Original Article

Toxic Human Islet Amyloid Polypeptide (h-IAPP) Oligomers Are Intracellular, and Vaccination to Induce Anti-Toxic Oligomer Antibodies Does Not Prevent h-IAPP–Induced β-Cell Apoptosis in h-IAPP Transgenic Mice

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OBJECTIVE—Islets in type 2 diabetes are characterized by a deficit in β-cells, increased β-cell apoptosis, and islet amyloid derived from islet amyloid polypeptide (IAPP). The toxic form of amyloidogenic protein oligomers are distinct and smaller than amyloid fibrils and act by disrupting membranes. Using antibodies that bind to toxic IAPP oligomers (but not IAPP monomers or fibrils) and a vaccination-based approach, we sought to establish whether IAPP toxic oligomers form intra- or extracellularly and whether vaccination to induce anti-toxic oligomer antibodies prevents IAPP-induced apoptosis in human IAPP (h-IAPP) transgenic mice.

RESEARCH DESIGN AND METHODS—Pancreas was sampled from two h-IAPP transgenic mouse models and examined by immunohistochemistry for toxic oligomers. The same murine models were vaccinated with toxic oligomers of Alzheimer β protein ($A\beta P_{1-40}$) and anti-oligomer titers, and blood glucose and islet pathology were monitored.

RESULTS—Toxic oligomers were detected intracellularly in ~20–40% of h-IAPP transgenic β-cells. Vaccine induced high titers of anti–h-IAPP toxic oligomers in both transgenic models, but β-cell apoptosis was, if anything, further increased in vaccinated mice, so that neither loss of β-cell mass nor diabetes onset was delayed.

CONCLUSIONS—IAPP toxic oligomers form in h-IAPP transgenic mouse models, and anti-toxic oligomer antibodies do not prevent h-IAPP–induced β-cell apoptosis. These data suggest that prevention of h-IAPP oligomer formation may be more useful than a vaccination-based approach in the prevention of type 2 diabetes. ***Diabetes* 56:1324–1332, 2007**

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AβP, Alzheimer's β protein; h-IAPP, human islet amyloid polypeptide; ELISA, enzyme-linked immunosorbent assay; IAPP, islet amyloid polypeptide; r-IAPP, rodent islet amyloid polypeptide; TBS, Tris-buffered saline.

Type 2 diabetes is characterized by progressive β-cell dysfunction (1). Morphologically, islets in type 2 diabetes have a deficit in β-cells, increased β-cell apoptosis, and local amyloid deposits of islet amyloid polypeptide (IAPP) (2,3). In health, IAPP is co-expressed and secreted with insulin from pancreatic β-cells and is believed to play a paracrine role in restraining insulin secretion (2,4–6).

Several explanations have been offered for the increased β-cell apoptosis in type 2 diabetes, including glucotoxicity and lipotoxicity (7,8). The focus here is on the potential role of the amyloidogenic peptide IAPP. The loss of β-cells in type 2 diabetes shares pathological features with several other degenerative diseases. For example, in Alzheimer's disease (9) and senile cardiac amyloidosis (10), tissue degeneration is characterized by deposits of locally expressed proteins, Alzheimer's β protein (AβP) and transthyretin, respectively. The role of these protein amyloid deposits in tissue loss has been controversial, in part because there is either no correlation or a weak correlation between the extent of amyloid deposits and degenerative changes (11–13).

However, it is increasingly apparent that amyloid per se is not the cytotoxic form of these proteins but rather much smaller oligomers that have the ability to induce membrane instability and apoptosis (14–16). The toxic oligomers of these amyloidogenic proteins apparently share close tertiary structural properties, since antibodies raised against toxic oligomers of $A\beta P_{1-40}$ bind to oligomers of IAPP, synuclein, transthyretin, and prion and negate the cytotoxicity of these oligomers when applied to cells in culture (17). We used these newly available toxic oligomer–specific antibodies to establish whether human IAPP (h-IAPP) toxic oligomers form intracellularly in h-IAPP transgenic mice. Also, because immunization of rabbits with the mimic $A\beta P_{1-40}$ oligomers raised antibodies protective against h-IAPP oligomer cytotoxicty, we also sought to establish whether immunization with the same $A\beta P_{1-40}$ oligomer preparation could prevent diabetes in h-IAPP transgenic mouse models of type 2 diabetes. The mimic $A\beta P_{1-40}$ oligomer preparation has several favorable properties for its use as a vaccine. The oligomers

are composed of aggregates of similar size and structural properties to naturally formed AβP (and h-IAPP) oligomeric intermediates. The mimic oligomers are stable, homogeneous, and without contamination from either low–molecular weight AβP or fibrils (17).

The propensity of h-IAPP to form oligomers and amyloid fibrils depends on a hydrophobic sequence in amino acid residues 20–29. This amino acid sequence is closely homologous in humans, monkeys, and cats, species that share a predisposition to spontaneously develop type 2 diabetes. The sequence of IAPP in mice and rats is identical and nonamyloidogenic, and neither of these species develop type 2 diabetes without genetic manipulation (18–22). For the present studies, we used two h-IAPP transgenic mouse models. One was a homozygous h-IAPP transgenic mouse model that develops diabetes characterized by rapid decline in β-cells at an early age (23). The period of high β-cell loss in this homozygous h-IAPP model is coincident with the presence of small intracellular nonfibril aggregates of h-IAPP (23). The second model we used is a derivative of the homozygous h-IAPP transgenic mouse model established by crossbreeding homozygous h-IAPP transgenic mice onto the Avy/agouti model of obesity (24,25). Obese h-IAPP hemizygous transgenic mice (but not lean h-IAPP transgenic or nontransgenic obese mice) develop diabetes characterized by extensive extracellular islet amyloid and increased β-cell apoptosis at ~20–30 weeks of age.

Using these two models, we first sought to establish whether h-IAPP toxic oligomers form in h-IAPP transgenic mice and, if so, intra- and/or extracellularly. We also vaccinated the same mouse models with the mimic of $A\beta P_{1-40}$ oligomers previously used in rabbits to raise antibodies that bind and neutralize h-IAPP oligomer–induced toxicity. Using this vaccination-based approach, we sought to establish whether h-IAPP toxicity in vivo acts primarily extracellularly, implying that a vaccine approach for prevention of type 2 diabetes might be feasible. Alternatively, if increased β-cell apoptosis in h-IAPP transgenic mice is not prevented despite the presence of neutralizing antibodies to toxic h-IAPP oligomers, this would imply that h-IAPP toxic oligomers act primarily intracellularly and would argue against the likely usefulness of a vaccine-based approach for the prevention of type 2 diabetes.

RESEARCH DESIGN AND METHODS

Human and rodent IAPP transgenic rodent models. Development and characterization of transgenic mice homozygous for h-IAPP [FVB-*Tg(IAPP)6Jdm/Tg(IAPP)6Jdm*] and rodent IAPP (r-IAPP) [FVB/N-*Tg(Iapp)6Wcs/Tg(Iapp)6Wcs*] have been described elsewhere (23,26). Likewise, we have previously reported crossbreeding homozygous h-IAPP transgenic mice with the A^{vy}/a mouse on the C57BL/6 background to generate obese mice hemizygous for h-IAPP, referred to as OTG mice [FVB-*tg(IAPP)6Jdm/-A^{vy}/A*] and their nontransgenic obese counterparts ONTG mice (24,25). Mice were maintained on a 12-h day/night rhythm with Harlan Teklad Rodent Diet 8604 and water ad libitum.

Toxic oligomers

Tissue collection. Before collection of mouse pancreata, the heart was perfused with 10 ml of 4% paraformaldehyde. The pancreas was dissected in cold PBS, the fat and lymph nodes were trimmed, and the pancreas was weighed, fixed in 4% paraformaldehyde at 4°C for 24 h, and then divided into equal parts. One portion was embedded in paraffin, the other frozen in optimal cutting temperature compound.

Immunofluorescence and confocal microscopy. Frozen sections (4 μm) of pancreas were washed with Tris-buffered saline (TBS)/0.1% Tween-20, then blocked with TBS/0.2% Triton X-100/3% BSA/2% normal donkey serum (Jackson Immunoresearch, West Grove, PA) for 3 h at room temperature. Sections were treated with oligomer-specific antibody (A11) raised as previously described against stable soluble oligomers of $A\beta P_{1-40}$ (17) (rabbit IgG, 15 μg/ml) for 20 h at +4°C, followed by 1-h treatment at room temperature with 1:200 diluted Cy3-F(ab)2 donkey anti-rabbit IgG (Jackson Immunoresearch). Insulin staining was performed using guinea pig anti-insulin Ab (Zymed, San Francisco, CA) diluted 1:100, applied for 1 h at room temperature, followed by 1-h treatment with 1:200 diluted fluorescein isothiocyanate-F(ab)2 donkey anti–guinea pig IgG (Jackson Immunoresearch). Antibodies were diluted in TBS/0.2% Tween-20/3% BSA. Between antibody treatments, slides were washed three times with TBS/0.1% Tween-20. Slides were coverslipped with Vectashield-DAPI mounting medium (Vector Laboratories, Burlingame, CA), stored in the dark at 4°C, and analyzed within 1–3 days after staining.

To confirm the specificity of oligomer staining, oligomer-specific antibody was preincubated for 5 h with 100 excess of preformed h-IAPP or AβP oligomers (prepared as described in ref. 17) or diluted with PBS. Blockage of antibody was confirmed by dot blot using neutrocellulose membranes blotted with preformed oligomers from h-IAPP and AβP (0.6 μg/spot). Congo Red staining was performed in 8 μmol/l paraffin sections.

Image analysis. Multiple images of pancreas were acquired with a Leica fluorescent microscope DM6000 at 20× magnification (Leica, Wetzlar , Germany) and analyzed using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). Since oligomer staining in the pancreas was confined to islets, further analysis focused on islets. A minimum of 20 islets per mouse was evaluated by two independent observers (A.E.B. and T.G.). For the purposes of this study, an islet was considered to be 10 or more insulin-positive cells. To measure relative islet amyloid area, Congo Red–stained sections were scanned using an Olympus IX 70 inverted microscope (Olympus America, Melville, NY) connected to a Hewlett Packard computer and analyzed using the Image-Pro Plus software.

Immunization. The synthetic molecular mimic of toxic $A\beta P_{1-40}$ oligomers used for immunization was prepared as previously described (17,27). In brief, colloidal gold particles (5.3 nm mean diameter from Ted Pella, Redding, CA) were mixed with a freshly prepared solution of 0.2 mg/ml $A\beta P_{1-40}$ thioester, (pH 5.0–5.5, 25 ml Aβ solution to 20 ml washed gold colloids in water [$\sim 1.0 \times 10^{14}$ particles per milliliter of water]). The initial peptide solution was in a monomeric or dimeric state, as determined by size exclusion chromatography. After 3 h of incubation at room temperature, the pH was adjusted to 7.4 with 100 mmol/l Tris, pH 8.0 (0.02% sodium azide). After incubation for 6 h at room temperature, the antigen was collected by centrifugation at 30,000*g* at 4° for 30 min, washed three times with PBS, pH 7.6, to remove any unincorporated Aβ, and then redispersed in distilled H_2O (0.02% sodium azide). The molecular mimic and naturally occurring spherical oligomers have very similar structural properties based on analysis by atomic force microscopy, electron microscopy, circular dichroism, thioflavin T fluorescence, and B anilino-sulfonic acid (data shown in ref. 27). Before use, the antigen was washed three times with PBS, pH 7.4, and resuspended in PBS at 250 mg/ml.

Protocol 1. Thirteen homozygous h-IAPP TG and 12 NTG mice were injected intraperitoneally with 100 μg $A\beta P_{1-40}$ oligomer micelles on four occasions, at 3, 4.5, 6.5, and 9 weeks of age. Another 13 TG and 12 NTG mice were injected intraperitoneally with PBS as vehicle control on four occasions at the same age as vaccinated animals. Mice from each group were randomly selected to be killed at different ages for pancreas and blood collection.

Protocol 2. Fifteen OTG and 15 ONTG mice were randomly assigned to control or experimental groups and then administered PBS or $A\beta P_{1-40}$ oligomer micelles, respectively. Experimental mice received 100 μg $A\beta P_{1-40}$ oligomer micelles at 4 weeks of age and then were boosted with the same amount of antigen at 6 and 8 weeks and every 4 weeks thereafter. Blood was collected by retro-orbital bleeding at 10 weeks and then every 4 weeks to monitor anti-oligomer antibody titer.

Monitoring of diabetes and tissue collection. Fasting body weight and blood glucose concentrations were measured after an 8-h fast every 2 weeks. Blood glucose values were measured from tail-tip blood sample by a Freestyle blood glucose meter (Therasense, Alameda, CA). Blood was obtained by cardiac puncture and allowed to clot and then separated to obtain serum for subsequent measurement of IAPP oligomer antibody titers. Pancreas was extracted and fat and lymph nodes removed, after which the pancreas was weighed, fixed in 4% paraformaldehyde, and embedded in paraffin. In protocol 1, pancreata were obtained for morphological studies at age 6 (n = 12 TG, n = 8 NTG), 8 (n = 8 TG, n = 8 NTG), and 12 (n = 6 TG, n = 8 NTG) weeks. In protocol 2, tissue was collected at 26 weeks of age (n = 15 OTG and n = 15 ONTG).

Enzyme-linked immunoassay and dot blot. Antibody levels in serum were determined by enzyme-linked immunosorbent assay (ELISA) using 96-well plates coated with preformed h-IAPP oligomers (40 ng/well) (17). Serial dilutions of serum from four to eight mice from each group were incubated overnight at 4°C followed by horseradish peroxidase–conjugated anti-mouse IgG (Zymed, diluted 1:3,000) for 1 h at 37°C. The plate was developed using 3,3′,5,5′-tetramethylbenzidine (TMB; KPL, Gaithersburg, MD). For protocol 2, the levels of anti-oligomer antibody were detected by dot blot using neutrocellulose membranes blotted with preformed h-IAPP oligomers

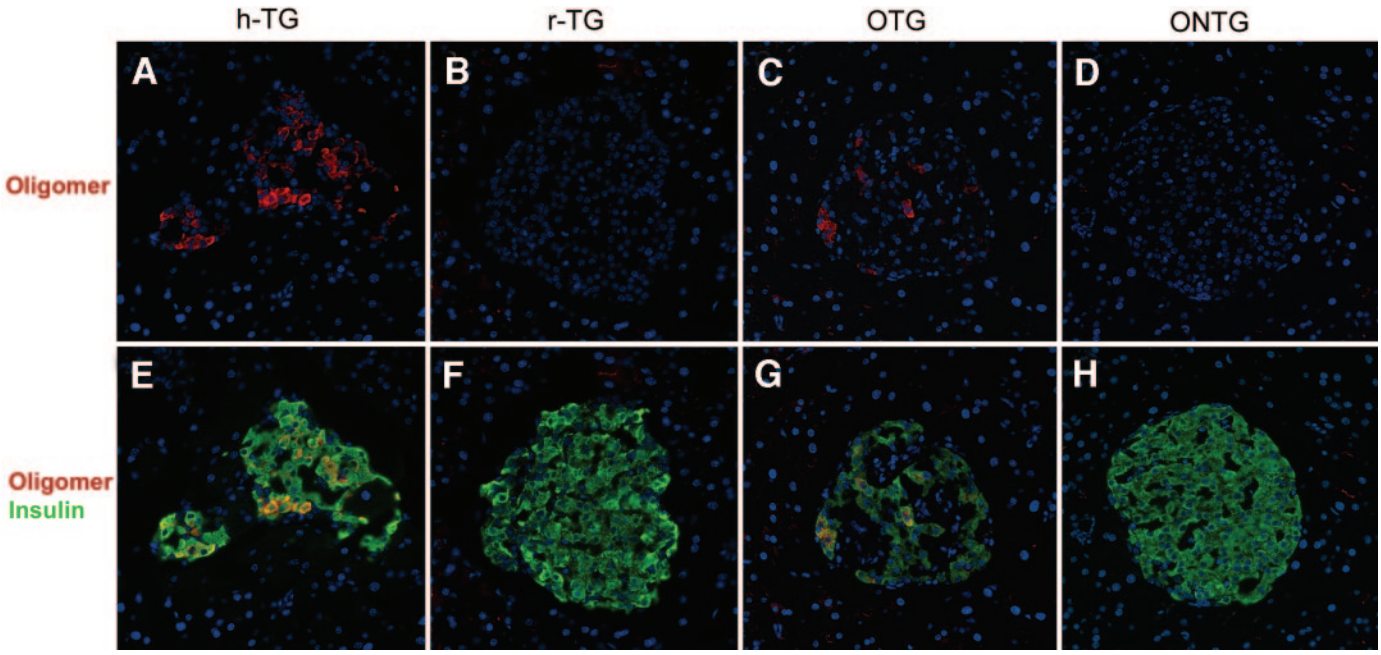

FIG. 1. h-IAPP toxic oligomers in h-IAPP versus r-IAPP transgenic and control mice. Representative islets from 10-week-old homozygous h-IAPP (h-TG; *A* and *E*), r-IAPP transgenic (r-TG; *B* and *F*), h-IAPP hemizygous obese (OTG; *C* and *G*), and nontransgenic obese (ONTG; *D* and *H*) mice showing immunofluorescence for h-IAPP toxic oligomers (red), insulin (green), and DAPI for nuclei (blue). Toxic oligomer immunoreactivity is confined to islets in h-IAPP transgenic mice and coincident with insulin staining in ~40% of β-cells in h-TG and ~15% of β-cells in OTG mice.

(0.6 μg/spot). Membranes were incubated in serial dilutions of serum samples overnight followed by horseradish peroxidase goat anti-mouse IgG (1:5,000; Zymed). Membranes were developed using an enhanced chemiluminescence kit from BioRad (Hercules, CA) and quantified using Un-Scan-It (Silk Scientific, Orem, UT).

β-Cell mass, β-cell apoptosis, and islet amyloid. Pancreas sections through the length of the pancreas were double immunostained for insulin (guinea pig anti-insulin polyclonal antibody 1:1,200; Dako, Carpinteria, CA) and the marker of replication Ki67 (rat anti-mouse Ki67 monoclonal antibody TEC-3, 1:45; Dako, Carpinteria, CA) or insulin (as above) and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling using the TdT-Frag El Kit from Oncogene Research Products (Cambridge, MA). Slides were evaluated independently by two observers (A.E.B. and C.Y.L.) with an Olympus IX 70 inverted microscope and analyzed using the Image-Pro Plus software to quantify β-cell mass, β-cell replication, β-cell apoptosis, and islet amyloid as described previously (25).

Calculations and statistical analysis. Statistical comparisons were performed using the nonpaired two-tailed Student's *t* test. A P value <0.05 was considered statistically significant. Data in graphs are presented as means ± SEM.

RESULTS

Toxic oligomers in h-IAPP transgenic mice

Homozygous h-IAPP transgenic versus r-IAPP transgenic mice. Of β-cells in h-IAPP homozygous mice, 46 ± 4.6% were positive for toxic oligomers at 10 weeks of age, whereas no immunoreactivity was detected against toxic oligomers in either the r-IAPP homozygous transgenic mice or nontransgenic controls (Fig. 1*A*, *E* and *B*, *F*, respectively). No oligomer staining was detected in exocrine tissue in any group.

Obese hemizygous h-IAPP transgenic mice. Toxic oligomer immunoreactivity was detected in 13.2 ± 1.4% β-cells in ~80% of islets from 24-week-old obese h-IAPP transgenic mice but none in β-cells of obese nontransgenic mice (Fig. 1*C*, *G* and *D*, *H*, respectively). Toxic oligomer immunoreactivity was not present in exocrine tissue in either group. As previously reported (24,25), obese h-IAPP transgenic mice developed extensive extracellular islet amyloid by ~24 weeks of age (Fig. 2*A*). Amyloid did not contain toxic oligomer immunoreactivity. Confocal imaging confirmed that most oligomer staining was intracellular and confined to β-cells (Fig. 2*B*). Within β-cells, the oligomer staining was both perinuclear and in frequent small discrete deposits, consistent with secretory vesicles. Specificity of oligomer staining was confirmed by blocking experiments. Preincubation of anti-oligomer antibody with preformed h-IAPP or $A\beta P_{1-40}$ oligomers blocked immunofluorescent staining, as well as the binding to oligomers blotted on the neutrocellulose membrane (Fig. 3).

Consistent with prior studies (17), addition of antibodies to synthetic toxic oligomers of $A\beta P_{1-40}$ prevented toxicity caused by application of h-IAPP peptide or preformed oligomers to rat insulinoma cells in culture (data not shown).

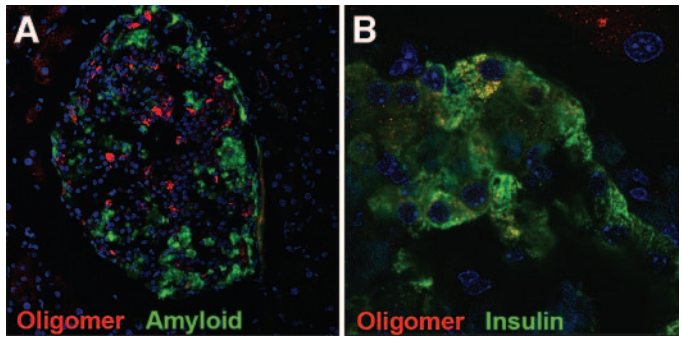

FIG. 2. h-IAPP toxic oligomers in hemizygous h-IAPP transgenic mice. Representative confocal images of islets from 24-week-old obese h-IAPP transgenic mouse (original magnification: *A*, 20×; *B*, 100×). *A*: Immunofluorescent staining for toxic h-IAPP oligomers (red), autofluorescence for amyloid (green), and nuclei DAPI (blue). h-IAPP toxic oligomer immunoreactivity does not coincide with amyloid. *B*: Immunofluorescent staining for toxic h-IAPP oligomers (red), insulin (green), and nuclei DAPI (blue). h-IAPP toxic oligomers are predominantly intracellular, confined to β-cells, and perinuclear or in vesicle-like structures.

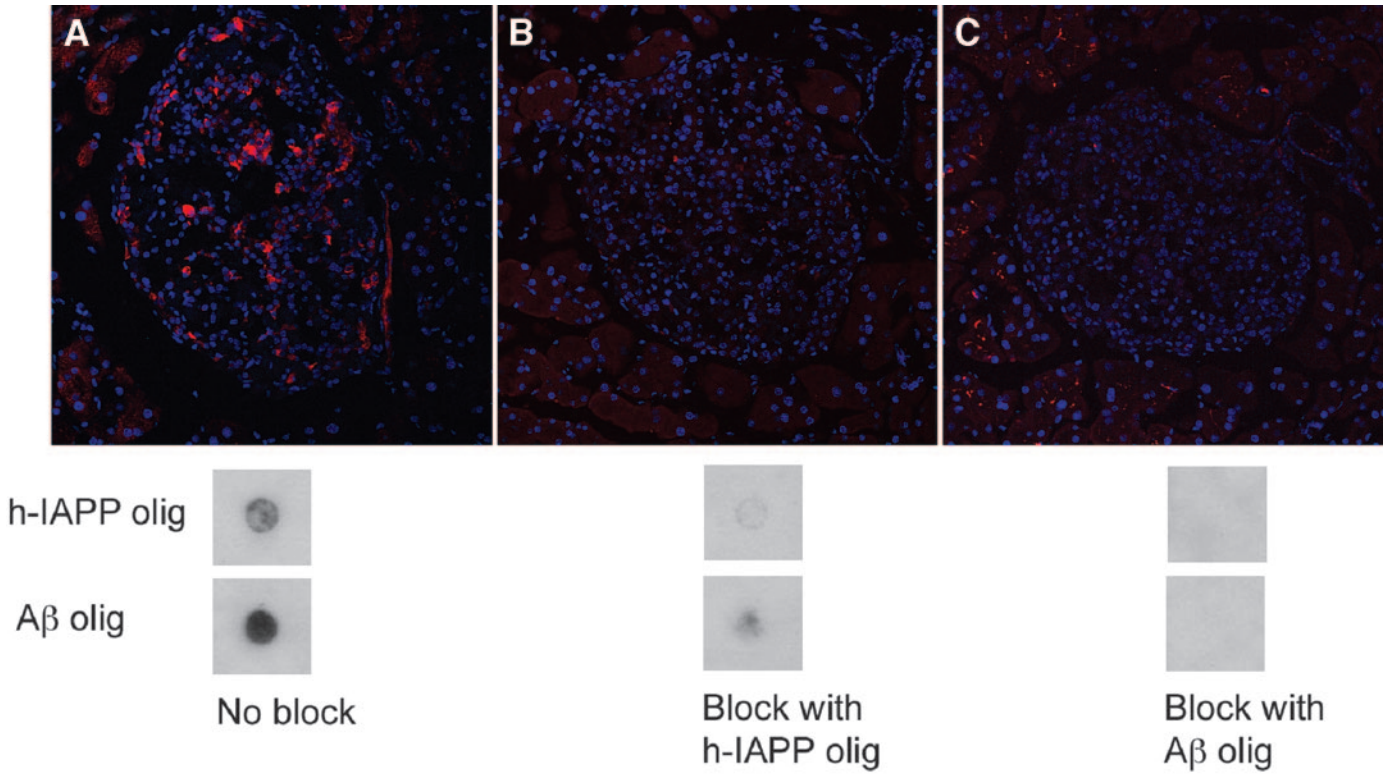

FIG. 3. Specificity of anti-toxic oligomer antibody. *A–C*: Confocal images (original magnification 20×) of representative islets from 24-week-old hemizygous h-IAPP transgenic obese (OTG) mouse stained with anti-oligomer Ab (A-11, red) and DAPI (blue). Anti-oligomer staining (*A*) was blocked by in vitro preformed oligomers of h-IAPP (*B*) or Aβ peptide (*C*). Antibody blockage was confirmed by dot blot using membranes spotted with preformed h-IAPP or Aβ peptide oligomers (*lower three panels*).

Vaccination protocol 1

Anti-oligomer h-IAPP antibody. Vaccination with the mimic of $A\beta P_{1-40}$ oligomer induced anti-oligomer h-IAPP antibody production in both TG and NTG mice after 6 weeks of age ($P < 0.005$) (Fig. 4). The background ELISA reading was no different in TG and NTG mice with PBS treatment.

Body weight and fasting blood glucose. As observed previously, body weight in TG mice did not increase as rapidly as in NTG mice. However, vaccination did not influence weight gain in either the TG or NTG mice (Fig. 5*A* and *B*). Fasting blood glucose concentrations were comparable in TG and NTG mice until 6 weeks of age, after which it increased in TG mice (Fig. 5*C*, $P < 0.05$).

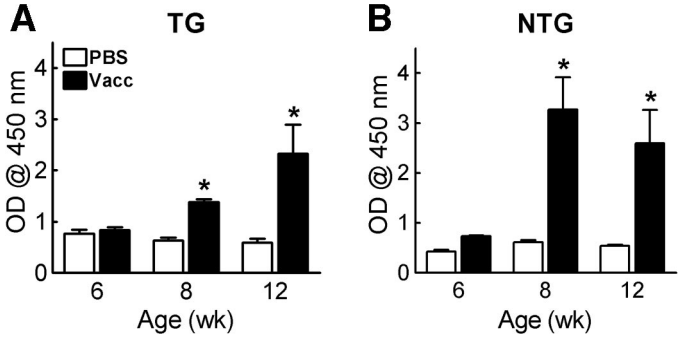

FIG. 4. Anti-toxic oligomer antibody titers in homozygous mice in protocol 1. Anti-oligomer h-IAPP antibody was increased after 6 weeks of age in both transgenic (TG) (*A*) and nontransgenic (NTG) (*B*) vaccinated (Vacc) mice (ELISA, sera dilution 1 in 400). Data are the mean ± SEM; **P* < 0.005 vs. PBS-treated mice of the same age. OD, optical density.

Vaccination with $A\beta P_{1-40}$ oligomers versus PBS had no impact on the blood glucose concentration with age in either the TG or NTG mice. Thus, vaccination with $A\beta P_{1-40}$ oligomers did not prevent diabetes in TG mice.

β-Cell mass. Pancreas weight did not differ between TG and NTG mice. β-Cell mass was comparable in TG and NTG mice at 6 weeks of age. Thereafter, while β-cell mass progressively increased in NTG mice, it declined in TG mice. By 12 weeks of age, β-cell mass in TG mice was <20% of that in NTG mice. Vaccination did not prevent this fall in β-cell mass in TG mice, with β-cell mass actually marginally lower in vaccinated TG mice compared with PBS-administered TG mice (Fig. 6*A*, $P < 0.05$).

β-Cell apoptosis and β-cell replication. β-Cell apoptosis per islet, normalized to insulin area, was more than eightfold higher in TG mice compared with NTG mice after 8 weeks of age ($P < 0.05$). However, immunization with $A\beta P_{1-40}$ oligomers did not decrease the frequency of β-cell apoptosis. To the contrary, β-cell apoptosis was higher in the h-IAPP transgenic mice vaccinated with $A\beta P_{1-40}$ oligomers versus PBS (Fig. 6*B*, $P < 0.05$). β-Cell replication was comparable in NTG and TG mice and was not influenced by vaccination (Fig. 6*C*).

Vaccination protocol 2

Anti-oligomer h-IAPP antibody. Vaccination induced anti-oligomer antibodies in both OTG and ONTG mice (Fig. 7). Antibodies were well detectable at 10 weeks of age, and the antibody titers increased with the following boosts. The background levels of immunoreactivity of serum from nonimmunized mice were not different in OTG and ONTG mice and did not change with age.

Body weight, blood glucose, and β-cell mass. Immunization did not affect weight gain with age (Fig. 8*A*). Both

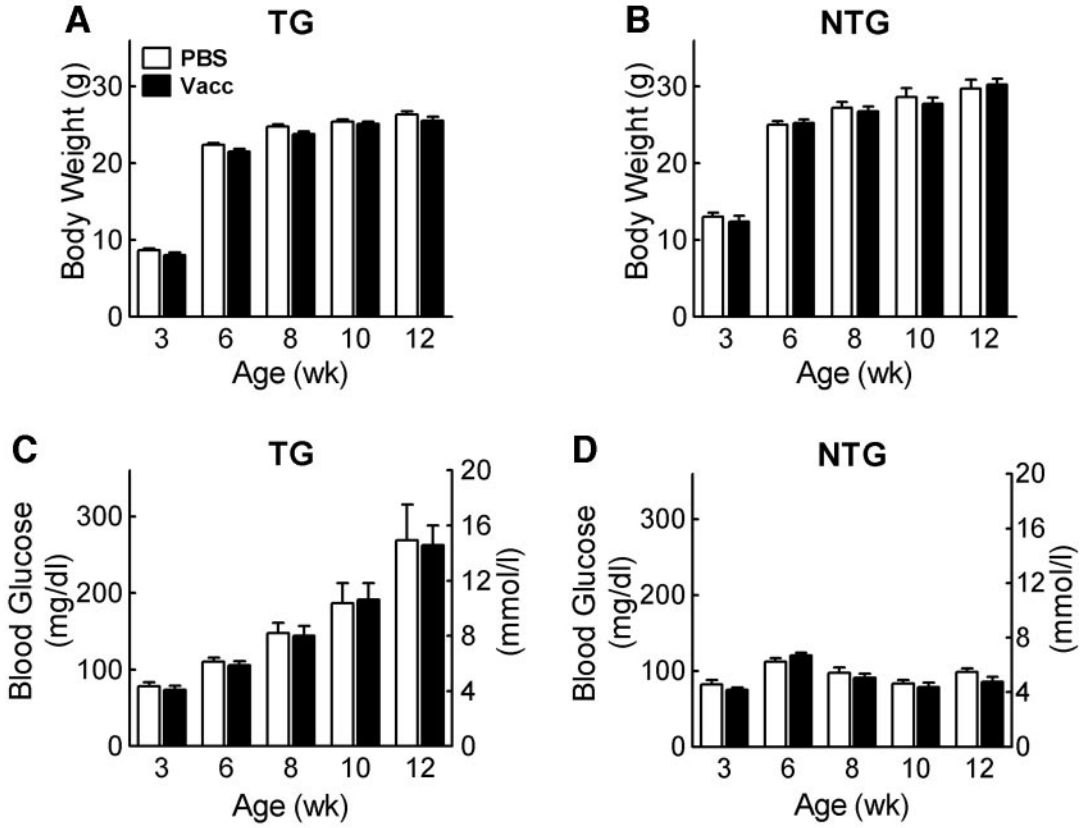

FIG. 5. Blood glucose and body weight in protocol 1. Fasting body weight and blood glucose levels in homozygous h-IAPP transgenic (TG) and nontransgenic (NTG) mice after vaccination with $A\beta P_{1-40}$ oligomers (Vacc) or PBS. (*A* and *B*). Fasting body weight did not differ between vaccinated and PBS-treated groups of TG or NTG mice. *C*: In TG mice, fasting blood glucose concentration was not different between vaccinated and PBS-treated mice; both groups developed diabetes by 10 weeks of age. *D*: In NTG mice, fasting blood glucose concentrations were within a normal range and similar in vaccinated and PBS-treated mice. Data are the mean ± SEM.

immunized and control OTG mice progressively developed hyperglycemia compared with ONTG mice (Fig. 8*B*). OTG mice had a 65% deficit ($P < 0.05$) in β-cell mass compared with ONTG mice by 26 weeks of age (Fig. 8*C*), with no protection against loss of β-cell mass afforded by vaccination. Both immunized and control OTG groups had comparable β-cell mass at the end of experiment (4.5 ± 0.3 and 4.9 ± 0.5 mg, respectively) (Fig. 8*C*). As described previously, islet amyloid deposits developed in the OTG but not in the ONTG mice. Neither frequency nor extent of islet amyloid was influenced by anti-oligomer vaccination (data not shown).

DISCUSSION

Our first goal in these studies was to establish whether IAPP toxic oligomers are formed intra- or extracellularly. In two h-IAPP transgenic mouse models, we report that toxic h-IAPP oligomers are formed intracellularly. The anti-oligomer antibody used to detect h-IAPP oligomers was raised against toxic oligomers of $A\beta P_{1-40}$ (17). As previously reported, this antibody binds to the toxic oligomers of several amyloidogenic proteins, including AβP, IAPP, prion, insulin, and transthyretin, while not binding to either monomers or amyloid fibrils of any of

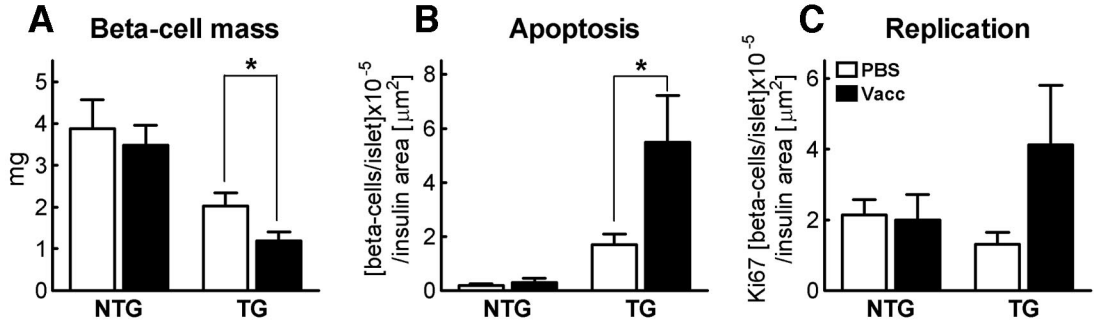

FIG. 6. β-Cell mass, apoptosis, and replication in protocol 1. β-Cell mass (*A*), β-cell apoptosis (*B*), and replication (*C*) in homozygous h-IAPP transgenic mice (TG) and nontransgenic (NTG) controls pooled from 6, 8, and 12 weeks of age. β-Cell mass was decreased in TG mice versus NTG mice ($P < 0.0001$) and was further decreased by vaccination (*$P < 0.05$) in TG versus PBS-treated TG mice (*A*). β-Cell apoptosis/insulin area was significantly higher in TG mice ($P < 0.05$) versus NTG mice and was further increased by vaccination (*$P < 0.05$) in TG versus PBS-treated TG mice (*B*). β-Cell replication/insulin area was not significantly different between TG and NTG mice or between vaccinated and PBS-treated TG mice (*C*).

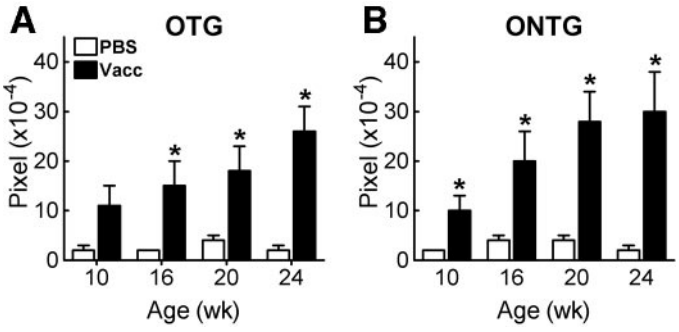

FIG. 7. Anti-toxic oligomers antibody titers in protocol 2. Anti-oligomer antibody was induced in vaccinated OTG (*A*) and ONTG (*B*) mice (dot blot, sera dilution 1 in 800). Data are the mean ± SEM; **P* < 0.05 vs. PBS-treated mice of the corresponding age.

these proteins (17,27). Since insulin and the precursor protein of AβP (28) are both expressed by β-cells, it is theoretically possible that the immunoreactivity detected in the β-cells of h-IAPP transgenic mice in the present study is due to formation of toxic oligomers of a protein other than h-IAPP. However, since no immunoreactivity was detected in either nontransgenic controls or controls transgenic for nonamyloidogenic r-IAPP, the most likely explanation for the detection of toxic oligomer immunostaining in h-IAPP transgenic mice is the formation of h-IAPP oligomers.

Toxic oligomer immunoreactivity was only detected intracellularly and was distinct from the extracellular amyloid deposits when present. These findings are consistent with a growing body of evidence that the amyloid fibril forms of amyloidogenic proteins are distinct from the toxic oligomers of these proteins (29,30), and others have also identified the latter to be intracellular (31,32). It is theoretically possible that toxic oligomers formed extracellularly and then were taken up, but this seems unlikely for several reasons. First, only β-cells were immunoreactive for toxic oligomers and no other islet cell types. Second, only a small subset of β-cells were immunoreactive for toxic oligomers. Third, we did not observe extracellular toxic oligomers. Finally, the toxic oligomers appeared to be present at least in part within insulin secretory granules, implying formation within the secretory pathway. This latter observation leaves open the possibility that the toxic oligomers may be shed as insulin is secreted; therefore, while forming intracellularly, they could theoretically also act extracellularly, for example, on the plasma membrane.

Our second goal was to establish whether vaccination with toxic oligomers to induce anti-toxic oligomer antibodies influenced the outcome of h-IAPP–induced β-cell apoptosis and development of diabetes, recognizing that even though the oligomers are formed intracellularly, they might still be secreted and act extracellularly. Vaccination with $A\beta P_{1-40}$ oligomer mimics successfully raised antibodies to toxic oligomers in both h-IAPP transgenic models tested. In vitro studies affirmed that antibodies against toxic oligomers are protective against the addition of h-IAPP toxic oligomers to cells. However, anti-toxic oligomer antibodies raised by vaccination did not afford any protection in vivo against increased β-cell apoptosis in h-IAPP transgenic mice. These data indirectly support the concept that cytotoxicity induced by high expression levels of h-IAPP is primarily mediated by intracellular actions.

The amyloid concept for neurodegenerative diseases and type 2 diabetes has been controversial because of a poor correlation between amyloid deposition and disease extent (11–13,23,25,33). This paradox has been somewhat clarified by the appreciation that proteins with a predisposition to form amyloid fibrils may also develop nonfibrillar protein oligomeric structures that are much smaller, membrane disruptive, and cytotoxic, while amyloid fibrils are relatively inert (14–16). Of interest, h-IAPP toxic oligomers were detected in many but not all β-cells, raising the question of what distinguishes between β-cells that develop detectable h-IAPP toxic oligomer immunoreactivity and those that do not? One possible explanation is that endoplasmic reticulum stress is induced by accumulation of unfolded proteins, and in particular aggregated proteins, in the endoplasmic reticulum (34). We suspect that induction of β-cell apoptosis in these h-IAPP transgenic models is consequent upon delivery of h-IAPP to the endoplasmic reticulum at a rate that exceeds the capacity of the β-cell to chaperone and traffic the h-IAPP, a hypothesis that gains support by an apparent threshold effect for h-IAPP expression to cause β-cell toxicity (23–26). Heterogeneity between β-cells for the formation of h-IAPP oligomers may depend on variance in the glucose concentration threshold for β-cell stimulation (35), ability to chaperone and traffic major client proteins, and ability to clear misfolded and aggregated proteins.

Vaccination has been explored as a potential therapy for Alzheimer's disease (36–39). Some mouse models transgenic for human AβP develop cerebral amyloid deposits associated with a cognitive decline (40). Active or passive immunization of these mouse models against AβP aggregated in the form of amyloid fibrils both reduced brain

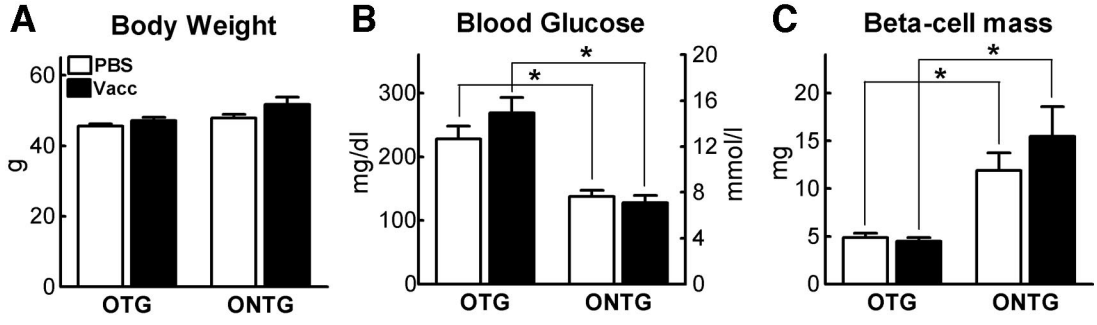

FIG. 8. Body weight, blood glucose, and β-cell mass in protocol 2. Body weight (*A*), blood glucose (*B*), and β-cell mass (*C*) in obese nontransgenic mice (ONTG) and obese h-IAPP hemizygous transgenic mice (OTG) at 26 weeks after vaccination or injections with PBS. Anti-oligomer vaccination did not effect weight gain (*A*) or protect OTG mice from developing hyperglycemia (P* < 0.01) (*B*). β-Cell mass was decreased in OTG compared with ONTG mice (**P* < 0.05) but was not affected by vaccination (*C*). Data are the mean ± SEM.**

pathology in the form of amyloid (41–44) and, if given early, reduced progression of cognitive decline (43,45,46). These findings were considered further evidence of the so-called amyloid hypothesis, implicating that the extracellular amyloid deposits characteristically present in Alzheimer's disease have a primary role in the associated neurodegeneration (47). Unfortunately, when the same vaccine approach was extended to humans with Alzheimer's disease, an unexpected side effect, clinical meningoencephalitis, occurred in 6% of cases and was subsequently confirmed at autopsy (48,49). The cerebral amyloid burden was decreased in these cases, however, supporting the concept that antibodies against amyloid fibrils would help clear the extracellular amyloid plaques, presumably by promoting microglial activation (42).

In the present studies, we tested a modified vaccine-based approach to prevent amyloid-related degenerative disease in mice transgenic for h-IAPP. Since it has become appreciated that the toxic form of amyloidogenic proteins is not amyloid fibrils but rather much smaller toxic forms of oligomer (17,50), we rationalized that induction of an immune response to these toxic oligomers might be an alterative approach to the prevention of h-IAPP oligomer–induced β-cell death. The toxic form of oligomers derived from amyloidogenic proteins appears to share a conformational-dependent structure that presumably dictates their toxicity. Because we had available a well-characterized synthetic mimic of $A\beta P_{1-40}$ toxic oligomers that had been previously successful in raising antibodies that protected cells from h-IAPP toxicity in vitro, we elected to use the same $A\beta P_{1-40}$ oligomer preparation in these vaccination studies.

Vaccination was successful in eliciting an immune response in the h-IAPP transgenic and nontransgenic mice. In mice homozygous for h-IAPP, as previously described (23), diabetes onset was by 8–10 weeks of age and associated with a rapid decline in β-cell mass due to increased β-cell apoptosis. When we first reported this model, we noted dissociation between loss of β-cell mass and formation of extracellular islet amyloid, with the former preceding the latter (23). At the time of maximal loss of β-cells, we noted intracellular IAPP oligomers that were nonfibrillar and suggested that the mechanism underpinning β-cell loss was more likely to be related to intracellular h-IAPP oligomers rather than extracellular islet amyloid. The distribution of nonfibrillar IAPP intracellular aggregates detected by electron microscopy in that study corresponds closely to the detection of toxic oligomers in the present study. Future studies are required with electron microscopy and double labeling of IAPP, and toxic oligomers will establish the exact relationship between these aggregates. In the present vaccination protocols, we did not see protection against loss of β-cells after vaccination in the homozygous h-IAPP transgenic model. Indeed, unexpectedly, we observed an increase in β-cell apoptosis perhaps consistent with the aggravated inflammatory response observed to the extent that it was obvious clinically as meningoencephalitis in humans after $A\beta P_{1-42}$ amyloid vaccination (49). In contrast to the cases that have come to autopsy after developing meningoencephalitis, we were unable to detect a T-cell inflammatory infiltrate in $A\beta P_{1-40}$ oligomer–vaccinated mice. One possible explanation for the increased β-cell apoptosis in vaccinated mice is enhanced complement-mediated destruction as a consequence of anti-oligomer binding with h-IAPP oligomers secreted or released from degenerating cells.

Beneficial effects of active immunization in mice against loss of cognitive function were best observed when vaccine was given early (37,43,45,46). We therefore recognized the possibility that we did not see delay or prevention of diabetes in the homozygous h-IAPP mouse model as a consequence of the temporal overlap between development of anti-oligomer antibodies and increased β-cell apoptosis. To overcome this, we undertook protocol 2, vaccinating obese heterozygous h-IAPP transgenic mice that do not develop diabetes until 20–30 weeks of age. These mice also develop islet pathology more reminiscent of humans with type 2 diabetes (24,25). Specifically, they develop large deposits of extracellular IAPP–derived islet amyloid associated with β-cell loss due to increased β-cell apoptosis. However, despite successfully eliciting an immune response in the obese h-IAPP heterozygous mice, there was again no delay in diabetes onset, and β-cell apoptosis was comparably increased in PBS-administered and $A\beta P_{1-40}$ oligomer–immunized mice. Moreover, immunization with $A\beta P_{1-40}$ oligomers did not influence the extent of extracellular amyloid. This is in contrast with mice vaccinated with $A\beta P_{1-42}$ amyloid fibrils, which had less brain amyloid, a change attributed to microglial-induced amyloid clearance (42,44).

Several possibilities exist to account for the failure of anti-oligomer antibodies to prevent h-IAPP oligomer–induced β-cell loss and development of diabetes in these transgenic h-IAPP mouse models. One is that the toxic form of IAPP is not recognized or bound by these antibodies. Against this, we repeated prior studies that showed that antibodies raised against $A\beta P_{1-40}$ oligomers do protect cells in vitro against the addition of preformed h-IAPP oligomers (17). The titers of anti-oligomer antibodies in plasma of immunized mice in both our protocols were lower but comparable to the titers detected in plasma of mice immunized with $A\beta P_{1-42}$ (43). In that study, the titers of anti-AβP were sufficient to improve cognitive function and to decrease the amyloid burden in the brain, despite the fact that only 0.1% of plasma antibodies can cross the blood-brain barrier (42). None the less, it is possible that the antibodies raised in mice in our protocols were not present in a sufficient enough concentration within the islet to be protective. An alterative explanation is that the initiation of apoptosis induced by h-IAPP oligomers is intracellular and therefore relatively inaccessible to antibodies. Although antibodies can gain access to cells, this is predominantly true for phagocytic cells (for example, microglia in brain). The adverse action of h-IAPP oligomers are believed to be mediated by their interactions with membranes (16).

It is important to consider the limitations of using h-IAPP transgenic mice rather than humans with type 2 diabetes in this study. It is possible that formation of toxic h-IAPP oligomers differs in both extent and location in humans with type 2 diabetes and mice transgenic for h-IAPP. Detection of toxic oligomers in the islet requires freshly frozen tissue, but available human tissue at autopsy is paraffin embedded. Also, vaccination studies in transgenic mouse models are not necessarily predictive of outcomes in humans (51). Vaccination with $A\beta P_{1-42}$ fibrils in mice was encouraging, but subsequent studies in humans revealed acceleration of disease in some patients (48,49). In vaccination protocol 1, we observed a vaccine-dependent acceleration of β-cell apoptosis in h-IAPP trans-

genic mice, cautioning that a similar adverse outcome might occur in humans with type 2 diabetes.

In conclusion, h-IAPP toxic oligomers were detected intracellularly in two h-IAPP transgenic mouse models. Immunization of the same h-IAPP transgenic mouse models with $A\beta P_{1-40}$ oligomers successfully induced an immune response generating antibodies that bound to synthetic h-IAPP oligomers. However, vaccination did not prevent or delay the onset of diabetes in either mouse model and was associated with increased β-cell apoptosis and loss of β-cells in homozygous mice transgenic for h-IAPP. These studies indirectly suggest that h-IAPP–induced toxicity in h-IAPP transgenic mice is likely initiated by an intracellular mechanism that may not be readily accessible to a vaccine-based approach. Moreover, the latter should perhaps be approached with caution, since it may exacerbate rather than prevent development of diabetes in humans, as has been described for Alzheimer's disease.

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