

# Islet Amyloid Polypeptide Is a Target Antigen for Diabetogenic CD4<sup>+</sup> T Cells

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**OBJECTIVE**—To investigate autoantigens in  $\beta$ -cells, we have used a panel of pathogenic T-cell clones that were derived from the NOD mouse. Our particular focus in this study was on the identification of the target antigen for the highly diabetogenic T-cell clone BDC-5.2.9.

**RESEARCH DESIGN AND METHODS**—To purify  $\beta$ -cell antigens, we applied sequential size exclusion chromatography and reverse-phase high-performance liquid chromatography to membrane preparations of  $\beta$ -cell tumors. The presence of antigen was monitored by measuring the interferon- $\gamma$  production of BDC-5.2.9 in response to chromatographic fractions in the presence of NOD antigen-presenting cells. Peak antigenic fractions were analyzed by ion-trap mass spectrometry, and candidate proteins were further investigated through peptide analysis and, where possible, testing of islet tissue from gene knockout mice.

**RESULTS**—Mass-spectrometric analysis revealed the presence of islet amyloid polypeptide (IAPP) in antigen-containing fractions. Confirmation of IAPP as the antigen target was demonstrated by the inability of islets from IAPP-deficient mice to stimulate BDC-5.2.9 in vitro and in vivo and by the existence of an IAPP-derived peptide that strongly stimulates BDC-5.2.9.

**CONCLUSIONS**—IAPP is the target antigen for the diabetogenic CD4 T-cell clone BDC-5.2.9. *Diabetes* 60:2325–2330, 2011

**C**D4 T cells are major immune players in the initiation and pathogenesis of type 1 diabetes, but the identification of the  $\beta$ -cell antigens that drive autoreactive T-cell responses has been a long and difficult quest. Although nearly 20 different proteins have been identified as targets for T cells in the NOD mouse, and at least 12 of these also are autoantigens in human patients (1), the impact of most of these proteins on the disease process is not well understood, particularly with regard to antigens for CD4 T cells. We previously have described islet-reactive CD4 T-cell clones that are representative of memory effector CD4 T cells arising during the progression of disease in the NOD mouse (2). In response to mouse islets from a variety of strains, clones from the panel produce Th1 inflammatory cytokines, especially interferon (IFN)- $\gamma$  (3), and are highly diabetogenic in vivo. The best-known T cell from this panel is BDC-2.5, the T-cell receptor of which was used to produce the BDC-2.5 T-cell receptor transgenic mouse (4), an animal used by

many investigators to probe mechanisms of pathogenic T-cell activity and regulation. The BDC T-cell clones initially were selected on the basis of their ability to respond to antigens in the form of whole islet cells, presented by NOD antigen-presenting cells. We recently identified the protein antigen for BDC-2.5 and two other T-cell clones (BDC-5.10.3 and BDC-10.1) as chromogranin A (ChgA), a secretory granule protein in islet  $\beta$ -cells (5). Here, we report that the antigen for another diabetogenic CD4 T-cell clone, BDC-5.2.9, is islet amyloid polypeptide (IAPP), a 37-amino acid peptide hormone cosecreted with insulin.

## RESEARCH DESIGN AND METHODS

**Mice.** NOD, NOD.*scid*, and NOD.RIP-Tag mice were bred and maintained in the Biological Resource Center at National Jewish Health. NOD.IAPP<sup>-/-</sup> mice were bred in our colony by backcrossing C57BL6.IAPP<sup>-/-</sup> mice (6) onto the NOD background; these mice have been fully backcrossed to the NOD mouse (10 generations). All experimental procedures were in accordance with the guidelines of the institutional animal care and use committee, National Jewish Health.

**Antigen purification.** NOD.RIP-Tag mice (7) were used as a source of  $\beta$ -cell tumors for the preparation of antigen; the enrichment of membrane proteins from  $\beta$ -cells isolated from NOD.RIP-Tag adenomas has been previously described (8). In brief, single-cell suspensions of  $\beta$ -cell adenomas were prepared by pressing whole-tumor tissue through a mesh screen, followed by several centrifugation steps to obtain a cellular membrane pellet; this pellet ( $\beta$ -Mem) is highly antigenic for the T-cell clones and serves as a positive control antigen in T-cell assays as well as the source of antigen for further purification. Membrane protein preparations were solubilized in detergent-containing buffer (20 mmol/L Tris, pH 8.0, and 1% octyl- $\beta$ -glucoside), followed by centrifugation at 18,400g (10 min at 4°C) to remove insoluble debris. Size-exclusion chromatography (SEC) was carried out on a Superdex 200 16/60 column (Amersham Biosciences) at 21°C (flow rate: 1 mL/min; fraction size: 1.25 mL; and injection volume: 2.0 mL), eluting with SEC buffer (20 mmol/L Tris, pH 8.0, and 150 mmol/L NaCl). Fractions from SEC were assayed for antigenic activity with the T-cell clones, and a peak antigenic fraction (950  $\mu$ L) was acidified with 20  $\mu$ L trifluoroacetic acid before adding 30  $\mu$ L acetonitrile. A total of 800  $\mu$ L of this mixture was then applied to a reverse-phase high-performance liquid chromatography (RP-HPLC) mRP-C18 high-recovery protein column (Agilent). A buffer gradient was used to elute proteins from the column, and a total of 36 fractions were collected between 0 and 72 min at a flow rate of 0.75 mL/min and a constant column temperature of 80°C. Antigenic peaks were again determined through assays with T-cell clones.

**Assays for antigen.** The antigenicity of islet cells, cellular and biochemical fractions, or peptides was assessed through the IFN- $\gamma$  responses of T-cell clones. In 96-well microtiter plates, assay cultures contained  $2 \times 10^4$  responder T cells,  $2.5 \times 10^4$  NOD peritoneal exudate cells as antigen-presenting cells, and  $\beta$ -cell antigen. IFN- $\gamma$  production by the responder T cells was determined by ELISA (BD Biosciences). IFN- $\gamma$  standards were added to the assay plates at concentrations between 0 and 100 ng/mL, but concentrations of the standard >50 ng/mL were above the assay detection range. An aliquot of  $\beta$ -Mem or whole NOD islet cells were used as positive controls; test wells contained SEC or RP-HPLC fractions, peptides, or islet cells. Organic solvents were removed from HPLC fractions by vacuum centrifugation prior to assay. Synthetic peptides were obtained from CHI Scientific.

**Mass spectrometry.** The protein-identification strategy we have previously described (5) was used for mass spectrometry. Proteins were digested with trypsin, and extracted peptides were resolved by chromatography online on a C18 column and a 1200 Series HPLC system (Agilent Technologies). Analysis was carried out with a 6340 LCMS ion-trap mass spectrometer in the National

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Jewish Health facility. Raw data were extracted and used to search the Swiss-Prot or National Center for Biotechnology Information databases with the Spectrum Mill search engine (Rev A.03.03.038 SR1; Agilent Technologies). Data were evaluated and protein identifications were considered significant if the following confidence thresholds were met: individual peptide scores of at least 10 and scored percent intensity of at least 70%. A reverse (random) database search was simultaneously conducted, and manual inspection of spectra was used to validate the match of the spectrum to the predicted peptide fragmentation pattern.

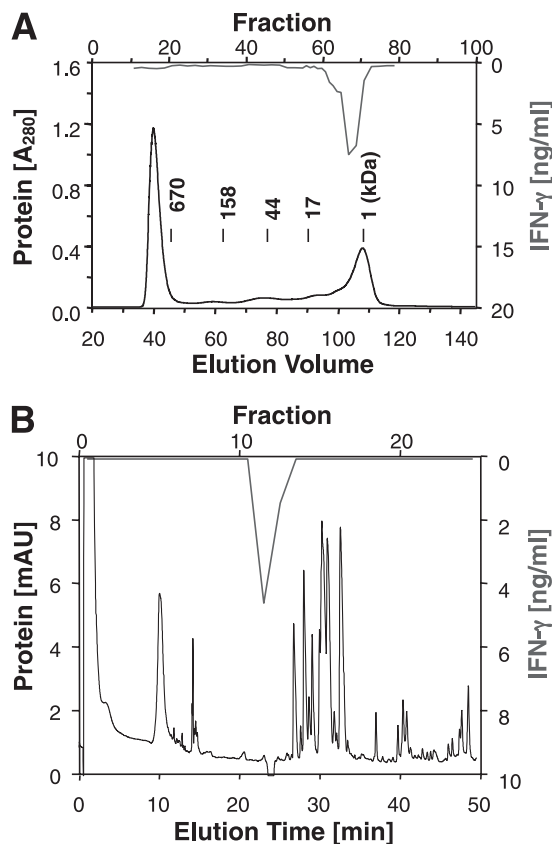
**Adoptive transfer of T-cell clones.** Cell cultures of BDC-2.5 and BDC-5.2.9 were expanded in interleukin-2, and after harvesting, T cells ( $1 \times 10^7$ ) were injected intraperitoneally into age-matched 6- to 14-day-old NOD or NOD.IAPP<sup>-/-</sup> recipient mice. Mice were monitored on a daily basis for urine glucose using Diastix (Bayer). The presence of urine glucose was followed by blood glucose monitoring using a OneTouch Ultra glucometer (LifeScan). Mice were considered diabetic when blood glucose levels were  $>18$  mmol/L (324 mg/dL). For disease-transfer experiments, a Wilcoxon rank sum test was used to determine statistical significance.

## RESULTS

**Purification and mass-spectrometric identification of IAPP in antigenic fractions.** As previously described (5), to identify candidate antigens for the T-cell clones, we carried out biochemical fractionation of  $\beta$ -cell tumors and mass spectrometric analysis of peak antigenic fractions. Similar to the other clones from the panel, BDC-5.2.9 responds *in vitro* to pancreatic islet cells or cell extracts of  $\beta$ -cell adenomas presented in the context of IA<sup>g7</sup> (2). Our starting source of antigen was  $\beta$ -cell tumor tissue excised from NOD.RIPTAg mice (7) and separated by differential centrifugation to yield a highly antigenic  $\beta$ -cell membrane pellet ( $\beta$ -Mem); this preparation also served as the positive control in T-cell activity assays. After lysis, the membrane pellet was separated by sequential chromatography, first through an SEC column and then by RP-HPLC. Illustrated in Fig. 1, the presence of antigen was tracked through the IFN- $\gamma$  response of the T-cell clone BDC-5.2.9 (*top axis*, gray lines) to protein (*bottom axis*, black lines) in individual fractions. Figure 1A is the chromatogram from SEC and shows a single antigenic peak that corresponds to a low-molecular weight protein region. The antigenic fractions from SEC were further purified by RP-HPLC, yielding another single peak of antigen activity (Fig. 1B).

The highly enriched and antigenic RP-HPLC fractions were subjected to *in-solution* tryptic digest and were then analyzed on an ion-trap mass spectrometer. Resulting spectra were sequenced and matched to proteins by searching the Swiss-Prot protein database using the search program Spectrum Mill. We identified a total of 21 proteins distributed over HPLC fractions 11–14 (Fig. 2A). Comparison of the antigen distribution profile with individual protein spectral-intensity values, an approximate indicator for relative protein abundances, singled out IAPP as the only candidate. Other proteins did not match the antigen distribution profile; for example, secretogranin 1 was detected only in non-antigenic fractions 11 and 14. We identified two IAPP peptides, representing 28% amino acid coverage of prepro-IAPP and 70% coverage of the 37-residue peptide hormone (Fig. 2B and C). The peptide LANFLVR was matched with high confidence and the peptide SNNLGPVLPPTNVGSNTY was matched with medium confidence.

**A peptide from IAPP contains a natural ligand for BDC-5.2.9.** To investigate the peptide(s) targeted by BDC-5.2.9, we analyzed a series of overlapping IAPP peptides, spanning the entire propeptide region (Fig. 3A). One peptide, KS20, elicited a vigorous response from BDC-5.2.9 and could stimulate the clone at concentrations as low as

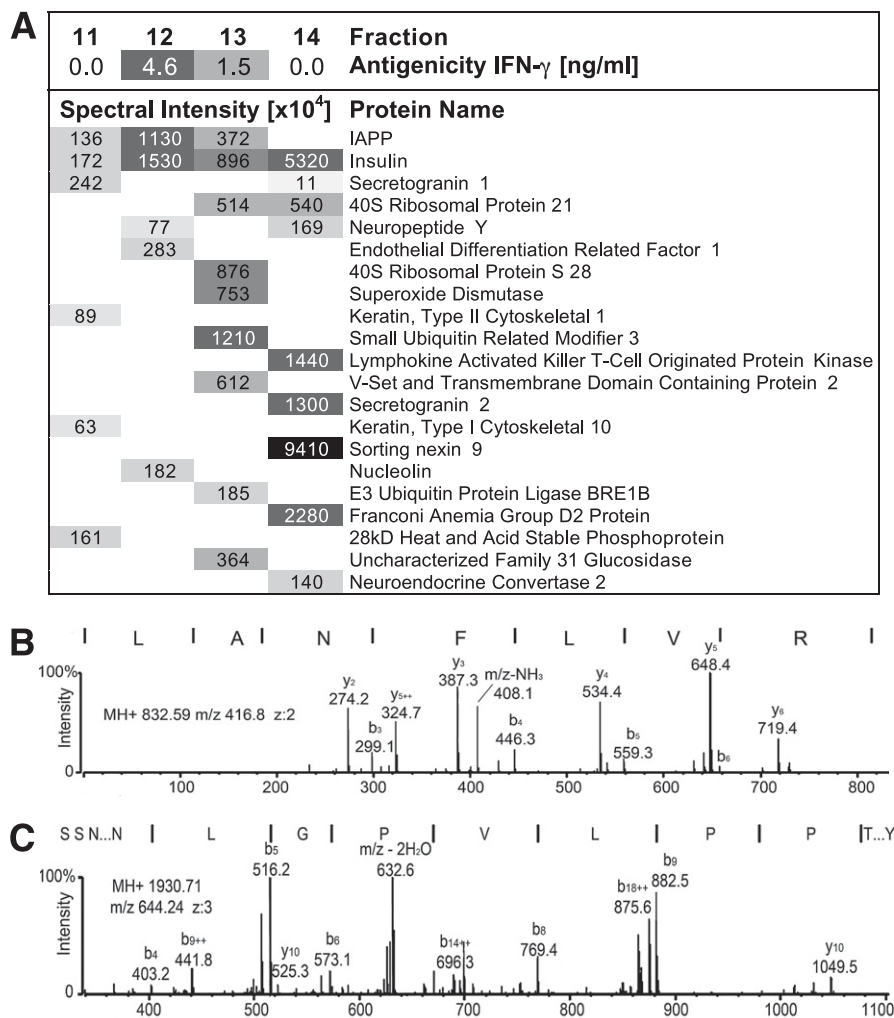


**FIG. 1.** Chromatographic purification of the antigen for the T-cell clone BDC-5.2.9. **A:** SEC of  $\beta$ -cell membrane lysate. **B:** RP-HPLC of antigenic SEC fraction no. 60. Protein elution was monitored by absorbance at 280 nm (black lines). Presence of antigen was determined by measuring the IFN- $\gamma$  response (gray lines) of the T-cell clone BDC-5.2.9 to small aliquots of individual fractions in the presence of peritoneal exudate cells used as antigen-presenting cells. Data are representative of at least four separate experiments.

10 ng/mL (4.6 nmol/L) (Fig. 3B). BDC-5.2.9 did not respond to any other IAPP peptide tested or to the insulin peptide B9–23. Two neighboring peptides, RT20 and GR20, share either the C- or N-terminus sequence with KS20, but neither of these peptides can stimulate BDC-5.2.9, indicating that the T-cell receptor of this clone recognizes the adjoining sequences. An insulin-reactive clone, PD-12.4.4 (9), was used as a negative control and showed no response to IAPP peptides. The fact that BDC-5.2.9 is stimulated by very low concentrations of the IAPP peptide KS20 suggests that this peptide may contain the natural ligand for BDC-5.2.9.

**Pancreatic islets lacking IAPP cannot activate BDC-5.2.9.** To further validate IAPP as the antigen for the BDC-5.2.9 T-cell clone, we analyzed IFN- $\gamma$  responses to islet cells from either NOD (IAPP<sup>+/+</sup>) or NOD.IAPP<sup>-/-</sup> mice. The T-cell clone BDC-2.5 was used as a positive control because it responds to a different  $\beta$ -cell antigen, ChgA (5). As illustrated in Fig. 4, both clones responded to antigen present in wild-type islets from NOD mice (IAPP<sup>+/+</sup>) (Fig. 4A), and the ChgA-reactive clone BDC-2.5 also responded to islets from NOD.IAPP<sup>-/-</sup> mice (Fig. 4B). In contrast, there was no stimulation of BDC-5.2.9 by IAPP-deficient islets (Fig. 4B), indicating that islets from IAPP<sup>-/-</sup> mice lack the antigen for this clone.

The highly aggressive nature of BDC-5.2.9 in disease induction can be demonstrated by adoptive transfer into



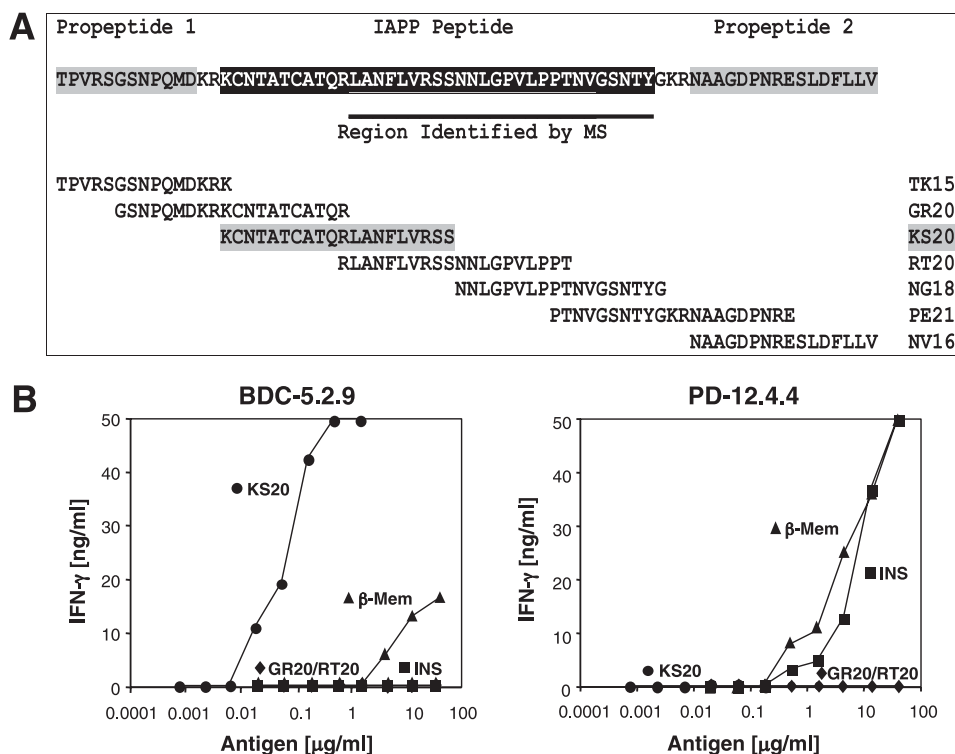
**FIG. 2.** Mass-spectrometric analysis of peak antigenic HPLC fractions. Antigenic chromatographic fractions were digested with trypsin, separated by RP-HPLC, and analyzed by ion-trap mass spectrometry; the resulting spectra were used to search a protein sequence database. **A:** Proteins identified (right column) in the highly purified antigenic RP-HPLC fraction no. 12 and neighboring fractions with lower antigen content (fractions 11, 13, and 14, as shown in Fig. 1B). The summarized spectral intensities (left) are indicative of the relative abundance of a specific protein in a fraction; darker shading indicates higher intensities. **B** and **C:** Ion-trap mass spectra matching the IAPP peptides LANFLVR (**B**) and SSNLG...P...V...L...P...P...T...Y (**C**) (SwissProt accession no. P12968). Data are representative of two separate experiments.

young NOD and NOD.*scid* mice. To investigate the in vivo islet reactivity of BDC-5.2.9 in IAPP-deficient mice, adoptive transfers with this clone were made into young (aged <2 weeks) IAPP<sup>-/-</sup> recipients. BDC-2.5 was again used as a positive control and rapidly induced disease in both IAPP<sup>+/+</sup> and IAPP<sup>-/-</sup> recipients (Fig. 5A). When BDC-5.2.9 was used in transfers, disease developed in most of the NOD.IAPP<sup>+/+</sup> mice within 3 weeks, but there was no diabetes in IAPP-deficient mice for the duration of the experiment, up to 45 days. We also conducted histological analysis of pancreatic sections from IAPP-deficient mice to assess leukocyte infiltration and granulation of islet  $\beta$ -cells (Fig. 5B). The spontaneous development of insulinitis and hyperglycemia in NOD.IAPP<sup>-/-</sup> mice is typical and very similar to that in NOD.IAPP<sup>+/+</sup> mice. Transfer experiments were terminated when recipients were ~2 months of age and the pancreatic histology of NOD.IAPP<sup>-/-</sup> mice receiving BDC-5.2.9 closely resembled that of aged-matched, uninjected IAPP<sup>-/-</sup> controls, with at least 60% of islets being well granulated and no infiltrate apparent. In contrast, histology from IAPP<sup>-/-</sup> mice that received the BDC-2.5 T-cell clone showed complete degranulation of  $\beta$ -cells.

These results further demonstrate that islets lacking IAPP do not contain the antigen for BDC-5.2.9.

## DISCUSSION

The activation of autoreactive CD4 T cells through T-cell receptor responses to peptide/major histocompatibility complexes is a major event in autoimmune diseases such as type 1 diabetes. The identification of  $\beta$ -cell antigens, and the peptide epitopes from these antigens, could have important implications for both diagnosis and therapy of type 1 diabetes. Our results reported here demonstrate that IAPP is now the third secretory granule protein in islet  $\beta$ -cells, after insulin and ChgA, to be targeted by pathogenic CD4 T cells, and it is possible that other secretory granule proteins (e.g., other secretogranins) also could be antigenic targets in type 1 diabetes. Similar to insulin, IAPP is uniquely expressed within the pancreatic  $\beta$ -cells. This peptide hormone is cosecreted with insulin from the  $\beta$ -cell granules at a molar ratio of ~1:100 IAPP:insulin (10). Similar to insulin and ChgA, IAPP is expressed as a larger precursor molecule (pro-IAPP), which is then posttranslationally



**FIG. 3.** BDC-5.2.9 responds to a natural peptide sequence from IAPP. **A:** Murine pro-IAPP amino acid sequence. The hormone peptide is highlighted (white lettering on black background) and the region identified by ion-trap mass spectrometry is underlined. Listed below are the peptide sequences synthesized for activity measurements. Antigenic peptide KS20 is highlighted. **B:** IFN- $\gamma$  responses of BDC-5.2.9 and the insulin-reactive clone PD-12.4.4 to various concentrations of the peptide antigens IAPP KS20 (●), GR20/RT20 (◆), INS B9-23 (■), or the  $\beta$ -cell membrane ( $\beta$ -Mem) positive control (▲). Although BDC-5.2.9 did not respond at all to the insulin peptide B9-23 or the IAPP-peptides GR20 and RT20, it responded to IAPP KS20 at concentrations as low as 0.01  $\mu$ g/mL. The insulin-reactive T-cell clone PD-12-4.4 responded to INS B9-23 and to the  $\beta$ -Mem control but not to any IAPP peptides. Data are representative of at least three separate experiments.

processed within the secretory granules by the enzymes prohormone convertase 2 and carboxypeptidase E (11).

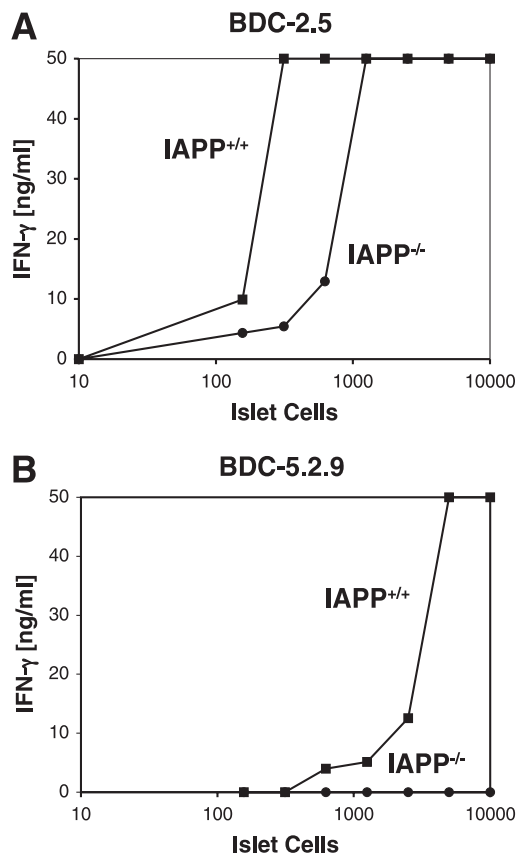
IAPP is well known as a component of the amyloid plaques found in the pancreatic islets of patients with type 2 diabetes (12–14). In type 1 diabetes, there has been documentation of autoantibodies to IAPP in patients, but there was no correlation to disease (15). To date, there has been little association of IAPP with type 1 diabetes. One major exception was the report from Panagiotopoulos et al. (16) in which it was found that a significant proportion of CD8 T cells from HLA-A\*0201 patients with recent-onset type 1 diabetes respond to a peptide derived from the leader sequence of prepro-IAPP. This group also contributed to a later study in which it was found that a second sequence of prepro-IAPP (ppIAPP 9–17) elicited responses from cytotoxic T-lymphocytes of type 1 diabetic patients (17). The other indicator that IAPP might have a role in type 1 diabetes, and the first evidence from the NOD mouse, was a genetic mapping study in which we reported linkage of the *iapp* gene with the antigen locus for a CD4 T-cell clone BDC-6.9 (18). Although we have not identified an IAPP peptide that stimulates this T-cell clone, strong corroborating evidence for IAPP as its antigen target was provided by islets from the NOD.IAPP<sup>-/-</sup> mouse. As observed with the BDC-5.2.9 clone, IAPP-deficient islets were unable to activate BDC-6.9 (K.H., unpublished data). In our new report, we have provided definitive evidence that diabetogenic CD4 T cells in the NOD mouse react to IAPP as an antigen, including the identification of a peptide containing a natural ligand for one of these T cells. The

sequence of secreted IAPP is quite similar between mouse and human forms of the molecule, and there is only one amino acid difference between the KS20 mouse and human sequences. It is not unreasonable, therefore, to assume that human CD4 T cells also may react to IAPP peptides presented, for example, by HLA-DQ8.

A major function of secretory granule proteins is to participate in the biogenesis of the secretory granules and in the process, these proteins tend to form aggregates (19,20). IAPP is of particular interest in this regard because of its propensity to form amyloid deposits in type 2 diabetic patients. Such deposits are not found in type 1 diabetic patients, but it has been demonstrated by others that the initial step in amyloid deposition is the formation of toxic IAPP oligomers (21). Soluble oligomers of IAPP also have been shown to be responsible for activation of the NLRP3 inflammasome that leads to increased interleukin-1 $\beta$  production in type 2 diabetes (22). In type 1 diabetes, similar smaller aggregates of IAPP could form under conditions of inflammatory stress. It is quite conceivable that such oligomers would be taken up by antigen-presenting cells and processed in a way that leads to the formation of neoantigenic epitopes.

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**FIG. 4.** Islets from IAPP<sup>-/-</sup> mice cannot stimulate BDC-5.2.9. IFN- $\gamma$  response of BDC-2.5 (ChgA reactive) (A) and BDC-5.2.9 (B) to various numbers of islet cells obtained from NOD.IAPP<sup>+/+</sup> (■) and NOD.IAPP<sup>-/-</sup> (●) mice. Data are representative of three separate experiments, in which each cell concentration was measured in duplicate.

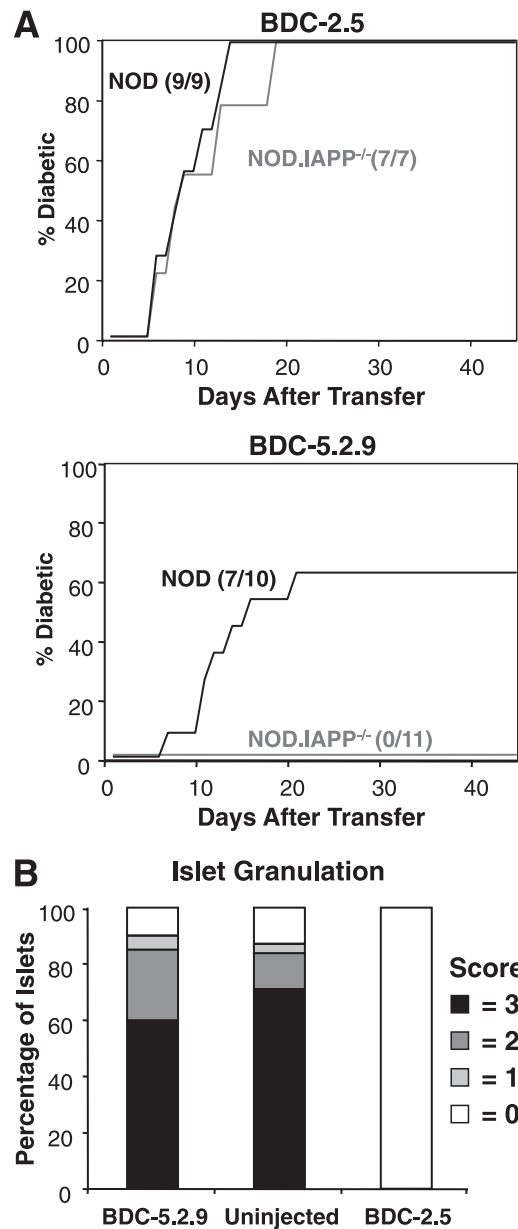
National Center for Research Resources (S10RR023703) to N.R., and NIH grant UL-1-RR-025780 to N.R. Islets were obtained from the DERC Islet Core at the Barbara Davis Center, supported by NIH grant P30-DK-057516.

No potential conflicts of interest relevant to this article were reported.

T.D. performed the biochemical purification and antigen analysis and wrote the manuscript. R.L.B. performed disease transfer experiments and analyzed histology. N.R. and R.R. supervised the mass spectrometry and contributed to discussion. R.L.P. and M.A. assisted with mass spectrometry and HPLC. G.B. maintained and assayed T-cell clones. B.B. runs the mouse colony and contributed to *in vivo* experiments. K.H., as the principal investigator, held overall responsibility and oversight of the project and wrote and edited the manuscript.

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**FIG. 5.** BDC-5.2.9 transfers disease only when its antigen is present. **A:** BDC-2.5 or BDC-5.2.9 T cells ( $1 \times 10^7$ ) were injected intraperitoneally into 6- to 14-day-old NOD or NOD.IAPP<sup>-/-</sup> mice, which were monitored for hyperglycemia. NOD or NOD.IAPP<sup>-/-</sup> mice that received the BDC-2.5 T-cell clone became diabetic within 3 weeks of transfer. BDC-5.2.9 transferred disease in NOD mice but not in NOD.IAPP<sup>-/-</sup> mice ( $P = 0.002$ ). Data summarize at least three independent experiments for each clone and indicate that BDC-5.2.9 is diabetogenic upon adoptive transfer in NOD mice only when IAPP is expressed in the islets. **B:** Pancreatic sections were stained with aldehyde/fuchsin for histological analysis. Islets in sections of NOD.IAPP<sup>-/-</sup> mice that received BDC-5.2.9 showed no infiltrate and were well granulated; in contrast, islets from mice that received BDC-2.5 were infiltrated and the islets were degranulated. Histology was based on two mice per group for each of the following groups: IAPP<sup>-/-</sup> mice injected with BDC-5.2.9; 3-month-old uninjected IAPP<sup>-/-</sup> mice; and IAPP<sup>-/-</sup> mice injected with BDC-2.5. The bar graph summarizes the islet granulation scores: 3 = well-granulated islet  $\beta$ -cells; 2 = 50-75% granulated; 1 = 20-50% granulated; and 0 = no granulation.

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