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Evidence for In Vivo Primed and Expanded Autoreactive T Cells as a Specific Feature of Patients with Type 1 Diabetes

Paolo Monti,* Miriam Scirpoli,* Andrea Rigamonti,† Anya Mayr,‡ Annika Jaeger,‡ Riccardo Bonfanti,‡ Giuseppe Chiumello,‡ Anette G. Ziegler,‡ and Ezio Bonifacio2*†

Identifying β cell autoantigen-reactive T cells that are involved in the pathogenesis of type 1 diabetes has been troublesome for many laboratories. Disease-relevant autoreactive T cells should be in vivo Ag experienced. The aim of this study was to test this hypothesis and then use this principle as a strategy for identifying diabetes-relevant autoreactive T cells. In this study, a CSFE dilution assay was used to detect glutamic acid decarboxylase 65 (GAD65)- and insulin-responsive T cells and HLA-0201*-GAD65114–122 pentamers were used to detect CD8+ GAD-responsive T cells in memory CD45RO+ and naive CD45RO− cell populations from patients with type 1 diabetes and healthy control subjects. T cell proliferative history was evaluated by flow cytometry telomere length measurement. CD4+ and CD8+ T cells specific for GAD65 and insulin were present in patients with type 1 diabetes and control subjects. Within the naive CD45RO− cells, CD4+ and CD8+ T cell responses were similar between patients and controls. Within the memory CD45RO+ cells, CD4+ T cell responses against whole GAD65 and insulin and HLA-0201*-GAD65114–122 pentamer-positive CD8+ T cells were found in patients with type 1 diabetes, but not in control subjects (p < 0.05 for all). Responding cells from the CD45RO+ T cell population had substantially shorter telomere lengths than responding cells from the CD45RO− population. Diabetes-specific autoreactive T cells in the circulation have uniquely undergone sustained in vivo proliferation and differentiation into memory T cells. Prior selection of these cells is possible and is a way to identify diabetes-relevant target Ags and epitopes. The Journal of Immunology, 2007, 179: 5785–5792.

Type 1 diabetes mellitus (T1DM) results from a chronic autoimmune destruction of insulin-producing β islet cells mediated by autoreactive T cells (1–3). Several β cell autoantigens are targets of islet autoimmunity. Of these, glutamic acid decarboxylase 65 (GAD65), insulinoma-associated protein 2, and (pro)insulin appear to be highly antigenic in humans both for T cells (4–6) and B cells (7, 8). To date, immune markers for T1DM have primarily centred on the presence of autoantibodies to β cell Ags and measurement of these Abs has been shown to be useful for prediction and diagnosis of type 1 diabetes (9). In contrast, T cell responses to islet cell Ags have been inconsistent in their diabetes specificity. Early studies reported that GAD65 and (pro)insulin-specific T cell responses were preferentially detectable in patients with T1DM and at-risk subjects, but rarely in healthy individuals (10–12). Other studies, however, showed that T cell responses against T1DM-associated autoantigens can be readily measured both in patients with T1DM and subjects without any sign of autoimmunity (13).

Recent studies indicate that autoreactive T cells in patients with type 1 diabetes can be distinguished by characteristics that are typical of cells that have already encountered Ag. These include proliferation to Ag in the absence of costimulatory signals (14) and the presence of specific late activation markers (15). These studies suggest that it may be possible to uniquely identify autoreactive T cell responses in at-risk subjects and patients using assays that selectively measure memory T cell responses. To test this hypothesis, we have investigated CD4 and CD8 T cell responses to autoantigens using whole PBMC, CD45RA−, and CD45RO+ T cell subsets. To test the hypothesis that autoreactive T cells in patients have undergone expansion in vivo, we have estimated proliferative history using telomere length. In human germline cells, telomeres are ~20-kb long (16) and most cells, including leukocytes, undergo the loss of 50–100 bp at each cell division (17) as a result of the balance between telomere erosion during cell division and the activity of telomerase, a unique reverse transcriptase that has the ability to extend the 3′ end of telomeres (18, 19). Telomeres consist of hexanucleotide repeats, and telomere length can be determined by hybridization of fluorescence peptide nucleic acid probes to these repeat sequences (19). By separately studying autoreactive-induced proliferation in CD45RA− and CD45RO+ T cell subsets and by examining the telomere length of proliferating cells, we show that the autoreactive-reactive T cells from at-risk subjects and patients have an Ag-experienced in vivo proliferation history and in this regard are distinct from those in healthy subjects.

Materials and Methods

Patients and controls

Patients with recent (<6 mo) onset T1DM, Ab-positive at-risk subjects, and healthy donors were recruited at the Department of Pediatrics (San
Table I. Characteristics of subjects

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* nt, Not tested; CTR, Control.

Raffaele Scientific Institute, Milan, Italy) and at the Diabetes Research Institute (Munich, Germany). A total of 68 subjects including 35 patients with T1DM (median age, 14 years; range, 8–29 years; 18 males) 13 Ab-positive at-risk subjects (median age, 18 years; range 2–62 years; 5 males), and 20 healthy control subjects (median age, 15.6 years; range, 7–40 years; 9 males) were provided as blind-coded samples for testing. Peripheral venous blood samples were obtained by venipuncture with informed consent and ethics committee approval. Patients characteristics are shown Table I.

PBMC isolation

PBMC were isolated by Ficoll-Hypaque (Amer sham Pharmacia Biotech) density gradient centrifugation from sodium-heparinized venous blood samples and washed twice in PBS.

CSFE proliferation assay

Cells were cultured in RPMI 1640 (BioWhittaker) supplemented with 10% pooled human serum (Sigma-Aldrich), 2 mM glutamine (Glutamax; Invitrogen Life Technologies), penicillin (100 U/ml), and streptomycin (100 U/ml; Invitrogen Life Technologies), and 100 μM nonessential amino acids (Invitrogen Life Technologies). Proliferation was determined using the CFSE (Mo- used Life Technologies), and 100 μM penicillin (100 U/ml), and streptomycin (100 U/ml; Invitrogen Life Technologies). Separation of CD4+ cells was per- formed using magnetic microbeads (Miltenyi Biotec) accord- ing to the manufacturer’s instructions. CD4+ cells were separated from PBMC using a CD4 multisort kit and the CD4+ fraction was further separated into CD4+CD45RO+ and CD4+CD45RO− using a CD45RO sort kit. The CD4+ fraction containing monocytes and B cells for optimal Ag presentation was also collected. CD4+CD45RO+ and CD4+CD45RO− cells were mixed 1:1 with the CD4+ fraction. Mixed cells were all labeled with CFSE and cultured with Ags as previously described (20).

[3H]Thymidine incorporation assay

In some experiments, we used a proliferation assay based on [3H]thymidine incorporation. CD4+ CFSEdim cells were mixed 1:1 with autologous irradiated (3000 rad) PBMC and cultured for 5 days with Ags at the concentrations used for CFSE dilution assay. [3H]Thymidine (1 μCi/ml) was added for the last 14 h.

Pentamer staining

Pentamer staining was performed both on fresh PBMC and on PBMC stimulated in vitro for 10 days in culture medium with the immunogenic GAD65114–122 peptide. The GAD65114–122 pentamer has been demonstrated to elicit CTLs in patients with T1DM (21). The following pentamers all to elicit CTLs in patients with T1DM (21). The following pentamers all

FVFTL), and HLA-A*0201-HIV-1 gag p17 7 6–8 4 (SLYNTVATL). For

Pentamer staining was performed both on fresh PBMC and on PBMC cultured for 15 days (BioWhittaker) supplemented with 10% pooled human serum (Sigma-Aldrich). Three hundred microliters of cell suspension were then incubated for 30 min at room temperature in the dark with 0.5 μg/ml pentamers, 1 μg/ml anti CD8 PE-Cy5 (mouse IgGlk, clone RPA-T8; BD Pharmingen), and 1 μg/ml anti CD45RO-allophycocyanin (clone IgG2a, k, clone UCHL-1; BD Pharmingen). For FACS analysis, cells were gated as live cells based on forward scatter (FSC) and side scatter (SSC) parameters. Gating of lymphocytes on FSC and SSC was used to minimize nonspecific staining. A total of 5 × 105 live lymphocytes were acquired from each sample.
FIGURE 1. A CSFE dilution assay was used to detect GAD65- and insulin-responsive T cells. PBMC from a patient with T1DM were labeled with CFSE and stimulated with GAD65 (5 μg/ml), insulin (5 μg/ml), or TT (10 LFU/ml) for 7 days. Proliferating CD4+CD45RA+ T cells were then sorted and used in a second round of stimulation (5 × 10^5 cells/100 μl/well) with medium alone, GAD65 (5 μg/ml), insulin (10 μg/ml), or PHA (1 μg/ml) and [3H]thymidine (B). Left panel, [3H]Thymidine incorporation of T cells stimulated for the first round with GAD65. Right panel, [3H]Thymidine incorporation of T cells stimulated for the first round with insulin. PBMC from either patients with recent onset T1DM (n = 29) and healthy subjects (n = 14) were stained with CFSE and incubated for 7 days with Ags GAD65 (C). The graph shows the percentage of CD4+CFSEdim T cells (y-axis) that have proliferated to medium, GAD65, and insulin (x-axis) in patients (T1DM, ●) and healthy controls (ctrls, ○). Comparisons (p value) were made using the Mann-Whitney U test.

FACS analysis

For the CSFE dilution assay, cells were harvested after 7 days of culture, washed in PBS, and stained on ice with an anti-human CD4 PerCP (mouse IgG1, clone SK3; BD Pharmingen). Optimal compensation and gain settings were determined for each sample based on unstained and single-stained samples. Propidium iodide was used to exclude dead cells. Ten thousand propidium-iodide negative, lineage-positive, CFSEdim cells were acquired from each sample. The number of cells that had proliferated was determined by gating on the lineage-positive, CFSEdim subset. Data were collected with a FACSCalibur flow cytometer and analyzed with the WinMDI software (Stanford University, Stanford, CA).

Telomeres length measurement by flow cytometry

Telomeres consist of noncoding G-rich hexanucleotide repeats (TTAGGG)n and associated proteins. We used a carboxylfluorescein-conjugated peptide nucleic acid probe (PNA; Primm) specific for telomere sequences that hybridized in a quantitative way to telomere repeats. The sequence of the carboxylfluorescein-labeled PNA probe was AATCGCGGCGCAGCAGCCAGCCC. For staining, 3 × 10^5 cells were resuspended 300 μl of hybridization solution (Hybmix: 75% formamide, 20 mM Tris buffer, 1% BSA, 20 mM sodium chloride, water) containing 0.3 μg/ml PNA probe and incubated for 10 min at room temperature. Samples were subjected to heat denaturation at 80°C for 15 min followed by hybridization at room temperature for 90 min. Samples were washed two times in Hybmix and PBS, respectively. As heat denaturation at 80°C also denaturates proteins, it is not possible to stain cells with Abs. Thus, the use of a FITC probe does not permit the use of CFSE as they have overlapping emission spectra. To overcome these problems, PBMC were separated into CD4+CD45RO+ and CD4+CD45RA+ cells, labeled with the lipophilic tracer DiD Vibrant cell labeling solution (Molecular Probes), and stimulated with Ags. After 7 days, the CD4+CD45RO+DiDdim and CD4+CD45RA+DiDdim cells were FACSorted and telomere lengths were measured. For DiD Vibrant cell labeling, cells were suspended at a density of 10^6/ml in serum-free RPMI 1640 plus 5 μl/ml DiD Vibrant solution for 15 min at room temperature in the dark. Cells were subsequently washed two times in RPMI 1640 supplemented with 10% FSC. As for CFSE, DiD Vibrant fluorescence is reduced by half in daughter cells upon each cell division and can be alternatively used with fluorochromes with emission spectra overlapping those of CFSE.

Statistical analysis

To evaluate the CD4+CFSEdim proliferative response in patients with T1DM and healthy subjects, the median percentage of CD4+CFSEdim cells...
and interquartile ranges (IQR) were calculated for each group. The Mann-Whitney U test was used to compare responses of patients with T1DM and healthy controls to the same Ag. For all analyses, a two-tailed p value of 0.05 was considered significant. Statistical analyses were performed using the Statistical Package for Social Science (SPSS 11.0).

Results
Detection of GAD65- and insulin-specific CD4+ T cells with the CFSE dilution assay
Proliferative responses to GAD65 and insulin were measured by the CFSE dilution assay. First, the capacity of the CFSE dilution assay to detect Ag specific T cell proliferation was assessed (Fig. 1A). PBMC were labeled with CFSE and stimulated for 1 wk with GAD65 (5 μg/ml), insulin (10 μg/ml), and TT (40 LFU/ml). CD4+ CFSEdim cells were detected for GAD65 (3.2%), insulin (2.7%), and the recall Ag TT (9.1%) as compared with negative control background (0.2%). To evaluate whether CD4+ CFSEdim cells were enriched for Ag-specific T cells, CD4+ CFSEdim cells obtained from stimulation with GAD65 and insulin were FACS sorted and then restimulated with the same Ag used in the first stimulation or with another Ag. [3H]Thymidine incorporation was used to measure proliferation (Fig. 1B). CD4+ CFSEdim obtained from GAD65-stimulated cells responded to rechallenge with GAD65 but not insulin. Similarly, CD4+ CFSEdim obtained from insulin-stimulated cells responded to rechallenge with insulin but not GAD65. These data suggest that the CFSE dilution assay detects GAD65- and insulin-specific CD4+ T cell responses in vitro.

CD4+ T cell responses to GAD65 and insulin are found in patients with T1DM and control subjects
Proliferative responses to GAD65 and insulin were measured in blinded samples from 43 subjects (Fig. 1C). No differences were observed between patients and control subjects with respect to the percentage of CD4+ CFSEdim cells in the absence of Ag (T1DM: median, 0.3%; IQR, 0.15–0.5%; vs controls: median 0.3%; IQR, 0.3–0.5%; p = 0.9), the percentage of CD4+ CFSEdim cells after incubation with GAD65 (T1DM: median, 1.7%; IQR, 1.05–3.05%; vs controls: median 1.7%; IQR, 0.3–2.9%; p = 0.6), and the percentage of CD4+ CFSEdim cells after incubation with insulin (T1DM: median, 1.05%; IQR, 0.65–1.75%; vs controls: median 0.5%; IQR, 0.3–2.05%; p = 0.3). These results provide evidence that autoreactive T cells responding to GAD65 and insulin are present both in patients with T1DM and in healthy control subjects.

Autoreactive CD4+ T cells have a memory phenotype in patients and at-risk subjects but not in control subjects
We reasoned that if GAD65 and insulin-reactive T cells were involved in the disease process, they should have been activated during the course of the disease and could have a memory T cell phenotype. Thirteen patients with T1DM (subject code numbers 003, 005, 006, 015, 018, 020, 028, 044, 046, 047, 050, 051, 053), 13 Ab-positive at-risk subjects (subject code numbers 056 to 068),...
and 13 age- and sex-matched healthy controls (subject code numbers 007, 009, 022, 026, 031, 040, 045, 048, 049, 052, 054, 055) were selected for separation into naive and memory T cells. Naive CD4+ T cells are CD45RA+/CD45RO− while memory T cells are CD45RA−/CD45RO+. Because all CFSE<sup>dim</sup> cells after 1 wk of stimulation with Ag express CD45RO (data not shown) CD4+</sup>/CD45RO<sup>−</sup> and CD4+</sup>/CD45RO<sup>−</sup> cells were isolated immediately after PBMC isolation and the two subsets were stimulated separately (Fig. 2A). T cells responding to the recall Ag TT were found in CD4+</sup>/CD45RO<sup>+</sup> and CD4+</sup>/CD45RO<sup>+</sup> negative populations in patients with T1DM, in at-risk subjects and in control subjects. Autointen-responding T cells in control subjects were detectable only in the CD4+</sup>/CD45RO<sup>−</sup> population, whereas patients with T1DM and at-risk subjects had autointen-responding cells both in the CD4+</sup>/CD45RO<sup>−</sup> and CD4+</sup>/CD45RO<sup>−</sup> cell subsets (Fig. 2B). Differences between patients and control subjects were observed in the proliferation of CD4+</sup>/CD45RO<sup>−</sup> T cells to GAD65 (p = 0.0001) and to insulin (p = 0.0001; Fig. 3). Differences were also observed between at-risk subjects and controls in the proliferation of CD4+</sup>/CD45RO<sup>−</sup> T cells to GAD65 (p = 0.012) and to insulin (p = 0.006). Notably, memory T cells responsive to GAD65 or insulin were not observed in the control subjects. Overall, 13 of 13 T1DM patients and 8 of 13 at-risk subjects had CD4+</sup>/CD45RO<sup>−</sup> T cell responses to GAD65 above those seen in control subjects, and 11 of 12 T1DM patients and 12 of 13 at-risk subjects had CD4+</sup>/CD45RO<sup>−</sup> T cell responses to insulin above those seen in control subjects. No significant differences between patients and control subjects were detected in the proliferation of CD4+</sup>/CD45RO<sup>−</sup> cells to GAD65 (p = 0.19) and insulin (p = 0.06). No differences between at-risk subjects and control subjects were detected for GAD65 (p = 0.71) while CD4+</sup>/CD45RO<sup>−</sup> cell responses to insulin were significantly higher in at-risk patients compared with control subjects (p = 0.006). Responses to TT were similar in patients, at-risk subjects and control subjects in both CD4+</sup>/CD45RO<sup>−</sup> cells and in CD4+</sup>/CD45RO<sup>−</sup> cells. These results suggest that patients with T1DM and at-risk subjects but not control subjects have autoreactive T cells that are fully Ag experienced.

**Autoreactive T cells from patients with T1DM and at-risk subjects have shorter telomere length**

To corroborate the findings of an Ag-experienced autoreactive memory T cell population in the peripheral blood of patients with T1DM and at-risk subjects, we examined telomere length of the proliferating memory and naive cells in 8 patients with T1DM (005, 006, 015, 018, 020, 028, 032, and 034), 13 at-risk subjects (from 056 to 068), and eight healthy controls (007, 009, 022, 026, 031, 039, 040, 041) (Fig. 4). If autoreactive T cells from diabetic individuals had been primed in vivo to differentiate into CD45RO<sup>+</sup> memory T cells, they were likely to have undergone cell division and therefore have reduced telomere length. Carboxyfluorescein-labeled PNA probes were used to measure telomere length by flow cytometry at the single-cell level (19). Because carboxyfluorescein and CFSE have similar emission spectra, PBMC were stained with the carbocyanine dye DiD Vibrant cell labeling solution. CD4+</sup>/CD45RO<sup>−</sup> and CD4+</sup>/CD45RO<sup>−</sup> were FACs sorted (Fig. 4A) and a 7-day proliferation assay in the presence of GAD65, insulin, or TT was performed as above. Telomere length was measured in sorted CD4+</sup>/DiD<sup>dim</sup> cells in a subset of subjects (Fig. 4B).

Sorted Ag-responding CD4+</sup>/DiD<sup>dim</sup> cells had similar DiD fluorescence intensities in CD4+</sup>/CD45RO<sup>−</sup> and CD4+</sup>/CD45RO<sup>−</sup> cells suggesting no differences in the in vitro cell division number
between these subsets (Fig. 4A). CD4+CD45RO- cells that proliferated in response to GAD65, insulin, and TT had similar telomere length in patients with T1DM, at-risk subjects, and in control subjects (Fig. 4C). Within these naïve T cells, the average mean fluorescence intensity (MFI) was 464 ± 29 for GAD65, 464 ± 26 for insulin, 464 ± 23 for TT in the control subjects, 488 ± 16 for GAD65, 495 ± 17 for insulin, and 491 ± 12 for TT in at-risk subjects and 446 ± 23 for GAD65, 447 ± 20 for insulin, and 448 ± 20 for TT in patients with T1DM (Fig. 4C). In control subjects, Ag-responding CD4+CD45RO- T cells were observed only for TT and they displayed considerable telomere shortening as compared with their naïve counterpart (average MFI 446 ± 23 naïve vs 366 ± 27 memory p = 0.0001). For both patients and at-risk subjects, CD4+CD45RO- cells responding to GAD65 and insulin had shorter telomeres compared with their naïve counterparts (patients: average 488 ± 16 naïve vs 454 ± 30 memory P = 0.009 for GAD65; average 495 ± 17 naïve vs 374 ± 35 memory p < 0.0001 for insulin; at-risk subjects: average MFI 447 ± 23 naïve vs 366 ± 27 memory p = 0.0003 for GAD65). In at-risk subjects, telomere shortening in memory GAD65-specific T cells was less pronounced than in T cells specific for insulin and tetanus (p < 0.0001 GAD65 vs insulin; p = 0.0008 GAD65 vs TT) while memory T cells specific for insulin had similar telomere shortening compared with TT (p = 0.69 insulin vs TT). In patients with T1DM telomere shortening of GAD65 and insulin-reactive memory T cells was similar (p = 0.23 GAD65 vs insulin) but significantly less than memory T cells specific for TT (p = 0.0025 GAD65 vs TT; p = 0.011 insulin vs TT). These results suggest that in patients with T1DM and in at-risk subjects GAD65 and insulin-specific T cells with a memory phenotype have undergone cell division in vivo.

GAD65-specific CD8+ T cells with a memory phenotype in patients with T1DM

Because we were able to detect T1DM-specific CD4+ T cell response phenotypes, we examined CD8+ T cell responses to a previously reported GAD65 epitope (21). HLA-A*0201-restricted GAD65114–122 pentamers were used to detect GAD65-specific CD8+ T cells. HLA-A*0201 influenza A matrix protein58–66 pentamers were used as a positive control and HLA-A*0201 HIV-1 gag p1776–84 pentamers were used as a negative control (all individuals tested were seronegative for HIV-1).

GAD65114–122-specific CD8+ T cells were abundant in PBMC stimulated in the presence of GAD65114–122 peptide for 10 days in both patients and control subjects (Fig. 5A). We therefore examined tetramer binding in fresh unstimulated, uncultured PBMC
In comparison with the control subjects, T cells specific for the HIV-related peptide gag p17 glycoprotein 76–84 were not detected. GAD65\(_{114-122}\)-specific CD8\(^+\) T cells were detected in all subjects studied (median 0.24% of CD8\(^+\) T cells in T1DM vs 0.24% in at-risk subjects vs 0.35% in controls). Consistent with CD4\(^+\) T cell data, GAD65\(_{114-122}\)-specific CD8\(^+\) T cells were observed in the CD45RO\(^-\) subset from patients, at-risk subjects, and control subjects whereas GAD65\(_{114-122}\)-specific CD8\(^+\) T cells in the CD45RO\(^+\) subset were significantly increased in patients as compared with control subjects \((p = 0.03\) T1DM vs controls; \(p = 0.012\) Ab\(^+\) vs control, Fig. 5C) and were also observed in the at-risk subjects.

Discussion

The presence of CD4\(^+\) and CD8\(^+\) autoreactive T cells responsive to β cell autoantigens are here demonstrated in the peripheral blood of both healthy individuals at-risk subjects and patients with T1DM. Unique to patients with T1DM and at-risk subjects were autoantigen responsive T cells that had been activated and expressed CD45RO and had undergone substantial cell division in vivo. The findings support the notion that autoantigen-responsive T cells are part of the normal T cell repertoire, that β cell autoantigen-responsive T cells are relevant to the disease process in T1DM, and that before and by the time of clinical disease onset these cells have received stimuli consistent with Ag-induced proliferation.

T cell responses to GAD65 and to insulin have been repeatedly shown in patients with T1DM. Responses have been inconsistently demonstrated to be more frequent in patients than in control subjects (22). Recent reports have shown that the T cells responding to β cell autoantigens and their peptides are phenotypically different to those in control subjects. Patients have proinsulin peptide-responsive T cells that produce IFN-γ whereas those in control subjects usually produce IL-10 (23). GAD65-reactive T cells in patients do not require costimulation indicating that they are Ag-experienced (14) and autoreactive memory T cells from patients have unique coexpression of CD25 and the late activation marker CD134 (15). In a recent report, Danke et al. (24) using class II tetramers showed that GAD65\(_{35-56}\)–specific T cells are present only in the CD45RA\(^-\) fraction in healthy subjects and both in CD45RA\(^+\) and CD45RO\(^+\) fraction in patients with T1DM. Our findings are consistent with each of these reports because they all highlight that the normal T cell repertoire includes potentially autoreactive cells and that patients have cells that have encountered and responded to Ag. Like these and other studies, we were unable to see differences in GAD and insulin-responsive cells between patients and control subjects unless we considered T cell phenotypes consistent with prior Ag priming.

One caveat in all studies that examine peripheral blood T cell responses in vitro is that culture assays have high Ag concentration and spatial restrictions. Such conditions may artificially break tolerance in vitro and the in vitro findings may not be operative in vivo. Moreover, such conditions may favor response to low-affinity noncognate Ag. We sought, therefore, to determine the proliferative history of the responding cells. As priming of naive T cells is accompanied by proliferation we measured telomere length of autoreactive T cells. Consistent with a prior history of proliferation, we found that memory T cells that proliferated in response to GAD65 and insulin had shorter telomere length as compared with the naive counterpart in the same subjects. Telomere shortening of 100 arbitrary units of fluorescence has been shown to correspond to a loss of ~1500 kb (19). As the telomere loss in T cells has been calculated to be 50–100 bp at each cell division, we can estimate that autoreactive memory cells have undergone 15–30 divisions more than the naive cells before sorting. In vitro proliferation is not expected to be more than four cell divisions and is likely to be similar in the memory and naive T cells populations. Thus, the majority of the divisions that have led to telomere shortening must have occurred in vivo. This provides evidence that the proliferative responses observed in the CD4\(^+\)CD45RO\(^-\) memory cells are representative of the in vivo immune response.

A possible consequence of telomere shortening in T cells undergoing repeated antigenic stimulation and replication is replicative senescence due to excessive telomere erosion. In terms of arbitrary units of fluorescence, autoreactive T cells appear to have longer telomeres than memory T cells responding to HTL. This implies that they probably still retain a considerable proliferative potential and the autoimmune immune response is not exhausted at the time of disease onset. Patients with T1DM also had autoreactive T cells with a naive phenotype and longer telomere length. Although these could potentially be recruited and involved in islet β cell destruction, they can also be differentiated into different T cell subsets (25), thereby providing therapeutic value. It is possible to differentiate naive autoreactive T cells into CD4\(^+\)CD25\(^+\) T regulatory cells (26), for example, and the presence of naive GAD and insulin-responsive cells in patients offers the potential to develop autoantigen-specific T reg cell therapy (27). In this context, it is important to determine whether the naive Ag-responsive cells are truly responding to cognate Ag or are simply responding as a result of low-affinity T cell cross-reactivity in the presence of high Ag concentration.

On the basis of findings with the CD4\(^+\) T cells, we examined whether CD8\(^+\) T cells could also be detected and had similar phenotypes. As a model, we used class HLA-A*0201-GAD65\(_{114-122}\) pentamers to detect CD8\(^+\) T cells specific for GAD65. The peptide GAD65\(_{114-122}\) has been described as immunogenic peptide restricted for HLA-A*0201 able to trigger a cytotoxic response in patients with T1DM (21). Here, we show as a proof of principle in a limited number of subjects that it is possible to detect diabetes-specific GAD65\(_{114-122}\)-specific CD8\(^+\) T cells in peripheral blood. We were able to find pentamer-positive cells directly from fresh unstimulated PBMC suggesting a relatively high frequency of T cells with this specificity. Similar to GAD65-specific CD4\(^+\) T cells, GAD65\(_{114-122}\)-specific CD8\(^+\) T cells exist with a naive phenotype in healthy individuals and with a naive and memory phenotype in patients with T1DM.

In conclusion, our data suggest that although T cells specific for GAD65 and insulin are present in the circulation of both patients and healthy subjects, a key step for the pathogenesis of T1DM is the proliferation of autoreactive T cells in vivo and their differentiation into memory clones. The findings provide potential avenues for identifying, measuring, and monitoring autoreactive T cells responses in T1DM and suggest that the naive autoreactive T cell component of patients is an opportunity for immunomodulating therapy.

Disclosures

The authors have no financial conflict of interest.

References


