Differential Presentation of Glutamic Acid Decarboxylase 65 (GAD65) T Cell Epitopes Among HLA-DRB1*0401-Positive Individuals

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Glutamic acid decarboxylase 65 (GAD65) is one of the major autoantigens in type 1 diabetes. We investigated whether there is variation in the processing of GAD65 epitopes between individuals with similar HLA backgrounds and whether the processing characteristics of certain immunogenic epitopes are different in distinct APC subpopulations. Using DR401-restricted T cell hybridomas specific for two immunogenic GAD65 epitopes (115–127 and 274–286), we demonstrate an epitope-specific presentation pattern in human B-lymphoblastoid cell lines (B-LCL). When pulsed with the GAD protein, some DRB1*0401-positive B-LCL, which presented GAD65 274–286 epitope efficiently, were unable to present the GAD65 115–127 epitope. However, all B-LCL presented synthetic peptides corresponding to either GAD epitope. In addition, when pulsed with human serum albumin, all cell lines gave equal stimulation of a DR4-restricted human serum albumin-specific T hybridoma. GAD65-transfected cell lines displayed the same presentation phenotype, showing that lack of the presentation of the 115–127 epitope was not due to inefficient uptake of the protein. Blood mononuclear adherent cells, B cells, or dendritic cells derived from the same individual displayed the same presentation pattern as observed in B cell lines, suggesting that the defect most likely is genetically determined. Therefore, individual differences in Ag processing may result in the presentation of distinct set of peptides derived from an autoantigen such as GAD65. This may be an important mechanism for the deviation of the immune response either into a regulatory pathway or into an inflammatory autoimmune reaction. The Journal of Immunology, 1999, 163: 1674–1681.

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3 Abbreviations used in this paper: GAD65, glutamic acid decarboxylase 65; B-LCL, B-lymphoblastoid cell line; HSA, human serum albumin; IDDM, insulin-dependent diabetes.

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B10.M/DR4 transgenic mice (11). It is interesting to note that epitopes overlapping with the ones in this study have been shown to be recognized also by human T cells. Endl et al. (6) described reactivity for GAD65 peptide 266–285, with T cells from a newly diagnosed DR401-positive IDDM patient. Another group demonstrated a cytotoxic HLA-A*0201-restricted T cell response for GAD 114–123 epitope naturally processed in target cells in one prediabetic and two IDDM subjects (14). In this study, we report a differential Ag presentation pattern for these two epitopes among APCs derived from DRB1*0401-positive individuals.

Materials and Methods

Cells

B-LCL were the following: BpH (DRB1*0401, 0301), BrDe (DRB1*0401, 0701), EmWi (DRB1*0401, 0301), JeBa (DRB1*0401, 0801), RePo (DRB1*0401, 0301), Bsm (DRB1*0401 homozygous), and Pries (DRB1*0401 homozygous). The cells were obtained from diabetic patients (BpH, BrDe, JeBa), subjects positive for autoantibodies against multiple β cell Ags (EmWi, RePo), and normal subjects (Bsm, Pries). The first two groups were also positive for GAD65 autoantibodies. Three of these individuals (EmWi, JeBa, RePo) were reactive for GAD65 peptide 266–285, with T cells from a newly diagnosed T cell hybridoma T33.1 specific for GAD65 recombinant retrovirus-producing cell lines and infection of B-lymphoblastoid cells

GAD65-expressing B cell lines were generated by retroviral-mediated gene transfer, as previously described (17). Briefly, GAD65-cDNA fragment was cloned into the parental retroviral construct plNCLC6. GAD protein-producing cell lines were generated by introduction of the retroviral constructs into the amphotrophic packaging line PE501. Next day supernatants from transduced PE501 cells were used to infect PG13 fibroblasts in the presence of polybrene (4 µg/ml) and 10% FCS. Infected PG13 fibroblasts were selected under a selective medium containing G418 (1 mg/ml), isolated, expanded, and determined for a viral titer. Virus-producing cells (5 × 10^6) were plated and cocultivated with B-lymphoblastoid cells (2 × 10^6) in 60-mm plates in the presence of polybrene (4 µg/ml). The nonadherent B cells were harvested after 24 h of coculture and were grown for another 24 h before placing them in a selective medium containing G418 (1 mg/ml). GAD65 expression was detected in a Western blot and RIA (18).

T cell proliferation assay

In some experiments, B-LCLs were first fixed with 1% paraformaldehyde in PBS for 10 min at room temperature, and washed three times with PBS with 1% FCS. In the protease inhibitor assay, APCs were pretreated with leupeptin (40–200 µg/ml) or pepstatin A (10–50 µg/ml) overnight at 37°C. Otherwise, B-LCL or primary APCs were pulsed with human GAD65 (3–25 µg/ml), HSA protein (0.375–6 mg/ml), or peptides GAD5 p115–127 or p274–286 (0.01–10 µM) for 4–12 h. APCs were washed after preincubation with Ag to avoid possible degradation or toxicity of the protein during a long incubation period, after which the cells were irradiated and plated onto 96-well microtiter plate at density of 5 × 10^3/well. Dendritic cells were used at a density of 5000/well. T cell hybridoma cells were added at 10^4/well, and incubated for 24 h. IL-2 production of T hybridomas was determined by a proliferation assay performed in HT-2 cells. The proliferation of HT-2 cells reached a plateau at 2–2.5 U/ml corresponding to 3.5–4 × 10^3 cpm of thymidine incorporation.

Results

Differential presentation of GAD65 epitopes by DRB1*0401-positive B-LCLs

Three DR401-positive B-LCLs pulsed with intact rGAD65 protein efficiently processed and presented both GAD 115–127 and 274–286 epitopes, but four other DR401 B-LCLs displayed an epitope-specific presentation pattern: the GAD 274–286 epitope was efficiently presented to the T33.1 hybridoma, whereas no stimulation of the T cell hybridoma T35 specific for GAD 115–127 was detected (Fig. 1, A and B). The level of proliferation of the hybridomas was dependent on the GAD65 concentration. When 25
mg/ml GAD65 was used to pulse the APCs, both hybridomas were efficiently stimulated. HLA-DRB1*0401 homozygous cell lines (Priess and Bsm) presented GAD65 274–286 epitope well at lower concentration than the heterozygous cell lines. Similarly, GAD65 115–127 was most efficiently presented by Priess, in contrast to Bsm, which displayed the presentation-deficient phenotype at all concentrations tested.

One possible explanation for the deficient presentation phenotype might have been a lack of essential factors in the processing machinery. This was investigated by testing the capacity of these cell lines to present another protein Ag, exogenously added HSA (Fig. 1C). All cell lines presented the HSA Ag equally well at several concentrations between 1–6 mg/ml, excluding the possibility of a general inability of processing exogenous Ags. Fixed cells did not present GAD protein to both T-35 and T-33.1 hybridomas, indicating that the presentation of epitopes from GAD65 protein required intracellular processing (data not shown).

GAD65 peptides are presented by all B-LCLs

All B cell lines (fixed and unfixed) efficiently presented peptides corresponding to the particular epitopes, demonstrating that the peptide-DR-401 complex can interact with the specific hybridoma and induce stimulation (Fig. 2). DR-401 homozygous cell lines (Priess and Bsm) presented the peptides most efficiently at low peptide concentration: <10 μM for the GAD 274–286 peptide and <1 μM for the GAD 115–127 peptide. This result also demonstrated that T cell hybridoma T-35 specific for the epitope 115–127 produced more IL-2 when stimulated by its cognate peptide at low concentration than did T cell hybridoma T-33.1 with peptide 274–286 under identical conditions, indicating that the deficiency to present the 115–127 epitope is not due to a lower sensitivity of the T35 hybridoma. The lower stimulation of T cell hybridomas by the heterozygous cell lines was not due to peptide stealing, because the other class II molecules present on those cells were not good binders for the given peptides (data not shown); differences may be a result of a higher density of the equivalent class II molecules on the cell surface of the homozygous cells.

Same presentation pattern occurs in the GAD65-transfected cell lines

To investigate the processing pattern in a quantitative manner, we stably transfected the APCs (Priess, Bsm) with rGAD65 cDNA. The intracellular expression of GAD65 was equivalent in both cell lines, as detected by RIA and Western blot (data not shown). However, the processing failure in terms of the GAD115–127 epitope was observed in Bsm, whereas Priess-transfected cells could present the relevant endogenous epitope (Fig. 3).

Differential presentation of GAD65 epitopes is not dependent on APC lineage

To address the question of whether lack of processing of the GAD65 115–127 epitope would be displayed in primary APCs and different APC lineages, Ag presentation was tested in distinct cell populations. PBL were obtained from the same four subjects from whom we had B cell lines (BrDe, EmWi, JeBa, and Repo used in the experiments presented above) and one healthy new subject as
When GAD65 protein was presented by these primary peripheral lymphocytes, the epitope-specific presentation phenotype was not altered (Fig. 4). To determine whether a distinct APC subpopulation would be capable of presenting the subdominant epitope 115–127, B cells and adherent cells were differentially separated from fresh peripheral blood. One-half of the adherent cells were subjected to GM-CSF and IL-4 in culture for 7–10 days to generate a population of dendritic cells. A population positive for CD40, DR, DQ, CD86, and CD1a; low for CD83; and negative for CD14, CD4/8, CD25, and CD19 was obtained. Once again, the epitope-specific presentation pattern did not change in any of these APC types, nor in dendritic cells, which have the greatest capacity to present Ag (Fig. 5). Most of the experiments were performed using two concentrations of GAD65 at optimal dose-response range. An example of GAD65 dose response in all APC lineages derived from Repo is shown in Fig. 5. Both hybridomas, T33.1 and T35, were highly stimulated by all APCs when pulsed with GAD65 p274–286 and p115–127 peptides, respectively (data not shown).

The effects of protease inhibitors on the presentation of GAD65 epitopes
To explore the mechanism behind the presentation-deficient phenotype, B cell lines were incubated in the presence of protease inhibitors, leupeptin and pepstatin A, for 20–24 h before the subsequent pulsing with GAD65 protein. The effect of the protease treatment on GAD65 epitopes was similar in all cell lines: leupeptin inhibited the responses, whereas pepstatin A augmented the response. However, the presentation of GAD65 115–127 was not rescued by either of the protease inhibitors (Fig. 6). A submaximal concentration of GAD65 (5–15 μg/ml) was used in these assays for each cell line and both T cell hybridomas, allowing the detection of either augmentation or inhibition of the epitope processing. To evaluate potential nonspecific toxicity of protease inhibitors at high concentrations, three concentrations of leupeptin (40, 100, and 200 μg/ml) and pepstatin A (10, 25, and 50 μg/ml) were used to define a dose-response effect. The highest concentration that gave complete inhibition or the strongest augmentation was tested for toxicity on the APCs pulsed with a synthetic peptide. Protease inhibitor treatment had no effect on peptide presentation (data not shown).
Discussion

We report differences between DRB1*0401-positive individuals in the processing of epitope 115–127 from human rGAD65, showing an epitope-specific deficiency in the processing of an important IDDM autoantigen.

Epitope GAD65 115–127 was not processed and presented by four of seven B-LCLs derived from different DR401-positive individuals. The differences in the presentation cannot be due to differences in the ability to internalize an Ag, first, because the other GAD65 epitope 274–286 was presented equally well by all APCs, and second, because the same processing pattern was observed in GAD65-transfected cell lines. The expression level of GAD65 in transfected cell lines was quantitated showing a comparable high level of expression in both APC phenotypes. We also tested the presentation of HSA to see whether the cell lines with defective processing of GAD65 epitope also displayed an inefficient processing of epitopes from other Ags. This was not the case, because all APCs induced similar stimulation of an HSA-specific T hybridoma, ruling out the possibility of a global defect in the processing machinery. Fixed cells efficiently presented peptides, but did not present the whole GAD65 Ag, which shows that the inability to present GAD65 115–127 epitope is at the processing level. Titration of the synthetic peptides corresponding to the T cell epitopes is also a good indication of the sensitivity of T hybridomas. Both T hybridomas were stimulated in a dose-dependent manner, and at low peptide concentration T-35 hybridoma specific for GAD65 115–127 was induced to produce more IL-2 than T-33.1 hybridoma, demonstrating that the processing difference was not due to a lower sensitivity of the T-35 hybridoma.

One hypothesis for the processing deficiency in the distinct cell lines is an inefficient loading of peptide onto a particular MHC class II, if in the peptide-loading compartment the other class II and possibly class I molecule(s) have a higher binding affinity to a given peptide (19–21). In our study, some of the cell lines were

![FIGURE 5. Presentation of human rGAD65 protein by distinct APC subpopulations: mononuclear adherent cells (A), primary CD19-positive B cells (B), and dendritic cells (C). GAD65 was used at 15 μg/ml (A and B) or 25 μg/ml (C). IL-2 production of the hybridomas is measured by the proliferation of IL-2-dependent HT-2 cells in the culture supernatant. Peptide control was included in each experiment (not shown); both GAD65 p274–286 and p115–127 were efficiently presented to given hybridomas (level of stimulation 173–295,000 cpm). A dose-response curve for different APC lineages derived from RePo (D) shows that dendritic cells present the Ag most efficiently. Each bar represents a mean of cpm [3H]thymidine incorporation in triplicate cultures. Error bars represent SEM. Data are representative of the results of two or more experiments.](http://www.jimmunol.org/)
FIGURE 6. The effect of protease inhibitors on the processing of GAD65 274–286 epitope to T33.1 hybridoma (A) and GAD65 115–127 epitope to T35 hybridoma (B). APCs were pulsed at the concentrations of GAD65, which gave a submaximal stimulation of T cell hybridomas (shown in Fig. 1). In A, Priess and Bsm were pulsed at 5 μg/ml GAD65, and all of the other cell lines at 12.5 μg/ml. In B, all B cell lines were pulsed at 25 μg/ml GAD65. IL-2 production of the hybridomas is measured by the proliferation of IL-2-dependent HT-2 cells in the culture supernatant. Each bar represents a mean of cpm [3H]thymidine incorporation in triplicate cultures. Error bars represent SEM. Data are representative of the results of two or more experiments.

HLA identical, but to exclude the possibility of epitope stealing, peptide binding was measured for all of the other HLA-DR or DQ molecules present on the B-LCLs. None of the other HLA class II molecules displayed a higher affinity for the p115–127 or p274–286 than DR401 molecule. Because HLA-A*0201 has been shown to bind and present an almost completely overlapping naturally processed GAD peptide 114–123 (14), all of the cell lines were tested for the positivity of HLA-A*02. Four of them were positive for HLA-A*02 (Bsm, BrDe, JeBa, Priess), but no correlation between the processing phenotype and genotype was observed. The processing phenotype did not associate with IDDM in our study, but this small number of subjects tested does not exclude a possible relationship with disease progression. Five subjects in our study were positive for GAD65 autoantibodies. Ag-specific Igs on the surface of B cells facilitate Ag uptake and may modulate the transportation and processing of Ag. However, in our study, GAD65 autoantibody positivity did not correlate with the epitope-specific presentation phenotype.

Another possible explanation for the lack of presentation of the 115–127 epitope is an inefficiency in the generation of that particular peptide in the processing compartment, or loading of the peptide onto MHC class II molecules. In an earlier study (11) when GAD-transfected Priess was used as a source of APC, more IL-2 was secreted by the T cells recognizing GAD65 274–286 compared with those recognizing GAD65 115–127. This might have been due to a lower expression level of GAD65 in these particular Priess-transfected cells than in our GAD65 transfectants. In a long-term culture without the G418 selection, the number of cells expressing high levels of GAD65 decreases. In this study, the level of GAD65 expression was quantitated from the cell pellets by RIA. However, consistent with the previous observation, a lower stimulation of T-35 than T-33.1 hybridoma was seen in response to a low concentration of exogenously added protein, as shown in Fig. 1. Therefore, in all experiments, a high enough concentration of exogenous GAD65 protein (15–25 μg/ml, depending on the APC) was added to ensure maximal presentation of the 115–127 epitope. At this concentration, the cells capable of presenting both epitopes induced a high IL-2 production in both T-35 and T-33.1 hybridomas.

We wanted to investigate whether the differential processing was characteristic only in one APC population, B cells, or whether it could be seen across the APC lineages. Cell proteases play a key role in Ag processing, and it has been shown that distinct APC lineages differ in their ability to process T cell epitopes from a single Ag (22, 23). Distinct APCs also internalize Ag by different mechanisms, and some chaperones are found only in certain APC populations, such as the recently described DO molecule in B cells (24). To test this hypothesis, we separated distinct APC populations, monocytes, and primary B cells, from peripheral blood from the same four subjects and one healthy control representing both processing phenotypes observed in B-LCL. In addition, we generated dendritic cells from adherent mononuclear cells, which allowed us to investigate the presentation of the GAD65 protein in this most efficient APC population. The dendritic cells generated from monocytes using GM-CSF and IL-4 have properties of immature dendritic cells (high expression of CD1a), and they have maximal capacity to capture and process an Ag (23–27). Upon maturation, the population of dendritic cells, characterized by CD83 expression (28), loses their Ag-processing capacity, but becomes efficient stimulators of T cells. Our results showed that all APC populations derived from the same individual displayed the same epitope-specific presentation pattern as observed in B-LCL. Although our results do not define the precise molecular basis for the defect in the processing of the GAD65 115–127 epitope in some individuals, the consistent presentation pattern through the APC lineages strongly suggests that the defect is most likely genetically determined.

One hypothesis for the differential processing is a lack of a chaperone essential for the presentation of the given epitope. Dependence of chaperones in terms of T cell epitope generation, class II intracellular trafficking, and peptide loading can vary in distinct processing compartments (29–32), where different epitopes can be generated from a single Ag (25–28). It is also possible that other competing peptides with higher affinity are generated. Different epitopes within one Ag may vary in their sensitivity to proteases and processing requirements. Certain epitopes may be processed in early endosomes, while others may require processing in a more acidic late endocytic compartment. Some studies have implicated distinct processing mechanisms for different epitopes (29–31). Walker et al. (33) tested a number of B-LCL for their ability to present keyhole limpet hemocyanin (KLH). The observation was that three cell lines presented KLH to only one particular KLH-specific T cell hybridoma, but not to the others. A differential ability to process various distinct Ags has been observed also in la-transfected fibroblasts (34–36). Michalek et al. (37) provided evidence that genetically identical APC clones processed differently the same immunodominant T cell determinant from native OVA. These findings all suggest that different epitopes, even from a single protein Ag, may vary in their dependence on particular
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References


