *Nature (Lond.).* 326:304.
63. Sarvetnick, N., J. Shizuru, D. Liggitt, L. Martin, B. McIntyre, A. Gregory, T. Parslow, and T. Stewart. 1990. Loss of pancreatic islet tolerance induced by β-cell expression of

interferon-γ. *Nature (Lond.).* 346:844.
64. Campbell, I.L., T.W.H. Kay, L. Oxbrow, and L.C. Harrison. 1991. Essential role for interferon-γ and interleukin-6 in autoimmune insulin-dependent diabetes in NOD/Wehi mice. *J. Clin. Invest.* 87:739.
65. Oldstone, M.B.A., M. Nerenberg, P. Southern, J. Price, and H. Lewicki. 1991. Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. *Cell.* 65:319.
66. Allison, J., L. Malcolm, N. Chosich, and J.F.A.P. Miller. 1992. Inflammation but not autoimmunity occurs in transgenic mice expressing constitutive levels of interleukin-2 in islet β cells. *Eur. J. Immunol.* 22:1115.
67. Gorsuch, A.N., K.M. Spencer, J. Lister, J.M. McNally, B.M. Dean, G.F. Bottazzo, and A.G. Cudworth. 1981. Evidence for a long prediabetic period in type I (insulin-dependent) diabetes mellitus. *Lancet.* 2:1363.