Toxic Human IAPP Oligomers are Intracellular, and Vaccination to Induce Anti-toxic Oligomer Antibodies Does not Prevent Human IAPP Induced Beta-Cell Apoptosis in Human IAPP Transgenic Mice.

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ABBREVIATIONS USED: h-IAPP - human islet amyloid polypeptide; r-IAPP - rodent islet amyloid polypeptide; AβP - Alzheimer Beta Protein; T2DM – type 2 diabetes mellitus; TUNEL -
ABSTRACT

Objective: The islet in Type 2 diabetes is characterized by deficit in beta-cells, increased beta-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 if IAPP toxic oligomers form intra- or extracellularly, and if vaccination to induce anti-toxic oligomer antibodies prevents IAPP induced apoptosis in human IAPP transgenic mice.

Methods: Pancreas was sampled from two human IAPP (h-IAPP) transgenic mouse models and examined by immunohistochemistry for toxic oligomers. The same murine models were vaccinated with toxic oligomers of Alzheimer beta protein (AβP_{1-40}) and anti-oligomer titers, blood glucose and islet pathology were monitored.

Results: Toxic oligomers were detected intracellularly in ~20-40% h-IAPP transgenic beta-cells. Vaccine induced high titers of anti-h-IAPP toxic oligomers in both transgenic models but beta-cell apoptosis was if anything further increased in vaccinated mice so that neither loss of beta-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 beta-cell apoptosis. These data suggest approaches that prevent h-IAPP oligomer formation may be more useful in prevention of type 2 diabetes than a vaccination based approach.
INTRODUCTION

Type-2 diabetes mellitus (T2DM) is characterized by progressive beta-cell dysfunction (1). Morphologically the islet in T2DM has a deficit in beta-cells, increased beta-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 beta-cells and is believed to play a paracrine role in restraining insulin secretion (2; 4-6).

Several explanations have been offered for the increased beta-cell apoptosis in T2DM, including glucotoxicity and lipotoxicity (7; 8). The focus here is on the potential role of the amyloidogenic peptide IAPP. The loss of beta-cells in T2DM 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 beta 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 property of inducing 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 AβP₁₋₄₀ 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 if human IAPP (h-IAPP) toxic oligomers form intra-cellularly in h-IAPP transgenic mice. Also, since immunization of rabbits with the mimic AβP₁₋₄₀ oligomers raised antibodies protective against h-IAPP oligomer cytotoxicity, we also sought to establish if immunization with the same AβP₁₋₄₀ oligomer preparation could prevent diabetes in h-IAPP transgenic mice models of T2DM. The mimic AβP₁₋₄₀ 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 T2DM. The sequence of IAPP in mice and rats is identical and non-amyloidogenic, and neither of these species develop T2DM 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 at an early age characterized by rapid decline in beta-cells (23). The period of high beta-cell loss in this homozygous h-IAPP model is coincident with the presence of small intracellular non-fibril aggregates of h-IAPP (23). The second model we used is a derivative of the homozygous h-IAPP transgenic mouse model established by
cross-breeding 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 non-transgenic obese mice) develop diabetes at ~20-30 weeks of age characterized by extensive extracellular islet amyloid and increased beta-cell apoptosis.

By use of these two models we first sought to establish if 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 AβP₁−₄₀ oligomers that were previously used in rabbits to raise antibodies that bind and neutralize h-IAPP oligomer induced toxicity. By this vaccination based approach we sought to establish if h-IAPP toxicity in vivo acts primarily extracellularly implying a vaccine approach for prevention of T2DM might be feasible. Alternatively, if increased beta-cell apoptosis in h-IAPP transgenic mice is not prevented despite the presence of neutralizing antibodies to toxic h-IAPP oligomers, this would imply h-IAPP toxic oligomers act primarily intra-cellularly, and would argue against the likely usefulness of a vaccine based approach to T2DM.

MATERIALS AND METHODS

Human and rodent IAPP transgenic rodent models.

Development and characterization of transgenic mice homozygous for human IAPP (FVB-Tg(IAPP)6Jdm/Tg(IAPP)6Jdm) and rodent IAPP (FVB/N-Tg(Iapp)6Wcs/Tg(Iapp)6Wcs) have been described elsewhere (23; 26). Likewise we have previously reported cross breeding homozygous h-IAPP transgenic mice with the A²²/a mouse on the C57BL/6 background to generate obese mice hemizygous for h-IAPP, referred to as OTG mice (FVB-tg(IAPP)6Jdm/-Avy/A) and their non-transgenic 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, fat and lymph nodes trimmed, the pancreas weighed, fixed in 4% paraformaldehyde at 4°C for 24 h, then divided into equal parts. One portion was embedded in paraffin, the other frozen in OCT.

Immunofluorescence and confocal microscopy. Four µm frozen sections of pancreas were washed with TBS/0.1% TW-20, then blocked with TBS/0.2%TX-100/3%BSA/2% Normal Donkey Serum (Jackson Immunoresearch, West GroveCity, PA) for 3 h at room temperature. Sections were treated with oligomer-specific antibody (A11) raised as previously described against stable soluble oligomers of AβP₁−₄₀ (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 FITC-F(ab)2 donkey anti-guinea pig IgG (Jackson Immunoresearch). Antibodies were diluted in TBS/0.2%TW-20/3% BSA. Between antibody treatments, slides were washed three times with TBS/0.1% TW-
20. Slides were coverslipped with Vectashield-DAPI mounting medium (Vector Laboratories, Inc., 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 pre-incubated for 5 h with 100 excess of pre-formed h-IAPP or AβP oligomers (prepared as described (17)), or diluted with PBS. Blockage of antibody was confirmed by dot blot, using nitrocellulose membranes blotted with pre-formed oligomers from h-IAPP and AβP (0.6 g per spot). Congo Red staining was performed in 8 µM paraffin sections.

Image analysis. Multiple images of pancreas were acquired with Leica fluorescent microscope DM6000 at 20x magnification (Leica, 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 were evaluated by two independent observers (AEB, TG). 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 AβP_{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 Inc.) were mixed with a freshly prepared solution of 0.2 mg/ml AβP_{1-40} thioester, (pH 5.0–5.5, 25 ml Aβ solution to 20 ml of washed gold colloids in water (approximately 1.0 × 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 mM Tris pH 8.0 (0.02% sodium azide). After incubation for 6 h at room temperature, the antigen was collected by centrifugation at 30,000g at 4°C for 30 min, washed three times with phosphate buffered saline (PBS), pH 7.6 to remove any unincorporated Aβ, and then re-dispersed in distilled H_{2}O (0.02% sodium azide). The molecular mimic and naturally occurring spherical oligomers have very similar structural properties based on analysis by atomic force microscopy (AFM), electron microscopy, circular dichroism (CD), thioflavin T fluorescence, and B is-Anilino-Sulfonic Acid (Bis-ANS) (data shown in (27)). Before use, the antigen was washed three times with PBS, pH 7.4 and re-suspended in PBS at 250 mg/ml.

Protocol one. 13 homozygous h-IAPP TG and 12 NTG mice were injected intra-peritoneally with 100 µg AβP_{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 intra-peritoneally with PBS as vehicle control on four occasions at the same age as vaccinated animals. Mice from each group were randomly selected to be euthanized at different ages for pancreas and blood collection.

Protocol two. Fifteen OTG and 15 ONTG mice were randomly assigned into control or experimental groups, and then administered PBS or AβP_{1-40} oligomer micelles respectively. Experimental mice
received 100 µg AβP₁₋₄₀ oligomer micelles at 4 wk of age, then boosted with the same amount of antigen at 6 and 8 wk, and every 4 wk thereafter. Blood was collected by retro orbital bleeding at 10 wk and then every 4 wk to monitor anti-oligomer antibody titer.

Monitoring of diabetes and tissue collection. Fasting body weight and blood glucose concentrations were measured after eight hour 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, allowed to clot and then separated to obtain serum for subsequent measurement of IAPP-oligomer antibody titers. Pancreas was extracted, fat and lymph nodes were removed, after which the pancreas was weighed, fixed in 4% PFA, and embedded in paraffin. In Protocol One, 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 Two, tissue was collected at 26 wk of age (n=15 OTG and n=15 ONTG).

ELISA and dot blot. Antibody levels in serum were determined by ELISA using 96 well plates coated with preformed h-IAPP oligomers (40 ng/well) (17). Serial dilutions of serum from 4 to 8 mice from each group were incubated overnight at 4°C followed by HRP-conjugated anti-mouse IgG (Zymed, diluted 1:3000) for 1 h at 37°C. The plate was developed using 3,3’,5,5’-tetramethylbenzidine (TMB, KPL Gaitherburg, MD). For protocol two, the levels of anti-oligomer antibody were detected by dot blot, using neutrocellulose membranes blotted with pre-formed h-IAPP oligomers (0.6 g per spot). Membranes were incubated in serial dilutions of serum samples overnight followed by HRP-goat-anti-mouse IgG (1:5000, Zymed). Membranes were developed using ECL kit from BioRad (Hercules, CA), and quantified using Un-Scan-It (Silk Scientific Inc., Orem, UT).

Beta-cell mass, beta-cell apoptosis and islet amyloid. Pancreas sections through the length of the pancreas were double immunostained for insulin (guinea-pig anti-insulin polyclonal antibody 1:1200, 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 (TUNEL) using the TdT-Frag El Kit from Oncogene Research Products (Cambridge, MA). Slides were evaluated independently by two observers (AEB, CYL) with an Olympus IX 70 inverted microscope and analyzed using the Image-Pro Plus software to quantify beta-cell mass, beta-cell replication, beta-cell apoptosis and islet amyloid as described previously (25).

Calculations and statistical analysis. Statistical comparisons were performed using the non-paired 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. 46±4.6% of beta-cells in h-IAPP homozygous mice were positive for toxic oligomers at 10 wk of age whereas no immunoreactivity was detected against toxic oligomers in either the r-IAPP homozygous transgenic mice
or non-transgenic 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% beta-cells in ~80% of islets from 24 wk old obese h-IAPP transgenic mice, but none in beta-cells of obese non-transgenic 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 extra-cellular islet amyloid by ~24 weeks of age (Fig. 2A) Amyloid did not contain toxic oligomer immunoreactivity. Confocal imaging confirmed that most oligomer staining was intra-cellular and confined to beta-cells (Fig 2B). Within beta-cells the oligomer staining was both peri-nuclear and in frequent small discrete deposits consistent with secretory vesicles. Specificity of oligomer staining was confirmed by blocking experiments. Pre-incubation of anti-oligomer antibody with pre-formed h-IAPP or AβP1-40 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 AβP1-40 prevented toxicity caused by application of h-IAPP peptide or preformed oligomers to RIN cells in culture (data not shown).

Vaccination Protocol One. 
**Anti-oligomer h-IAPP antibody.** Vaccination with the mimic of AβP1-40 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, 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. 5A and 5B). The fasting blood glucose concentration was comparable in TG and NTG mice until 6 weeks of age after which it increased in TG Mice (Fig. 5C, p < 0.05). Vaccination with AβP1-40 oligomers versus PBS had no impact on the blood glucose concentration with age in either the TG or NTG mice. Thus vaccination with AβP1-40 oligomers did not prevent diabetes in TG mice.

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

**Beta-cell apoptosis and beta-cell replication.** Beta-cell apoptosis per islet, normalized to insulin area, was more than 8 fold higher in TG mice compared to NTG mice after 8 weeks of age (p < 0.05). However, immunization with AβP1-40 oligomers did not decrease the frequency of beta-cell apoptosis. To the contrary, beta-cell apoptosis was higher in the h-IAPP transgenic mice vaccinated with AβP1-40 oligomers versus PBS (Fig. 6B, p < 0.05). Beta-cell replication was comparable in NTG and TG mice, and
was not influenced by vaccination (Fig. 6C).

Vaccination Protocol Two.

Anti-oligomer h-IAPP antibody.

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

Body weight, blood glucose and beta-cell mass. Immunization did not affect weight gain with age (Fig. 8A). Both immunized and control OTG mice progressively developed hyperglycemia compared to ONTG mice (Fig. 8B). OTG mice had a 65% deficit (p<0.05) in beta-cell mass compared to ONTG mice by 26 weeks of age (Fig. 8C), with no protection against loss of beta-cell mass afforded by vaccination. Both immunized and control OTG groups had comparable beta-cell mass at the end of experiment: 4.5+0.3 mg and 4.9+0.5 mg respectively (Fig. 8C). As described previously, islet amyloid deposits developed in the OTG mice but not 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 if IAPP toxic oligomers are formed intra- or extra-cellularly. In two h-IAPP transgenic mouse models we report that toxic h-IAPP oligomers are formed intra-cellularly. The anti-oligomer antibody used to detect h-IAPP oligomers was raised against toxic oligomers of AβP1-40 (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 these proteins (17; 27). Since insulin and the precursor protein of AβP (28) are both expressed by beta-cells it is theoretically possible that the immunoreactivity detected in the beta-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 non-transgenic controls or controls transgenic for non-amyloidogenic r-IAPP, the most likely explanation for the detection of toxic oligomer immunostaining in h-IAPP transgenic mice only is the formation of h-IAPP oligomers.

Toxic oligomer immunoreactivity was only detected intra-cellularly and was distinct from the extra-cellular 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 intra-cellular (31; 32). It is theoretically possible that toxic oligomers formed extra-cellularly and then were taken up but this seems unlikely for several reasons. First only beta-cells were immunoreactive for toxic oligomers, and no other islet cell types. Second only a small subset of beta-cells were immunoreactive for toxic oligomers. Third, we did not observe extra-cellular 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 and so while forming intra-
cellularly, they could theoretically also act extra-cellularly, for example on the plasma membrane.

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

The amyloid concept for neurodegenerative diseases and T2DM 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 non-fibrillar 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 beta-cells, raising the question what distinguishes between beta-cells that develop detectable h-IAPP toxic oligomer immunoreactivity and those that do not? One possible explanation is 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 beta-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 beta-cell to chaperone and traffic the h-IAPP, a hypothesis that gains support by an apparent threshold effect for h-IAPP expression to cause beta-cell toxicity (23-26). Heterogeneity between beta-cells for formation of h-IAPP oligomers may depend on variance in the glucose concentration threshold for beta-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 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 the extra-cellular 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 occurred in 6% of cases, clinical meningoencephalitis 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 extra-cellular 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 beta-cell death. The toxic form of oligomers derived from amyloidogenic proteins appear to share a conformational dependent structure that presumably dictates their toxicity. As we had available well characterized synthetic mimic of AβP1-40 toxic oligomers which had been previously successful in raising antibodies that protected cells from h-IAPP toxicity in vitro, we elected to use the same AβP1-40 oligomer preparation in these vaccination studies.

Vaccination was successful in eliciting an immune response in the h-IAPP transgenic and non-transgenic 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 beta-cell mass due to increased beta-cell apoptosis. When we first reported this model we noted dissociation between loss of beta-cell mass and formation of extra-cellular islet amyloid, with the former preceding the latter (23). At the time of maximal loss of beta-cells we noted intra-cellular IAPP oligomers that were non fibrillar, and suggested that the mechanism underpinning beta-cell loss was more likely to be related to intra-cellular h-IAPP oligomers rather than extra-cellular islet amyloid. The distribution of non fibrillar IAPP intra-cellular aggregates detected by electron microscopy in that study correspond 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 beta-cells after vaccination in the homozygous h-IAPP transgenic model. Indeed unexpectedly we observed an increase in beta-cell apoptosis, perhaps consistent with the aggravated inflammatory response observed to an extent that it was obvious clinically as meningoencephalitis in humans after AβP1-42 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 AβP1-40 oligomer vaccinated mice. One possible explanation for the increased beta-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 beta-cell apoptosis. To overcome this we undertook protocol two, 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 T2DM (24; 25). Specifically they develop large deposits of extra-cellular IAPP derived islet amyloid associated with beta-cell loss due to increased beta-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 beta-cell apoptosis was comparably increased in PBS and AβP_{1-40} oligomer immunized mice. Moreover immunization with AβP_{1-40} oligomers did not influence the extent of extra-cellular amyloid. This is in contrast mice vaccinated with AβP_{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 beta-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 AβP_{1-40} oligomers do protect cells in vitro against addition of pre-formed 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 P_{1-42} (43). In that study the titers of anti-A P were sufficient to improve cognitive function and to decrease the amyloid burden in the brain despite of 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 sufficient concentration within the islet to be protective. An alterative explanation is that the initiation of apoptosis induced by h-IAPP oligomers is intra-cellular and so 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 T2DM in this study. It is possible that formation of toxic h-IAPP oligomers differs in both extent and location in humans with T2DM 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 AβP_{1-42} fibrils in human was encouraging, but subsequent studies in humans revealed acceleration of disease in some patients (48; 49). In vaccination protocol one, we observed a vaccine dependent acceleration of beta-cell apoptosis in h-IAPP transgenic mice cautioning that a similar adverse outcome might occur in humans with T2DM.

In conclusion, h-IAPP toxic oligomers were detected intra-cellularly in two h-IAPP transgenic mouse models. Immunization of the same h-IAPP transgenic mouse models with AβP_{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 beta-cell apoptosis and loss of beta-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 intra-cellular mechanism that may not be readily accessible to a vaccine-based approach. Moreover, the latter should perhaps be approached with caution since it might possibly exacerbate rather than prevent development of diabetes in humans as has been described for Alzheimer’s disease.

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FIG. 1. h-IAPP toxic oligomers in h-IAPP versus r-IAPP transgenic and control mice.
Representative islets from 10 wk old homozygous h-IAPP (h-TG; A,E), r-IAPP transgenic (r-TG; B,F), h-IAPP hemizygous obese (OTG; C,G) and non-tramsgenic obese (ONTG; D,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 beta-cells in h-TG and ~15% of beta-cells in OTG mice.
FIG. 2. h-IAPP toxic oligomers in hemizygous h-IAPP transgenic mice. Representative confocal images of islets from 24 wk old obese h-IAPP transgenic mouse (a 20x, b 100x).
(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 beta-cells, and it is perinuclear or in vesicle like structures.
FIG. 3. Specificity of anti toxic oligomer antibody.
Upper three panels, confocal images (20x) of representative islets from 24 week old hemizygous h-IAPP transgenic obese (OTG) mouse immunofluorescence staining with anti-oligomer Ab (A-11, red) and DAPI (blue).
Anti-oligomer staining (A) was blocked by *in-vitro* pre-formed oligomers of h-IAPP (B) or Aβ peptide (C). Antibody blockage was confirmed by dot blot using membranes spotted with pre-formed h-IAPP or Aβ peptide oligomers (lower three panels).
FIG. 4. Anti toxic oligomer antibody titers in homozygous mice in protocol one. Anti-oligomer h-IAPP antibody was increased after 6 weeks of age in both TG (A) and NTG (B) vaccinated (Vacc) mice (ELISA, sera dilution 1 in 400). Data are the mean + SEM; *p<0.005 versus PBS treated mice of the same age.
FIG. 5. Blood glucose and body weight in protocol one.
Fasting body weight and blood glucose levels in homozygous h-IAPP transgenic (TG) and non-transgenic (NTG) mice after vaccination with AβP₁₋₄₀ oligomers (Vacc) or PBS.

(A,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 wk 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.
FIG. 6. Beta-cell mass, apoptosis and replication in protocol one.
Beta-cell mass (A), beta-cell apoptosis (B) and replication (C) in homozygous h-IAPP transgenic mice (TG) and non-transgenic controls pooled from 6, 8 and 12 weeks of age. Beta-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). Beta-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). Beta-cell replication/insulin area was not significantly different between TG mice and NTG mice, and between vaccinated and PBS treated TG mice (C).
FIG. 7. Anti toxic oligomers antibody titers in protocol two. 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 versus PBS treated mice of the corresponding age.
Body weight, blood glucose and beta-cell mass in protocol two

Body weight (A), blood glucose (B) and beta-cell mass (C) in obese non-transgenic 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 hyperglycaemia (*p<0.01) (B). Beta-cell mass decreased in OTG compared to ONTG mice (*p<0.05), but was not affected by vaccination (C). Data are the mean ± SEM.