Immunobiology 217 (2012) 920–925

Contents lists available at SciVerse ScienceDirect

Immunobiology

journal homepage: www.elsevier.de/imbio

T-cell receptor repertoire analysis in monozygotic twins concordant and discordant for type 1 diabetes

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ARTICLE INFO

Article history:
Received 21 May 2011
Received in revised form
28 December 2011
Accepted 1 January 2012

Keywords:
T-cell receptor repertoire
Monozygotic twins
Type 1 diabetes
Spectratyping
Flow cytometry

ABSTRACT

Several data suggest that stochastic rearrangements of the TCR could play a pathogenic role in both disease predisposition and protection in type 1 diabetes (T1D). As twin sets offer an enormous potential in evaluating the role of genetic and environmental factors in susceptibility to disease, the main goal of this study was to assess whether the degree of sharing of the expressed TCR repertoire of twin pairs discordant for T1D differs from that of disease concordant pairs. We performed our analysis in 5 pairs of monozygotic twins, 3 of which were concordant and 2 discordant for T1D, by combining flow cytometry and CDR3 spectratyping on both CD4+ and CD8+ T-cells. Our data show that TCR repertoires show increased level of concordance within each twin pair, especially in CD8+ cells, in terms of mean BV expression levels on flow cytometry as well as of CDR3 patterns and frequencies of skewed or oligoclonal BV subfamilies on spectratyping. It is worth noting that the degree of similarity among twins seems to be independent of concordance or discordance for T1D. Our findings seem to suggest that in monozygotic twins with T1D the TCR repertoire is influenced by genetic factors more than by the presence of the autoimmune disorder itself.

Introduction

Type 1 diabetes (T1D) results from a T lymphocyte-dependent, selective destruction of the insulin-producing pancreatic beta-cells. A number of genetic and biological data support a model in which the affinity of the HLA class II molecules for a preproinsulin-derived peptide, the levels of this peptide and the state of activation of CD8+ T-cells in the thymus act jointly as primary events of the disease pathogenesis [reviewed in (Todd 2010)]. The T-cell receptor (TCR), being mainly involved in the recognition of the peptide-MHC complex represents one of the key-molecule in the immune system. This is especially true of the third complementarity determining region (CDR3) of the variable portion of the beta chain (BV). The CDR3 loop, because of the rearrangement among V-D-J gene segments at the junction level, shows the highest structural variability (Nikolich-Zugich et al. 2004). Chiefly involved in foreign antigen specific T-cell responses, this CDR3 diversity derives from both amino acid number and sequence variations and is determined in an inherited as well as stochastic manner. Heterogeneous biases of the TCR repertoire have been found in several auto-immune diseases, both in CD4+ and CD8+ T-cell responses [reviewed in (Miles et al. 2011)]. In particular the occurrence of a so-called public TCR BV bias against a specific peptide expressed by the islet cells has been recently described in murine models of T1D (Quinn et al. 2006). Moreover, a clonotypic identity between the restricted repertoires of T-cells obtained from peripheral blood and from Langerhans islets has been previously demonstrated (Wong et al. 2007).

Twin sets offer an enormous potential in evaluating the role of genetic variations in susceptibility to disease as well as in revealing the interactions between genotype and environmental factors [reviewed in (Boomsma et al. 2002)]. More specifically, twin sets concordant or not for T1D represent the ideal scenario to explore the stochastic factors involved in the TCR repertoire shaping and in T1D risk. It is worth noting that studies on twins affected by different immune-mediated conditions suggest that homozygosity plays a predominant role in determining the concordance of the

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doi:10.1016/j.imbio.2012.01.002

overall TCR BV repertoire (Gulwani-Akolkar et al. 1994; Kuwana et al. 2001; Nanki et al. 1996; Somma et al. 2007).

As there are scarce data on the TCR BV repertoire usage in T1D concordant and discordant twins, we evaluated the degree of sharing of the expressed TCR repertoires in five twin pairs of Sardinian origin in both CD4+ and CD8+ peripheral blood lymphocytes, by combining flow cytometry and CDR3 spectratyping. To compare subject by subject the TCR repertoires determined by spectratyping, we employed the similarity score which allows the comparison of individual CDR3 TCR BV profiles (Fozza et al. 2007).

Methods

Patients

We performed our analysis in 5 pairs of monozygotic twins (2 females and 8 males), 3 of which were concordant and 2 discordant for T1D. The age of the twin pairs ranges from 18 to 45 and mean age at onset of T1D ranged from 2 to 12. As controls, we analyzed samples from 15 age-matched healthy volunteers (10 males and 5 females). All patients and controls were of Sardinian origin for at least three generations. All patients and donors had given informed consent. The study had been approved by the local ethics committee.

Flow-cytometry

Flow-cytometric analysis of the TCR-BV repertoire was performed with the IOTest Beta Mark Kit (Beckman Coulter, San Diego, CA), according to the manufacturer's instructions. Briefly, 1×10^6 cells/sample were stained with a panel of 24 BV family-specific antibodies, combined in groups of 3 in eight tubes, one antibody being conjugated to FITC, another to PE and the third to both FITC and PE. Costaining was performed with anti-CD4 or anti-CD8 PerCp (all antibodies from Becton Dickinson, San Jose, CA). Samples were acquired by a FACSort flow-cytometer using CELLQuest software. A lymphocyte gate was established on the basis of forward and side scatter characteristics. The relative representation of a given BV family was expressed as the percentage of cells stained with the family-specific antibody among CD4+ or CD8+ cells. A BV expansion was defined any value of BV family expression higher than the mean + 3 standard deviations (SD) calculated in normal controls.

CD4+ and CD8+ cell separation

Peripheral blood mononuclear cells were prepared by Ficoll-Hypaque (Sigma Diagnostic, St. Louis, USA) gradient centrifugation. The cells were separated into CD4+ and CD8+ subsets by positive selection using antibody-coated immunomagnetic beads (Dynabeads; Dynal, Oslo, Norway) for 30 min at 4 °C on a rotating shaker followed by magnetic isolation. TRIzol (Invitrogen, Paisley, UK) was added to both cell fraction pellets and they were kept at −80 °C until RNA extraction.

CDR3 spectratyping

The RNA was isolated from each subset of cells as described elsewhere (Chomczynski and Sacchi 1987). Complementary DNA was synthesized by using Superscript III reverse transcriptase and random hexamer primers (Invitrogen, Paisley, UK) according to the manufacturer's instructions. PCR was performed in a volume of 25 μL comprising 1× PCR buffer, 2.5 mM $MgCl_2$, 1 U AmpliTaq Gold (Applied Biosystems, Foster City, CA), 200 μM deoxyribonucleoside triphosphate (dNTPs), and 500 nM of 1 of 24 TCR-BV primers combined with 1 Beta-Constant (BC) primer conjugated to the fluorescent dye 6-carboxyfluorescein amino hexy (6-FAM). The sequences of the TCR-BV and BC primers were described previously (Gorski et al. 1994). The PCR conditions were: 95 °C for 10 min followed by 36 cycles of 94 °C for 20 s, 55 °C for 40 s, 72 °C for 40 s and a final extension of 72 °C for 5 min (Chen et al. 2005). The PCR fragments were then run on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) and data were collected and analyzed by the ABI Prism GeneScan Analysis software version 3.7 (Applied Biosystems, Foster City, CA).

Spectratyping analysis

Analysis of spectratyping data was performed by analyzing profiles by peak area and shape, in order to establish the degree of skewing and oligoclonality. In particular, each spectratyping profile was assessed following previously described criteria (Lu et al. 2004). A profile was defined as normal if showing a Gaussian bell-shaped distribution, with discrete peaks spaced by 3 nucleotides. Evidence of oligoclonal expansion or skewing within each BV was assessed by calculating the relative fluorescence intensity (RI) of each peak (RI = peak area/total BV peak area). A profile was defined as skewed if: either (a) a dominant peak with a RI greater than 50% of the total peak area was observed; or (b) 2 dominant peaks were present and RI of each peak was greater than 25% of the total peak area; or (c) there were multiple peaks differing from a Gaussian pattern and the RI of dominant peaks was greater than 25% of the total peak area. The first of these three criteria (i.e. the presence of a dominant peak with a RI greater than 50% of the total peak area) was used to specifically identify oligoclonal BVs. The percentages of skewed and oligoclonal BV subfamilies were calculated among the total number of BVs analyzed in each patient.

We specifically compared the spectratyping profiles within different pairs of subjects by using the similarity score (Fozza et al. 2007). Within each BV subfamily, the spectratype profiles were considered to be similar if: (a) both showed a Gaussian bell-shaped distribution; or (b) both showed the same skewed pattern, according to the criteria previously defined; or (c) an oligoclonal peak was observed in the same position in both cell subpopulations. The similarity score was calculated in both CD4+ and CD8+ cells within each pair of subjects as the ratio between the number of BVs with coincident profile between two sets of spectratyping profiles and the total number of BVs available in both sets. For example, a pair of subjects showing 14 BVs with coincident profile out of 22 BVs available in both subjects would have a 14/22 ratio, equivalent to a score of 0.64.

Statistical analysis

The Student *t*-test was used to assess the differences, between the 10 twins and the control group as well as between healthy and diabetic twins, in the percentage of BV expansions determined by flow-cytometry as well as in the percentage of skewed or oligoclonal BV subfamilies determined by spectratyping. The level of concordance among the mean BV expression levels detected by flow cytometry within each pair of twins as well as within each any other possible pair of subjects was determined by calculating the Pearson's correlation coefficient. The correlation coefficients obtained within the 5 pairs of twins were then compared by the Chi Square test with those obtained within the other 40 possible pairs of subjects, obtained by comparing each subject with all the other twins. Similarly the similarity scores calculated by comparing the spectratyping profiles of the 5 pairs of twins were compared by the Chi Square tests with those obtained within the other 40 possible pairs of subjects. The comparisons among correlation coefficients derived from flow cytometry data and among similarity scores calculated on spectratyping data were then performed on both the