Brief Definitive Report

Cytotoxic T Cells Specific for Glutamic Acid Decarboxylase in Autoimmune Diabetes

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Summary

Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease that results in the destruction of the pancreatic islet β cells. Glutamic acid decarboxylase (GAD) has been recently indicated as a key autoantigen in the induction of IDDM in nonobese diabetic mice. In human diabetes, the mechanism by which the β cells are destroyed is still unknown. Here we report the first evidence for the presence of GAD-specific cytotoxic T cells in asymptomatic and recent diabetic patients. GAD65 peptides displaying the human histocompatibility leukocyte antigen (HLA)-A*0201 binding motif have been synthesized. One of these peptides, GAD114-123, binds to HLA-A*0201 molecules in an HLA assembly assay. Peripheral blood mononuclear cells from individuals with preclinical IDDM, recent-onset IDDM, and from healthy controls were stimulated in vitro with the selected peptide in the presence of autologous antigen-presenting cells. In three cases (one preclinical IDDM and two recent-onset IDDM), we detected specific killing of autologous antigen-presenting cells when incubated with GAD114-123 peptide or when infected with a recombinant vaccinia virus expressing GAD65. These patients were the only three carrying the HLA-A*0201 allele among the subjects studied. Our finding suggests that GAD-specific cytotoxic T lymphocytes may play a critical role in the initial events of IDDM.

Insulin-dependent diabetes mellitus (IDDM) is caused by pathological T cell responses that lead to the destruction of β cells in the pancreatic islets. Recent studies suggest that MHC class II–restricted $CD4^+$ T cells specific for peptides derived from glutamic acid decarboxylase (GAD65) play a crucial role in the initial phase of IDDM in nonobese diabetic (NOD) mice (1, 2). GAD-specific $CD4^+$ T cells have also been observed in recent-onset IDDM patients and in relatives of IDDM patients at risk to develop diabetes (3). In addition to $CD4^+$, $CD8^+$ T cells have also been shown to be involved in the pathogenesis of the disease. Induction of diabetes in adoptive transfer experiments requires both $CD4^+$ and $CD8^+$ T cells (4, 5). In addition, $CD8^+$ T lymphocytes were the predominant cells in the inflamed islets of an acutely diabetic patient (6).

In this study we demonstrate that MHC class I HLA-A*0201–restricted $CD8^+$ CTLs specific for a GAD peptide (GAD114–123) are present in the peripheral blood of subjects with recent-onset IDDM and at high risk to develop IDDM. This peptide is generated by natural processing of human GAD65. GAD-specific CTLs were not found in healthy individuals expressing HLA-A*0201. These findings argue for a critical role of GAD-specific CTLs in the initial events of IDDM.

Materials and Methods

Peptide Synthesis. Nine peptides conforming to the A*0201 motif were identified and synthesized by the solid-phase peptide methodology using a synthesizer (Fmoc/tBu chemistry) (431A; Applied Biosystems, Inc., Foster City, CA). Crude peptides were purified by reverse-phase HPLC. The identities of the purified peptides were confirmed by amino acid analysis and fast atom bombardment mass spectrometry. Peptides were dissolved in DMSO at a concentration of 2 mM.

Subjects. Preclinical IDDM subjects (2 female, 2 male, mean age 29.5 yr, range 13–56), defined operationally as asymptomatic, were first-degree relatives of patients with IDDM and were found to have islet cell antibodies at a level ≥20 Juvenile Diabetes Foundation U. Recent-onset IDDM subjects (14 female, 8 male, mean age 25.4 yr, range 14–44) were insulin dependent, having presented with typical clinical features of IDDM in the previous 2 wk. Con-

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trol subjects were normal volunteers (4 female, 2 male, mean age 32.5 yr, range 26–45). Peripheral blood was taken after informed consent was obtained.

HLA Typing. All of the individuals analyzed were initially HLA-typed by standard serological techniques (7). To those found to be HLA-A2$^+$ (11 out of 26), the A2 subtypes were assigned according to the hybridization pattern obtained with subtype-specific probes after specific amplification of the A2 locus (8).

HLA Assembly Assay. The T2 cells were washed twice in serum-free medium. 1 × 10^5 cells in 40 μl serum-free medium were put into a U-bottom 96-well plate together with 10 μl of the peptide at a final concentration of 100 μM and 15 μg/ml of human β_2-microglobulin (Sigma Chemical Co., St. Louis, MO). After overnight incubation at 37°C, the cells were washed once and stained by indirect immunofluorescence. The HLA-A2.1–specific mAb BB7.2 (50 μl of hybridoma supernatant) was added for 30 min on ice. The cells were washed and 10 μl FITC-labeled goat anti–mouse Ig (Becton Dickinson and Co., Cockeysville, MD) was added. After 30 min on ice, the cells were washed, and fluorescence was measured on a FACScan® flow cytometer (Becton Dickinson and Co.).

Generation of GAD-specific Cytotoxic T Cell Lines. Peripheral blood lymphocytes were separated from whole blood and incubated at 2 × 10^6 cells/ml in a 24-well plate together with autologous adherent cells (obtained by a 2-h adherence) that had been pulsed overnight with 100 μM of peptides that had bound to HLA-A2 in the HLA assembly assay. The influenza matrix peptide M58-66 was included as control. On day 5, 10 U/ml human recombinant IL-2 (Hoffmann-La Roche, Inc., Nutley, NJ) was added, and CTL assay was done on day 8. ^{51}Cr-labeled PHA-blasts or EBV-B cells were preincubated for 1 h with 25 μM of peptide and without peptide, and a 4-h assay was done as described (9). Assays were done at effector to target cell ratios ranging from 100:1 to 1:1.

Construction of a Recombinant Vaccinia–expressing GAD. For the production of GAD65 recombinant vaccinia, a full-length human GAD65 cDNA was generated from two overlapping cDNA fragments obtained from A. Lernmark (University of Washington, Seattle, WA). A 5′ GAD fragment (contained in clone 11) comprising the translation initiation codon was amplified in 20 cycles using the primers (sense) 5′.CTGGATCCAACCATGGCATCTCCGGGCTCTG.3′ and (antisense) 5′.GTTGGTCTGCCAATTCCCAATTA.3′. The PCR product was digested with BamHI and PstI, and the resulting 260-bp fragment was used to replace the corresponding fragment in clone 11. After transfer of a 3′ XbaI/BglII fragment from clone 1.9 (containing the 3′ fragment of GAD cDNA) into Bluescript SKII+ (Stratagene, Inc., La Jolla, CA), the XbaI site in the resulting plasmid was opened, blunt-ended, and converted into an EcoRI site by linker ligation. The plasmid was then digested with BglII/KpnI and ligated in a triple ligation to a KpnI/NsiI 5′ fragment from the modified clone 11 and to a central NsiI/BglII fragment from clone 1.9. To create a suitable transfer vector for the full-length cDNA, the vaccinia virus vector pSC65 (obtained from Dr. B. Moss, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) was modified by insertion of an oligonucleotide containing SpeI, NotI, and ScaII sites between the PacI and KpnI sites in the polylinker. GAD cDNA was inserted into modified pSC65 as a KpnI/NotI fragment. The construction of recombinant vaccinia virus controlling GAD65 expression was performed using standard methods. To verify expression of the human GAD65 protein by human cells infected with recombinant Vacc/hGAD, immunoblotting and immunoprecipitation experiments with the mAb GAD6 were performed. Human EBV-transformed B cells infected with Vacc/hGAD 24 h before analysis expressed large amounts of a recombinant 65-kD protein that could be precipitated by GAD6 and also reacted with GAD6 in Western blots. No GAD6 immunoreactivity was detected in control cells that had been infected with a control virus (Vacc/TAP1) that contained the human TAP1 gene. Vacc/TAP1 had been constructed similarly to Vacc/hGAD, using modified pSC65 and the vaccinia strain WR (P. M. Van Endert, unpublished results). The cloning of the human TAP1 cDNA is described elsewhere (10). EBV-B cells at 3 × 10^6 cells/ml were infected with Vacc/hGAD or Vacc/TAP for 2 h at 37°C at a multiplicity of 5 PFU/cell in medium containing 2.5% FCS. The cells were then washed twice and let to stand overnight at 37°C. The next day, cells were pelleted and labeled with 50 μl of ^{51}Cr. CTL assay was performed as described above.

IFN-γ Production. IFN-γ production was evaluated by a solid-phase sandwich immunoenzymatic assay as described (11).

Results and Discussion

GAD65 is highly expressed in the cytoplasm of pancreatic β cells (12). GAD-derived peptides, therefore, may be presented by MHC class I molecules and recognized by CD8$^+$ T cells on the surface of β cells. To test this possibility, we first determined the potential of GAD-derived peptides to bind to HLA-A*0201, an MHC class I molecule with well-characterized peptide binding properties (13–15). HLA*0201 molecules bind peptides that are 9 or 10 residues long and contain 2 major anchor residues: either leucine, isoleucine, or methionine at position two, and a hydrophobic aliphatic residue at the COOH terminus. Secondary anchor positions were also identified at positions one, three, and seven. In human GAD65, nine peptides corresponding to the proposed motif were identified. These peptides were synthesized and tested for binding by an HLA assembly assay using the T2 cell line (16, 17).

T2 cells showed HLA*0201 expression after incubation with peptide GAD114-123 and with the influenza peptide M58-66 used as a positive control (Fig. 1). We therefore added GAD114-123 peptide to cultures containing PBMC from four individuals with preclinical IDDM, including one expressing A*0201; 22 diabetic patients, including two expressing A*0201; and six healthy A*0201$^+$ donors. 8 d later the cultures were tested for cytolytic activity against peptide-pulsed autologous targets. Peptide-specific cytotoxicity was observed in cultures containing cells from the three A*0201$^+$ individuals with preclinical IDDM and recent-onset IDDM, but not in any other culture. The data shown in Fig. 2 are representative of results obtained with GAD114-123–stimulated cultures from one subject with preclinical IDDM and one patient with recent-onset IDDM. The CTL activity observed from a second patient with recent-onset IDDM was lower but clearly detectable (13% specific lysis at an effector to target ratio of 10:1, 9% at 3:1, and 6% at 1:1) (data not shown). The CTL activity was GAD114-123–specific, since autologous targets incubated with an A*0201-binding control peptide were not lysed (Fig. 2, *A* and *B*). As expected, the peptide recognition was A*0201 restricted, since killing was inhibited by an A*0201-specific mAb (not shown), and, in à panel of EBV-B cell lines, only A*0201$^+$ cells were lysed (Fig. 2, *C* and *D*).

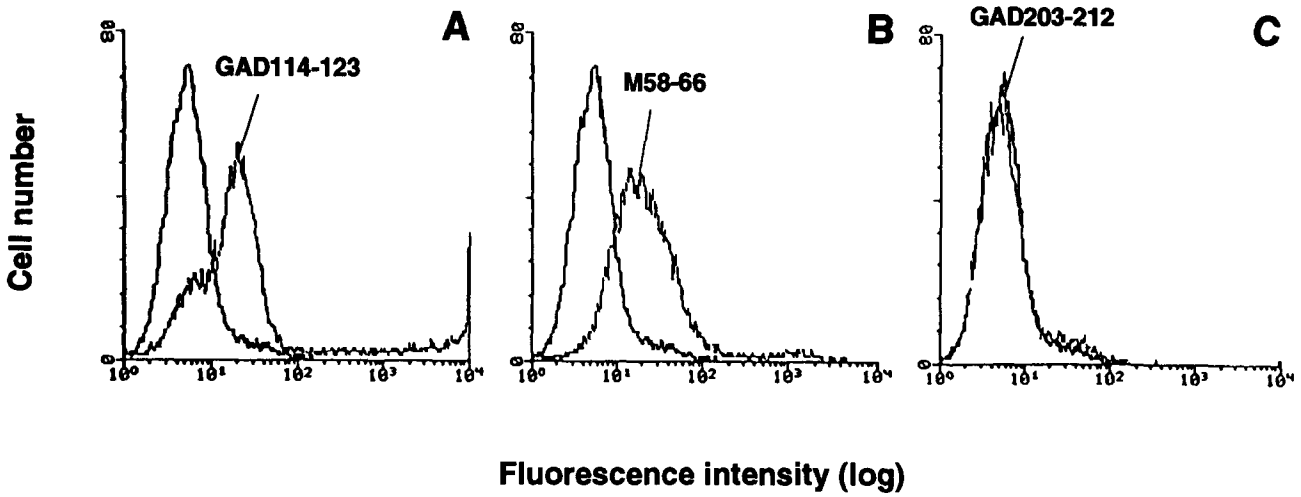

Figure 1. Induction of surface HLA-A2 expression in T2 cells. Aliquots of T2 cells (2×10^5) were incubated overnight at 37°C in medium containing 1% DMSO or in medium containing 0.5 μM β_2-microglobulin and (*A*) 100 μM GAD114-123 peptide (sequence: VMNILLQYVV); (*B*) 100 μM M58-66 peptide (sequence: GILGFVFTL); or (*C*) 100 μM GAD203-212 peptide (sequence: NMFTYEIAPV). Surface HLA-A2 expression was detected by FACS® analysis of cells stained with the A2.1-specific mAb BB7.2 (ATCC HB 82). The logarithm of fluorescence intensity is plotted on an arbitrary scale from 1 to 80. One representative experiment out of five is shown.

All of the individuals analyzed were initially HLA-typed by standard serological techniques. To those found to be HLA-A2$^+$ (11 out of 26), the A2 subtypes were assigned according to the hybridization pattern obtained with subtype-specific probes after specific amplification of the A2 locus. To date, 11 subtypes of HLA-A2 have been defined, differing from one another in one to six amino acid residues. Most of the variations are located in the peptide-binding cleft and scattered in the six peptide side chain–binding pockets. With the oligonucleotide probes we used, we were unable to distinguish between the two closely related subtypes A*0201 and A*0206. However, A*0201 only differs from A*0206 by the conservative substitution F → Y at position 9. Previous analysis of peptide binding between some of the HLA-A2 subtypes has indicated that a broad cross-reactivity exists between HLA-A*0201 and A*0206 (18).

An important issue is whether the GAD114-123–specific CTLs can recognize target cells expressing naturally processed native GAD65. Since A*0201-expressing islet cell lines are not available, we infected autologous EBV-B cell lines with a recombinant vaccinia virus expressing the GAD65 gene (Vacc/hGAD). As shown in Fig. 2 (*A* and *B*), the Vacc/hGAD-infected targets were lysed. No lysis was observed with control targets infected with a recombinant vaccinia virus expressing

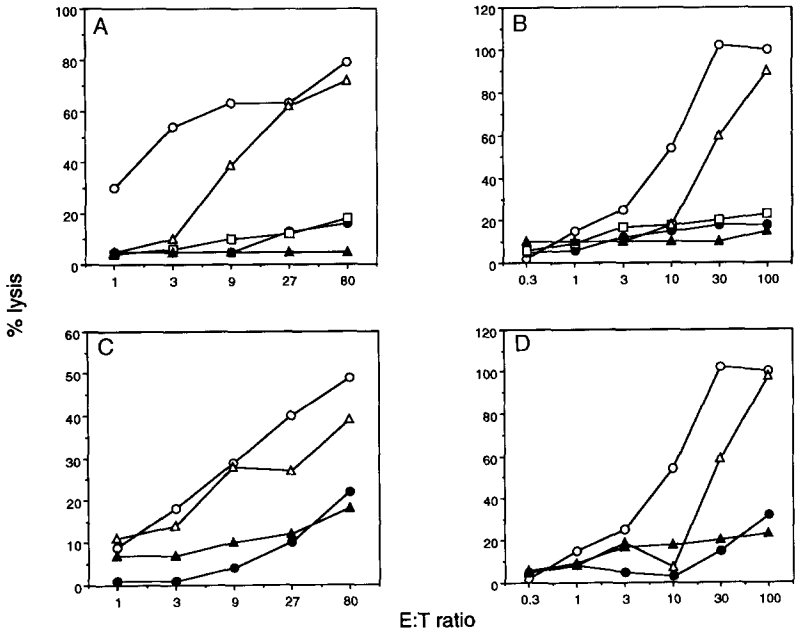

Figure 2. In vitro lytic activity of CTL from one subject with preclinical IDDM (*A* and *C*) and one patient with newly diagnosed IDDM (*B* and *D*). (*A* and *B*) Cells from both subjects showed cytolytic activity toward autologous PHA-blasts when incubated with GAD114-123 peptide (○), but not when incubated with medium alone (□) or with M58-66 peptide (●). Autologous PHA-blasts infected with a recombinant vaccinia virus that express human GAD65 were also lysed (△). No cytolysis was observed when autologous PHA-blasts were infected with a recombinant vaccinia virus that expresses the human transporter TAP-1 (▲). (*C* and *D*) Cells from both subjects showed cytolytic activity toward autologous EBV-B cells (○, *C*), autologous PHA-blasts (○, *D*), and A*0201$^+$ EBV-B cell line BSM (△) pulsed with GAD114-123 peptide, but not towards the EBV-B cell lines SWEIG (HLA-A29) (●), DKB (HLA-A24) (▲, *D*), or FC (HLA-A1,3) (▲, *C*) pulsed with GAD114-123 peptide. In both cell lines, >80% of the T cells were CD8$^+$, as revealed by FACS® analysis.

Table 1. *IFN-γ Production by GAD114-123-specific CTLs**

Ag	IFN-γ (pg/ml)	
	Pre-IDDM	Early IDDM
none	≤6	≤6
GAD114-123	520	170
GAD-VAC	155	60
GAD203-212	≤6	≤6

* GAD114-123–specific CTLs described in Fig. 2 were incubated in the presence of irradiated autologous EBV-B cells that had been pulsed with peptide GAD114-123 or peptide GAD203-212 or infected with Vacc/hGAD.

an irrelevant control gene (Vacc/TAP1). This indicates that GAD114-123 peptide is generated by the processing of the GAD protein and represents a natural dominant epitope.

Our finding of $CD8^+$ GAD-specific cytolytic T cells in asymptomatic as well as in early diabetic patients suggests that $CD8^+$ T cells are involved in the initial stages of the diabetogenic process. Interestingly, the GAD-specific CTLs seem to be activated only in the early development of the disease, perhaps as early as $CD4^+$ T cell responses. This is suggested by the fact that we have not been able to isolate CTLs specific for GAD from peripheral blood of the same subjects collected 2 and 6 mo after the onset of diabetes (data not shown).

MHC class II–restricted T cells are thought to play a pivotal role in the pathogenesis of IDDM. This view is supported by several lines of evidence. First, IDDM only occurs in mice and humans expressing certain MHC class II alleles (19). Second, the disease can be transferred by a $CD4^+$ T cell clone (20). Third, the incidence of IDDM is increased in NOD mice expressing a transgenic TCR cloned from a diabetogenic $CD4^+$ T cell line (21). Fourth, IDDM does not occur in NOD mice in which T cell tolerance has been induced by intravenous or intrathymic injection of GAD (1, 2). None of these findings, however, exclude a pathogenic role of $CD8^+$ cytotoxic T cells. Indeed, experiments involving the transfer of T cell subsets into neonatal (4) or athymic NOD mice (5), as well as the depletion of T cell subsets by the in vivo administration of antibodies (for review see reference 22), do indicate that both $CD4^+$ and $CD8^+$ T cells are involved in the development of IDDM. Furthermore, these and other reports (23–27) suggest that class I–restricted recognition is as important as class II–restricted recognition.

The CTLs described here kill GAD-expressing cells and may thus contribute to the development of IDDM by killing β cells. In addition to their cytolytic potential, GAD-specific CTLs produced IFN-γ in response to GAD114-123 peptide-pulsed, as well as to Vacc/hGAD-infected, HLA-A*-0201–expressing cells (Table 1). The early production of IFN-γ might play a role in promoting the development of type I diabetes. Indeed, it has been shown that expression of the gene encoding IFN-γ under the control of the insulin promoter resulted in inflammation in the islets and diabetes in transgenic mice (28). Furthermore, IFN-γ might have an important immunoregulatory role in vivo by promoting the development of autoreactive $CD4^+$ T cells able to induce IDDM in genetically susceptible individuals (29). In this context, IFN-γ has been shown to be a strong inducer of Th1 responses while inhibiting the differentiation and effector function of the Th2 cells. Evidence suggesting a pathogenetic role for Th1 cells in IDDM has recently been provided by studies in NOD mice (30–32).

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