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14. We used whole-cell and patch recording methods (*29*) and a conventional patch clamp amplifier. Patch electrodes contained 130 m*M* K^+, 5 m*M* EGTA-KOH, 1 m*M* Mg^{2+}, 114.5 m*M* Cl^-, 10 m*M* Hepes-HCl (*p*H 7.2). In some cases less EGTA was used (1 m*M*), which did not affect the results. Cells were usually bathed in a solution containing 3 m*M* K^+, 127 m*M* Na^+, 5 m*M* EGTA-NaOH, 1 m*M* Mg^{2+}, 114.5 m*M* Cl^-, 10 m*M* Hepes-NaOH, 10 m*M* glucose (*p*H 7.8). Exceptions are noted in the figure legends. Elevation of bath K^+ concentrations was achieved by substitution of K^+ for Na^+. FA solutions were prepared by either direct dispersal of the FA oil or dispersal of concentrated FA in dimethyl sulfoxide (DMSO) into the appropriate aqueous salt solution. DMSO (0.1%) alone in bathing solution had no effect. Because of the limited solubility of FAs in physiological salt solutions, the actual concentrations in these experiments are likely to be lower than the reported values. FA solutions were applied to cells by either pressure ejection from a micropipette (*30*) or, less often, by superfusion. For the former, concentrations given are those in the application pipette. FAs (>99% pure) were obtained from Nu Check Prep with the exception of ETYA (98% pure), which was obtained from Fluka and Biomol. For all whole-cell voltage clamp recordings shown, capacitive transients were removed for clarity. Membrane potentials are given with respect to the zero current reference potential before contact of the cell by the pipette. Thus the true membrane potential is likely to be somewhat more negative, giving recorded reversal potentials that are more positive than those calculated for a channel permeable only to K^+ (*31, 32*).
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18. Consistent with this interpretation, the more hydrophilic FA, myristoleic acid, was found to weakly activate K^+ current at a concentration of 80 μM (four experiments: two whole-cell recordings, two outside-out patches). This result suggests that higher concentrations of the more hydrophilic FAs result in interaction with a hydrophobic site associated with K^+ channel activation.
19. The reproducibility of whole-cell K^+ current activation by FAs is indicated by the following summary of the experimental results (expressed as the ratio of positive results to the number of cells tested): Arachidonic acid (29/30 cells); palmitoleic acid (4/4 cells); linoelaidic acid (5/5 cells); linolenic acid (3/3 cells); oleic acid (5/5 cells); myristic acid (12/12 cells); ETYA (7/7 cells). Negative results (expressed as the total number of cells tested): Myristoleic acid (20 to 40 μM) (8 cells); caprylic acid (8 cells) (in 2 additional cells, caprylic acid weakly increased K^+ current after prolonged, high-pressure applications); palmitic acid (two cells).
20. The reproducibility of K^+ channel activation in membrane patches is indicated by the following summary of the experimental results [expressed as in (*19*)]: Arachidonic acid (5/6 outside-out, 11/12 cell-attached, 2/2 inside-out); myristic acid (5/5 outside-out, 4/4 cell-attached, 2/2 inside-out); linoelaidic acid (4/4 outside-out); linolenic acid (2/3 outside-out); palmitoleic acid (3/3 outside-out); ETYA (3/3 outside-out).
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33. The results indicate the range of responses and do not necessarily reflect the relative potencies of these FAs.
34. A second channel type, which can be seen as a smaller amplitude channel current in the upper trace of Fig. 3A, was activated by AA in some patches. Because this channel was seen infrequently, we have not yet characterized it or its activation by FAs.
35. Supported by NSF DCB-8511674 and DCB-8819750 and NIH DK-31620. We thank C. Baker, P. Tilander, and P. Beane for assistance, and M. Kirber, T. Honeyman, K. Madden, D. Melchior, and D. Small for helpful comments and suggestions.

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Transgenic Mice with I-A on Islet Cells Are Normoglycemic But Immunologically Intolerant

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Insulin-dependent diabetes mellitus (IDDM) is caused by a specific loss of the insulin-producing beta cells from pancreatic Langerhans islets. It has been proposed that aberrant expression of major histocompatibility complex (MHC) class II molecules on these cells could be a triggering factor for their autoimmune destruction. This proposal was tested in transgenic mice that express allogeneic or syngeneic class II molecules on the surface of islet cells at a level comparable with that normally found on resting B lymphocytes. These animals do not develop diabetes, nor is lymphocyte infiltration of the islets observed. This immunological inactivity does not result from tolerance to the "foreign" class II molecules.

THE DESTRUCTION OF PANCREATIC beta cells in IDDM and the consequent abrogation of insulin secretion appear to have an autoimmune origin (*1*). Lymphocyte infiltration is common in islets from diabetic patients and is usual in islets from two animal models of the disease, the BioBreeding (BB) rat and the nonobese diabetic (NOD) mouse (*1–3*). In addition, the rodent diseases can be transmitted from a diabetic to a healthy individual by transferring lymphocyte populations (*4*).

As is the case with several other autoimmune diseases, IDDM seems to be associated with particular MHC alleles (*5, 6*). The reasons for this association are much in debate. Some diabetic patients express MHC class II molecules on their islet cells, as do diabetic BB rats and NOD mice (*7–9*). This observation has provoked the hypothesis that aberrant expression of MHC molecules may play a role in the autoimmune destruction of beta cells, possibly by allowing the presentation of previously ignored islet cell antigens (*5, 10*). The recent finding (*11*) that cultured beta cells can be induced to express class II molecules in vitro by lymphokine stimulation suggests an in vivo mechanism for such aberrant expression. An alternative interpretation, that the appearance of class II molecules on islet cells is secondary to the inflammatory response, rests on the timing of expression in BB rats (*8*).

The two interpretations are potentially

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distinguishable by studying transgenic mice with ectopic expression of class II molecules. Reports have recently appeared of transgenic mice expressing class I (*12*) or class II I-E (*13*) or I-A (*14*) molecules under the dictates of the insulin promoter. In all three cases, the animals developed diabetes, but the disease showed no sign of immune system involvement. In all cases, the MHC molecules seemed to be expressed at higher levels than on normal class II–positive cells, which has prompted the hypothesis that the beta cells become nonfunctional and die by a "clogging" of their secretory pathways (*15*). We report the construction of transgenic mouse lines that express class II molecules on their islet cells at levels comparable with those on resting B lymphocytes.

The transgene expression constructs consisted of A^k_α and A^k_β cDNAs carried in a pKCR3-derived vector (*16*) (Fig. 1A). The β-globin segments of the pKCR3 module provide splice donor and acceptor sites and a polyadenylation site. Transcription is driven by a 660-bp rat insulin (II) promoter fragment, which can target expression of a juxtaposed protein-coding segment to pancreatic beta cells (*17*). Purified fragments from the A_α and A_β constructs (IPAAK and IPABK, respectively) were injected into (C57Bl/6 × SJL)F_2 fertilized oocytes. Eight transgenic mice resulted from the various injections, and these founders were backcrossed to C57Bl/6 animals. The progeny of several of the founders did not inherit the transgene or expressed it very weakly or not at all; these lines will not be discussed further.

Two lines, IPAAK-25 and IPABK-22, had detectable transgene transcription in the pancreas, as evidenced by quantitative S1 nuclease analysis of RNA from various tissues (Fig. 1, B and C, lanes Pa). Expression of the IPABK transgene was specific to the pancreas among the organs tested, but small amounts of IPAAK-25 RNA were also detected in the kidney. Occasional leakiness of the insulin promoter in kidney has been observed by others (*13*) but, in the absence of IPABK expression, is probably of no consequence for this study. That the pancreatic IPAAK-25 transcripts were localized to the islets was confirmed by the situ hybridization (*18*).

To determine whether bona fide A^k_α and A^k_β proteins are translated from the pancreas transcripts, we mated mice from the IPAAK-25 and IPABK-22 lines to produce double-positive transgenics expressing $A^k_\alpha A^k_\beta$ complexes. Immunofluorescence and immunoperoxidase stainings of pancreatic sections were performed with the monoclonal antibodies (MAbs) 39E (A^k_β-specific), 2A2 (A_α-specific), and H116-32 (A^k_α-specific) (*16*). With each antibody, the islets of transgene double-positive animals stained positively, whereas the islets of single-positives or double-negatives failed to do so. Typical immunoperoxidase staining (with 39E) of pancreas sections from an $\alpha^+\beta^+$ double-positive transgenic and a control $\alpha^-\beta^+$ littermate is shown in Fig. 2. The islet from the double-positive animal (Fig. 2A) is positive for A complex (Fig. 2B), whereas the islet from the negative animal (Fig. 2C) appears to be negative for A complex (Fig. 2D). The spleen section from an A^k-positive C57Bl/10.A mouse serves as a standard (Fig. 2E). It was included on the same slide

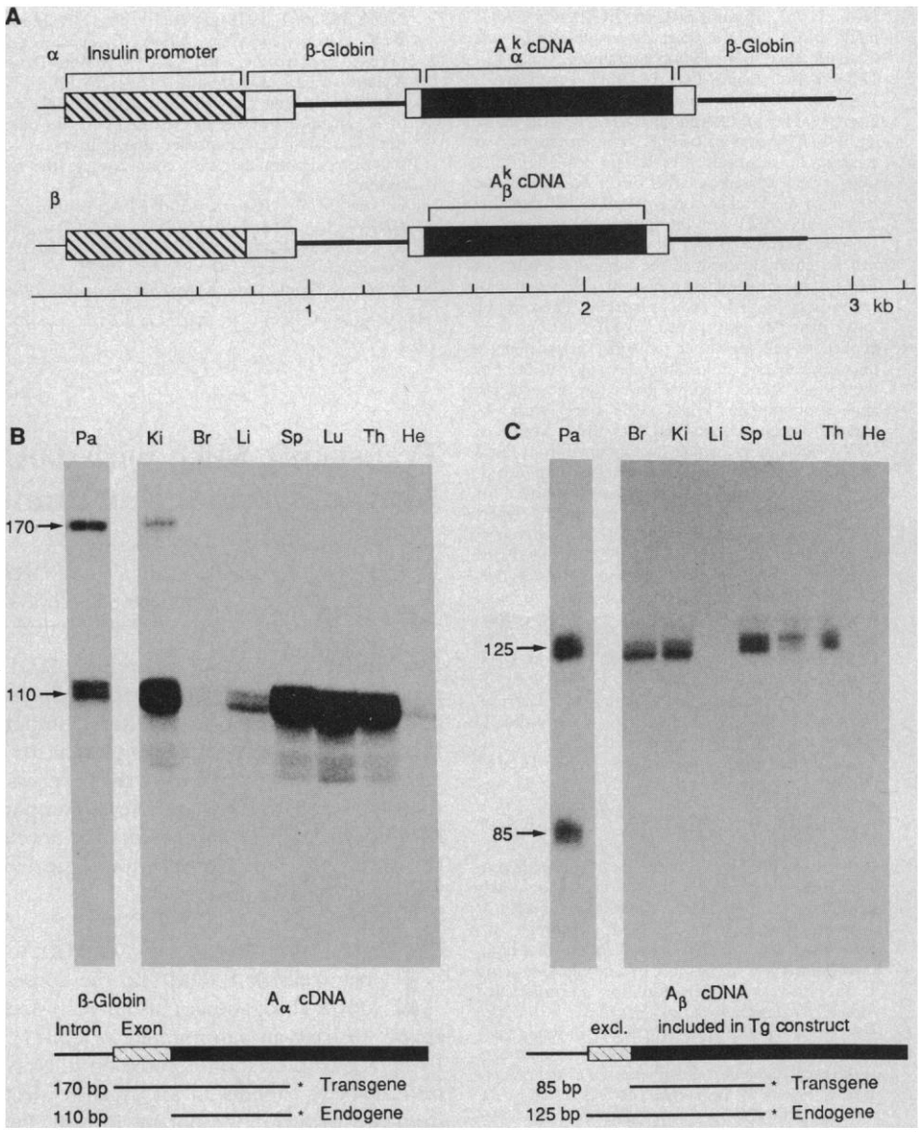

Fig. 1. Design of and transcription from transgene fragments. (**A**) The injected IPAAK and IPABK fragments. Briefly, full-length A^k_α and A^k_β cDNAs were inserted individually into the pKCR3 expression vector (*16*). The SV40 promoter of that vector was then replaced by a 660-bp Bam HI fragment carrying the rat insulin (II) promoter (*17*). The excised and injected fragments carry the first intron and 3′ termination region of the rabbit β-globin gene. The fine lines at both ends of each diagram indicate included pBR322 sequence: for IPAAK, the Hha I–Eco RI stretch at the 5′ and the Sal I–Hha I segment at the 3′ end; for IPABK, the Aat II–Eco RI stretch at the 5′ and the Sal I–Nru I segment at the 3′ end. (**B** and **C**). Transcription from IPAAK-25 and IPABK-22, respectively, as visualized by S1 nuclease analysis. The abbreviations for the tissue source are as follows: Pa, pancreas; Ki, kidney; Li, liver; Br, brain; Sp, spleen; Lu, lung; Th, thymus; and He, heart. Polyadenylated RNA (50 μg) from pancreas, kidney, liver, and brain; 10 μg of total RNA from spleen; 20 μg of total RNA from lung and thymus; and as much RNA as could be obtained from one heart (typically 20 to 40 μg of total RNA) were hybridized with 10^4 cpm of 5′ end-labeled, single-stranded DNA probe, as described (*30*). The samples were digested for 10 min with 30 U of S1 nuclease (Appligene, Strasbourg) and run on an 8% acrylamide-urea sequencing gel. The molecular weight values shown to the left of each panel derive from a comparison of band position with the migration of known single-stranded DNA markers. Diagrams of the probes and the expected protected fragments are shown at the bottom of (B) and (C); excl, excluded from transgene construct; Tg, transgene.

as the pancreas sections and thus was subject to exactly the same staining and development procedures. The intensity of 39E staining of the follicular B cells is similar to that of the transgenic islet cells, reflecting an A expression level of the same order of magnitude.

We next showed that the A complexes detected by immunohistology were indeed present on the surface of islet cells. A number of mice that were double-positive, single-positive, or negative for the transgene were evaluated for surface A expression by immunofluorescent staining of live, isolated islet cells. Because of the small number of cells obtained from individual mice, scoring was initially by fluorescence microscopy. The results of several independent experiments revealed that islet cells from all of the mice that were double-positive for the transgene (four individuals) stained with the broadly reactive MAb 40B, directed against A_β, whereas cells isolated from mice that were single-positive or negative for the transgene (eight individuals) consistently gave negative results (*19*). The level of surface A staining was again similar to that detected for splenic B cells. In several experiments, the specificity and level of staining were corroborated by cytofluorimetric analysis. Typical histograms show a clear and homogenous staining of cells from the double-positive mouse but not from the single-positive control (Fig. 2, F and G). The shift in peak channel number [102 channels on a 1024 channel log scale (three decades)] is similar to that routinely observed with splenic B cells (70 channels) (*19*).

These experiments demonstrate that a bona fide $A^k_\alpha A^k_\beta$ complex is expressed on the Langerhans islet cells of double-positive transgenics. The transgene-encoded protein occurs on the islet cell surface at levels comparable with those on splenic B cells. Thus, these transgenic mice seem a valid model for testing the hypothesis that diabetes is triggered by the aberrant expression of MHC class II molecules.

Diabetes was initially evaluated by measurement of blood glucose. Transgene-positive and -negative littermates showed no difference in blood sugar levels up to at least 8 months of age, whereas the value for the control diabetic NOD mouse was five times greater than the highest value for a test animal (Fig. 3A). Because lymphocyte infiltration can be observed in NOD mouse islets well before the onset of overt diabetes (*3*), histological sections from 11 double-positive animals (aged $2\frac{1}{2}$ to $8\frac{1}{2}$ months) were studied for evidence of lymphocytes in the islets. None of the sections showed any infiltration, as illustrated by the representative example in Fig. 3B. In contrast, lymphocyte infiltration of the islet was obvious in the section from a 9-week-old NOD female displaying no overt signs of diabetes (Fig. 3C).

The $A^k_\alpha A^k_\beta$ complexes in the pancreases of IPAAK/IPABK mice are allogeneic with respect to the MHC of the transgene-recipient strain ($H\text{-}2^b$). Since the IPAAK-25 and IPABK-22 transgenes are expressed at birth (*18*), one explanation for the absence of diabetes and of lymphocytic infiltration of islets in double-positive animals could be

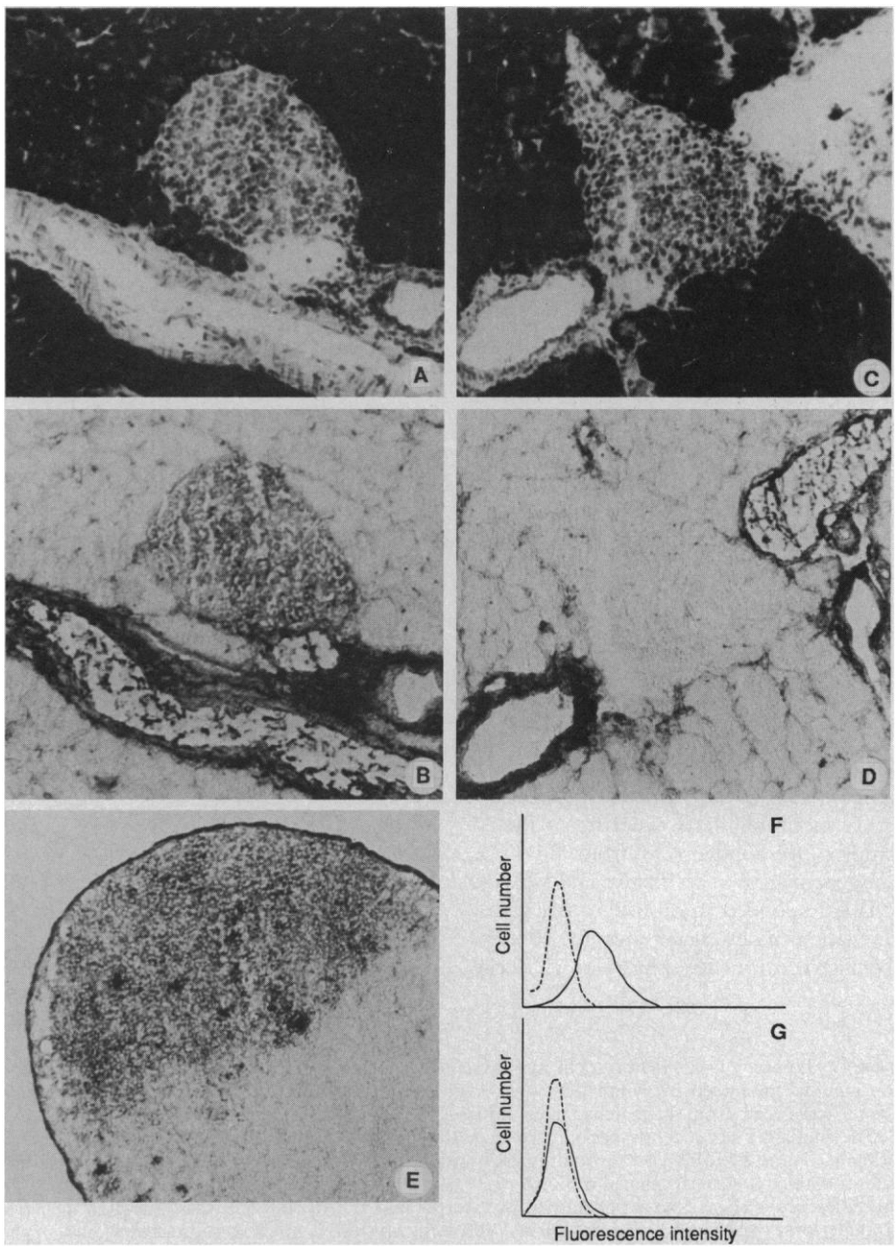

Fig. 2. A-complex expression in IPAAK-25/IPABK-22 double-positive transgenics. (**A** and **C**) Stainings with 1% methylene blue. Exocrine pancreas stains darkly, while the Langerhans islets appear only lightly stained. (A) Double-positive transgenic; (C) control littermate. (**B** and **D**) Immunoperoxidase stainings of sections parallel to those of (A) and (C) performed essentially as described (*29*) with the A^k_β-specific MAb 39E (*16*) as a first-step reagent. The second-step reagent was an immunoglobulin fraction of a rabbit antibody to a mouse Ig serum coupled to horseradish peroxidase (Dako). Rat serum (1%) was included in the second step to reduce nonspecific background. (B) Double-positive transgenic; (D) control littermate. (**E**) Immunoperoxidase staining, as in (B) and (D), of a spleen section from a C57Bl/10.A mouse. The unstained area consists of red pulp. This section was on the same slide as the (B) and (D) sections, and so all received exactly the same treatment. (**F** and **G**) Fluorescence intensity profiles of islet cells from a transgene double-positive mouse (F) and an $\alpha^+\beta^-$ littermate (G). Islet cells were prepared as described and stained (*31*). Stained cells (4×10^4 to 5×10^4) were suspended to 0.25 ml in wash buffer and analyzed on an Epics C flow cytometer. The first-step antibody was 40B, which is reactive with A_β molecules of several H-2 haplotypes (*16*).

that they are tolerant to the class II molecules on their islet cells. Mixed lymphocyte reactions (MLR) were set up to evaluate this explanation. In the first set of experiments, the critical responders were lymph node cells from IPAAK/IPABK double-positive transgenics of their transgene-negative littermates. The stimulators were irradiated spleen cells from B6 mice or from a line of transgenics that carries A^k_α and A^k_β transgenes on a B6 background. These transgenes are driven by their own promoter and are thus expressed in all the usual class II–positive cells (*20*). With this responder-stimulator combination, essentially all MLR reactivity is to the A^k complex. There is only a very low response to haplotype-mismatched molecules (that is, $A^k_\alpha A^b_\beta$ or $A^b_\alpha A^k_\beta$) because, as a result of severe pairing problems, there are very few such complexes in these transgenic mice (*20, 21*). As indicated by the representative experiment presented in Table 1, C57Bl/6 mice or transgene-negative littermates responded to the A^k complex with a stimulation index of about 6 to 10; this is a reproducibly lower response than that observed across whole MHC differences. Perhaps surprisingly, lymph node cells from IPAAK/IPABK double-positive mice also responded vigorously to the A^k stimulators.

In a second type of experiment, the stimulators were irradiated spleen cells from B6 or B10.A(4R) mice. The critical responders were CD8-depleted lymph node cells from IPAAK/IPABK double-positive transgenics and their negative littermates. This combination specifically tests reactivity to the A^k complex. Responder cells from B6 mice, transgene-negative littermates, and IPAAK/IPABK responded vigorously to A^k, with a stimulation index again around 10. The tolerance control, B6/$A^k_\alpha A^k_\beta$ responder cells, which should not react to A^k, showed a stimulation index of only 2.3. Thus, according to both MLR experiments, the IPAAK/IPABK mice are not tolerant to the MHC class II molecules they express on their pancreatic islet cells.

Considering the phenomenon of MHC restriction, it also seemed important to evaluate the effect of aberrant pancreatic expression of syngeneic class II molecules. Thus, by conducting matings to C57Bl/10.A mice, we produced animals that had transgene-encoded $A^k_\alpha A^k_\beta$ molecules on islet cells and endogenously encoded A^k complexes on the usual array of class II–positive cells. Although fewer have been analyzed, we have not seen evidence of diabetes or insulitis in these mice either (*18*).

Some of the results obtained with the IPAAK/IPABK transgenic mice are provocative because they bring into question the Bottazzo-Feldmann hypothesis or because they contrast with recent findings with similar transgenics (*12–14*). First, our experiments show that ectopic expression of the A^k complex on pancreatic islet cells does not necessarily engender autoimmune attack of the pancreas. This conclusion, in good agreement with the other transgenic mouse experiments, contradicts the Bottazzo-Feld-

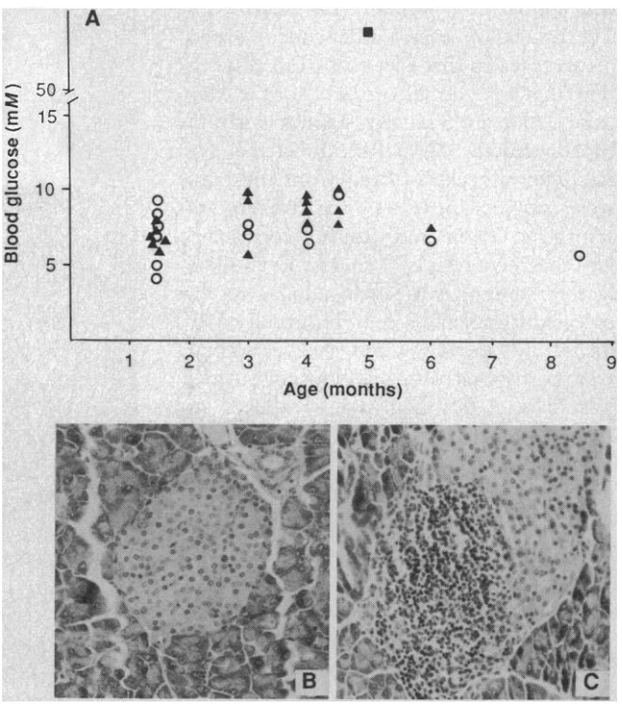

Fig. 3. Normal blood glucose levels in and absence of lymphocyte infiltration into the islets of double-positive transgenics. (**A**) Blood glucose values for double-positive transgenics, transgene-negative littermates, and a diabetic NOD mouse (as a control for diabetic mouse blood glucose levels). (○) Double-positive transgenics; (▲) transgene-negative littermates; (■) diabetic NOD mouse. An Ames glucometer was used for measuring glucose values on blood from overnight-fasting animals. The serum from the diabetic NOD mouse was diluted prior to analysis. (**B**) Hematoxylin and eosin staining of a paraffin-embedded Bouin-fixed pancreas from an 8½-month-old IPAAK/IPABK double-positive transgenic, visualized at a magnification of ×160. (**C**) Section treated in the same way from a 9-week-old NOD female that did not manifest overt signs of diabetes. Lymphocytes are seen as a dense dark cell mass occupying half of the islet space.

Table 1. Alloreactivity of splenocytes from transgenic and control mice against A^k stimulators. Two representative A^k-directed MLRs are shown. Responders were inguinal and mesenteric lymph node cells. In experiment 2, $CD8^+$ cells were removed by treatment with anti-Lyt2 and complement. Stimulators were irradiated spleen cells from sex-matched mice. B6/IP-A^k transgenic mice carry both the IPAAK and IPABK transgenes. They come from crosses of IPAAK and IPABK animals, independently backcrossed onto the C57B1/6 genetic background. B6/$A^k_\alpha A^k_\beta$ transgenic mice carry genomic A^k_α and A^k_β transgenes, also backcrossed onto a C57Bl/6 background; these transgenes specify expression of A^k molecules in all the usual class II–positive cells. Individual wells of 96-well plates contained 3×10^5 irradiated stimulator cells, and proliferation was assayed by incorporation of [^{3}H]thymidine after 4 days in culture, as described (*29*). The values are average counts per minute of incorporated ^{3}H in triplicate wells (standard deviations were 20% or less of the mean). Incorporation in responder-only wells ranged between 300 and 900 cpm, which have not been subtracted. Values in parentheses represent the stimulation index, standardized for each responder cell type against its response to B6 stimulator cells. The experiments include parallel sets with different responder-stimulator ratios or different culture times. These sets all gave similar results. Underlined values are significantly different from the negative control.

Stimulators	[^{3}H]Thymidine incorporation (cpm) when responders are lymph node cells from			Stimulators	[^{3}H]Thymidine incorporation (cpm) when responders are anti-CD8 + C′–treated lymph node cells from			
	Negative littermate	B6/IP-A^k	B6/$A^k_\alpha A^k_\beta$		Negative littermate	B6/IP-A^k	B6	B6/$A^k_\alpha A^k_\beta$
	Experiment 1				*Experiment 2*			
B6	1,194 (1)	2,537 (1)	2,333 (1)	B6	4,546 (1)	4,120 (1)	2,310 (1)	6,247 (1)
B6/$A^k_\alpha A^k_\beta$	9,625 (8.1)	17,265 (6.8)	2,735 (1.2)	B10A.(4R)	61,526 (13.5)	34,990 (8.5)	33,140 (14.3)	14,743 (2.3)
B6/IP-A^k	1,002 (0.8)	2,172 (0.9)	3,796 (1.6)	Balb/c	72,380 (15.9)	39,070 (9.5)	53,480 (23)	38,070 (6.1)
Balb/c	38,199 (32)	102,364 (40)	107,836 (46)					

mann hypothesis in its simplest form. It remains possible that the timing of the appearance of A complex in the transgenics is inappropriate, IDDM developing only if class II molecule expression appears later than the neonatal [tolerance-inducing(?)] stage. Alternatively, the haplotypes of the MHC molecules used in our and other experiments (*13, 14*) may not have been appropriate. Autoimmune IDDM could require ectopic expression of the proper class II allele [A molecules from NOD mice, for example, although the rejection of B10 islets transplanted into NOD→NOD × B10 chimeras argues against this (*22*)]. In any case, the transgenic mouse experiments put clear limitations on the Bottazzo-Feldmann hypothesis.

Second, our transgenic mice do not get diabetes. This result contrasts with earlier reports of transgenic mice that synthesize MHC molecules in pancreatic islets (*12–14*); however, the drastic reduction in insulin secretion and beta-cell degeneration in these mice were not of autoimmune origin but were most likely due to intracellular perturbations (*12–15*). Why are our mice so different? One source of the difference could be variation in the amount of A complex synthesized in the islets. The previously described lines carried simpler insulin promoter–MHC gene constructs, and these mice were reported to express transgene-encoded protein at higher levels than normal class II– or class I–positive cells. Our rather complicated cDNA expression construct—designed to facilitate the analysis of different class II alleles—results in much lower expression levels, similar to those of splenic B cells. Another possible explanation for the different results with the different transgenic lines centers on allelic variation. Parham (*15*) speculated that the toxicity of MHC molecules for beta cells could be due to their binding and sequestering of important intracellular proteins or peptides (for example, proinsulin). The A*k* molecules in our mice may not bind the same proteins as the A*d* or E*d* molecules targeted by others. One should recall, perhaps, that A*k* mice are nonresponders to procine and bovine insulins.

Third, the lack of tolerance to the transgene-encoded A^k complexes in our mice is striking, especially since Lo *et al.* (*13*) report that their mice are tolerant to transgene-encoded E^d complexes. This E^d tolerance (also revealed in MLR studies) seems paradoxical, since the transgenic mouse thymocytes are also tolerant. One is forced to envisage transport of pancreatic MHC molecules to the thymus or feedback transfer of information by peripheral T cells (*13, 15*). Again, the difference in results might be due to the constructs used. Our cDNA constructs, stripped of all introns, could lack regulatory elements that predispose to expression, even if at minute levels, in the thymus. The difference might also reflect an A versus E functional dichotomy, as suggested previously in another context (*23*).

There remains the paradox of A^k-reactive T cells coexisting with A^k-positive islet cells. Precedents can be cited for such a state of split tolerance: thymic epithelium grafts are generally not rejected, but do not induce tolerance as measured by MLR (*24*); neonatal tolerance induction prevents the rejection of subsequent skin allografts, but does not abrogate MLR reactivity (*25*); most relevant to this work, passenger leukocyte–depleted thyroid and pancreatic islet grafts are well tolerated, but do not induce MLR unresponsiveness (*26*). There may, however, be an important difference between our transgenic mice and islet-grafted mice; while graft rejection can be induced by injecting the tolerant mice with donor-type spleen cells, three attempts to induce autoimmune attack of IPAAK/IPABK islet cells by priming with A^k-positive splenocyte have failed (although one could argue that the transgenic mice present a situation akin to the priming-resistant tolerant state that develops with time in grafted animals) (*27*).

At least four resolutions to this paradox seem possible: (i) the pancreatic islets are anatomically "hidden" from the immune system and may only be recognized after a precipitating "insult"; (ii) pancreatic beta cells are poor T cell stimulators, perhaps being unable to deliver a costimulatory signal (for example, interleukin-1); (iii) suppressor cells downregulate the T cell response to islets; (iv) although the mice are not tolerant to the A^k expressed on splenocytes (as measured in an MLR), they may in fact be tolerant to the A^k molecules on islet cells. This explanation invokes the recently proposed idea that alloreactivity involves the recognition of intracellular peptides presented by MHC molecules (*28*). The peptides may well vary with different cell types.

In summary, our results demonstrate that immunologically relevant amounts of MHC class II molecules on pancreatic beta cells do not necessarily provoke diabetes, either by autoimmune attack or by simple toxicity. Islets that display allogeneic A complexes are well tolerated by the immune system, but they do not induce wholesale tolerance to the transgene-encoded MHC molecule.

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31. Single-cell islet cell suspensions were prepared as per K. Haskins, M. Portas, B. Bradley, D. Wageman, and K. Lafferty (*Diabetes*, in press). The suspensions were normally divided into portions of 5,000 to 10,000 cells for fluorescence microscopy. They were centrifuged at 1000 rpm at 4°C for 10 min, and the pellets were resuspended in a solution containing the primary antibody—typically, 100 μl of antibody culture supernatants containing 0.1% NaN_3. After incubation for 1 hour at 4°C, the cells were washed twice in 0.5 ml of cold wash buffer (2% fetal bovine serum and 0.2% NaN_3 in phosphate-buffered saline). Washed cell pellets were resuspended in 100 μl of the secondary antibody (Boehringer Mannheim goat antibodies to mouse immunoglobulins IgG and IgM, fluorescein-conjugated) diluted 1:100 in wash buffer. The cells were mixed and incubated for 30 to 45 min at 4°C, washed twice in cold wash buffer, and resuspended in 50 to 100 μl of wash buffer. A 5-μl sample was placed on a glass slide and was scored immediately with a Zeiss microscope equipped with an ultraviolet lamp. Cells that were positive for binding could be distinguished by a faint uniform glow and were always "weak positive." Cells that were negative for binding exhibited no fluorescence.

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