A diabetes-associated T-cell autoantigen maps to a telomeric locus on mouse chromosome 6

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ABSTRACT Identification of diabetes-associated T-cell autoantigens is important for understanding the immunopathology of diabetes and developing improved therapeutic strategies. We have used a genetic approach to move toward identifying the autoantigen recognized by a diabeticogenic islet-specific T-cell clone from a nonobese diabetic (NOD) mouse. The unique antigen recognition pattern of this clone was utilized to map the gene encoding the antigen (or its expression) by genetic linkage analysis. In vitro analysis of T-cell proliferation by this clone showed that the capacity of the islets to stimulate T cells segregates as a single codominant gene in BALB/cByJ × (BALB/cByJ × NOD/Bdc) backcross mice. This phenotype was tightly linked to two microsatellites in the telomeric region of mouse chromosome 6.

Type I or insulin-dependent diabetes mellitus (IDDM) is a complex T-cell-mediated autoimmune disease affecting 0.3–0.5% of the population. The nonobese diabetic (NOD) mouse is a spontaneous animal model of diabetes, in which the course of disease is marked by a cellular infiltration of the pancreatic islets, leading to β-cell degranulation and destruction. A number of studies have indicated the importance of the CD4+ T-cell subset in pathogenesis (1), and CD4+ T-cell clones have been demonstrated to accelerate diabetes in young NOD recipients (2). Considerable effort is being expended on identification and characterization of the antigens that activate these cells, since antigens defined in murine IDDM may be relevant to human disease. Based on data from NOD T-cell proliferation assays and tolerance induction experiments, several intriguing autoantigen candidates have been put forth, including insulin (3, 4), heat shock proteins (5), and glutamic acid decarboxylase (6, 7), but it is not clear that any one antigen is key to the disease process. Therefore, the characterization of islet antigens remains an important goal in diabetes research.

Recent developments in genetic linkage analysis provide a powerful approach to identifying diabetes-related antigens. Linkage describes the concept that the closer a known marker locus lies to a particular gene, the less likely it is that a recombination event will occur between the two loci, and thus, the inheritance patterns of the two loci will correlate more perfectly. The application of polymerase chain reaction (PCR) techniques to linkage analysis has greatly simplified this methodology by facilitating the rapid analysis of intra- and interspecific mouse crosses with DNA markers called microsatellites (8–10). Microsatellites, a subset of simple sequence repeats, are abundant and often polymorphic in length. They are therefore very useful for genotyping mouse crosses and identifying linkage with an unknown gene of interest.

Most of the current linkage research in diabetes has focused on disease susceptibility loci. Studies in the NOD mouse have shown that diabetes is associated with multiple susceptibility genes, such as Idd1 within the H-2 complex (11–13). Although at least 10 murine IDDM susceptibility loci have now been identified (14), most of these have not yet been associated with gene products or specific phenotypes. We have used genetic linkage analysis to map a specific diabetes-related locus, a gene encoding the antigen (or a product that regulates the expression of the antigen) which is recognized by an islet-specific and diabeticogenic T-cell clone.

The T-cell clones, BDC-6.9 and BDC-2.5, are two members of a panel of CD4+ islet-specific T-cell clones derived from newly diabetic NOD mice (15, 16). These clones proliferate and produce interleukin 2 and interferon γ in response to whole islet cell antigen. In vivo, the clones can induce diabetes and insulinitis in young NOD recipients (2) and in various non-diabetes-prone NOD F1; strain combinations (17). Significantly, BDC-6.9 can also transfer diabetes into the NOD-scid mouse, demonstrating that this clone can initiate disease with no requirement for host CD8+ T cells (J. D. Peterson and K.H., unpublished data). Whereas most of the T-cell clones, including BDC-2.5, react in vitro to islet cells isolated from all mouse strains tested (NOD, NON, NOR, BALB/c, SWR, C57BL/6, SJL, CBA, AKR, and C57/L), the BDC-6.9 clone is unusual in that it responds to islet antigen from only the NOD mouse and its related strains (NOR and NON) and the SWR strain. If other strains such as BALB/c are crossed to the NOD however, islet cells from the F1 progeny are antigenic for BDC-6.9, although to a lesser extent than the NOD parental islets. The observation that antigen expression is not inhibited in the F1 progeny suggests that allele expression is codominant. We sought to take advantage of the unique antigen specificity of the BDC-6.9 clone by determining the presence or absence of the stimulating islet antigen in a BALB/cByJ × (BALB/cByJ × NOD/Bdc) backcross and then mapping the gene encoding this phenotype by linkage analysis.

MATERIALS AND METHODS

Mice. Female BALB/cByJ mice (The Jackson Laboratory) were bred to male (BALB/cByJ × NOD/Bdc)F1 mice to produce first-generation backcross progeny (BC). NOD/Bdc mice were used as a source of antigen-presenting cells (APCs). NOD/Bdc, (BALB/cByJ × NOD/Bdc)F1, and backcross mice were bred and housed in the pathogen-free facility of the Barbara Davis Center for Childhood Diabetes.

Propagation and Assay of T-Cell Clones. T-cell clones were derived from the spleens and lymph nodes of newly diabetic female NOD mice as described (15, 16). Cultures were maintained on a biweekly basis by combining 106 responding T cells, 2.5 × 107 irradiated (3000 rad from a 60Co source; 1 rad = 0.01 Gy) NOD spleen cells as APCs, 5 × 105 NOD islet cells as antigen, and 2.5% (vol/vol) EL-4 cell culture supernatant as a

Abbreviations: NOD, nonobese diabetic; IDDM, insulin-dependent diabetes mellitus; APC, antigen-presenting cell; cM, centimorgan(s).
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source of interleukin 2 in culture medium [20 ml of DMEM (GIBCO/BRL) supplemented with 44 mM NaHCO3, 0.55 mM L-arginine hydrochloride, 0.27 mM L-asparagine, 1.5 mM L-glutamine, 1 mM sodium pyruvate, gentamicin sulfate (50 mg/liter), 50 μM 2-mercaptoethanol, 10 mM Hepes, and 10% (vol/vol) fetal bovine serum]. Islet cell antigen was isolated from individual mice by collagenase digestion of pancreatic tissue as described (15, 18). T-cell proliferation assays were carried out by combining in culture 2 × 104 T-cell clones, 5 × 105 irradiated NOD spleen cells as APCs, and 5000 islet cells in 96-well culture plates. Negative controls were responders or responders and APCs in the absence of antigen. After 3 days, assay wells were pulse-labeled for 6 h with [3H]thymidine (0.5 μCi per well; 1 Ci = 37 GBq) and then harvested.

Preparation of Genomic DNA and Microsatellite PCR Analysis. Genomic DNA was prepared from mice by overnight incubation (60°C) of liver sections in lysis buffer [1 M Tris-HCl, pH 8.3/500 mM KCl/15 mM MgCl2/4.5% (vol/vol) Nonidet P-40/4.5% (vol/vol) Tween 20/proteinase K (4 mg/ml)], followed by phenol/chloroform extraction. The DNA (0.25 μg) was then analyzed by PCR with Mouse MapPairs primers (Research Genetics, Huntsville, AL). The reaction mixture contained 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM MgCl2, all four NTPs (each at 400 μM), primers (each at 0.264 μM), and 1 unit of Taq polymerase. The DNA sequences were amplified for 40 cycles (30 sec at 92°C, 60 sec at 55°C, and 30 sec at 72°C, with a 10-min extension period). PCR products were analyzed on a 4% agarose gel (1:1 ultrapure agarose and low-melting-temperature agarose) and products were visualized with ethidium bromide (0.5 μg/ml).

Linkage Analysis. The χ² test for independence in 2 × 2 contingency tables (1 df) was used to analyze differences in genotyping in the antigen-positive and the antigen-negative groups (INSTAT Version 2.0, GraphPad Software). P values ≤ 0.001 were considered to be significant. The frequency of mice with recombinations between microsatellite primers was calculated, and by using Haldane’s mapping function as described (19), the approximate positions of the microsatellite markers were estimated.

RESULTS AND DISCUSSION

Phenotypic Analysis of Backcross Mice Shows That BDC-6.9 Antigen Expression Depends on the Inheritance of a NOD Allele at a Single Genetic Locus. For these experiments, female BALB/cByJ mice were bred to male (BALB/cByJ × NOD/Bdc) F1 mice to produce a total of 105 first-generation backcross progeny (BC1). At every genetic locus, each BC1 mouse will inherit a BALB/c allele from the BALB/c parent and either a NOD or a BALB/c allele from the F1 parent. Those mice that inherit NOD DNA from the F1 parent at the “antigen locus” are heterozygous, or “Fl-like,” and will have islets that are antigenic for the BDC-6.9 clone. Conversely, BC1 mice that inherit BALB/c DNA from the F1 parent at the “antigen locus” are homozygous, or “BALB/c-like,” and their islet cells will not bear the BDC-6.9 antigen.

Individual BC1 mice were screened for inheritance of the heterozygous BDC-6.9 antigen locus by testing islet cells from each mouse in a T-cell proliferation assay with the T-cell clones BDC-6.9 and BDC-2.5 as responders. At the time each animal was sacrificed (at 7–10 weeks of age), the pancreas was removed for islet isolation and the liver was removed for preparation of genomic DNA. A maximum of 15 backcross mice were screened in any one assay; NOD/Bdc and F1 islet cells were included as controls in every assay. In Fig. 1A, results are shown from a representative experiment in which islet cells from 14 individual backcross mice were tested as antigen. The control clone BDC-2.5, which responds to islet antigen from all mouse strains, responded to islet cells isolated from both control and BC1 mice. BDC-6.9 showed no reactivity to BALB/c islets or to islets isolated from 9 of the BC1 mice; only islets from mice BC-12, -27, -30, -34, and -35 were able to

Fig. 1. Microsatellite marker D6Mit58 is linked to the expression of the BDC-6.9 antigen in BC1 mice. (A) In this representative T-cell proliferation assay, the clone BDC-6.9 (solid bars) and control clone BDC-2.5 (hatched bars) were used to analyze islet cells from 14 individual backcross mice and 3 controls for the presence or absence of the BDC-6.9 antigen. BALB/c data are from a separate experiment. Background responses were typically less than 200 cpm. (B) Each mouse was typed for inheritance of the D6Mit58 marker by PCR. The inheritance of the F1 genotype was indicated by two bands, one at 108 bp for the NOD allele and one at 116 bp for the BALB/c allele, whereas the inheritance of the homozygous BALB/c genotype was denoted by a single band at 116 bp. Data are summarized by classifying individual mice as heterozygous for inheritance of the antigen phenotype and the microsatellite genotype (+ or NB) or homozygous negative (− or BB).
stimulate the NOD-specific BDC-6.9 response. Therefore, of the 14 backcross mice tested in this experiment, 5 were positive for the BDC-6.9 islet antigen. Upon completing the analysis of all 105 BC1 mice, it was found that islet cells from 50 mice (47.6%) stimulated the BDC-6.9 clone, and thus these mice were heterozygous for the antigen locus. These data indicated that the BDC-6.9 antigen expression depends on the inheritance of a NOD allele at a single genetic locus.

The Gene Associated with the Expression of the BDC-6.9 Antigen Maps to a Telomeric Region of Mouse Chromosome 6. In the second part of this study, each backcross mouse was genotyped to establish linkage of the BDC-6.9 antigen phenotype with a known genetic locus. Genomic DNA was isolated from the liver of each animal, and two to three polymorphic microsatellites per chromosome were identified and typed in the backcross mice. Results for each locus were then statistically compared with the T-cell proliferation data to determine correlation, or linkage, between the inheritance of the F1 genotype and the inheritance of the BDC-6.9 antigen.

We first conducted a preliminary screen on 45 BC1 mice with a selection of microsatellite primers. Results from the testing of loci on chromosomes 1–5 indicated that the inheritance of each allele was randomly distributed in both the antigen-positive and antigen-negative mice (data not shown), so these chromosomes were eliminated from further investigation. With chromosome 6, however, significant linkage ($\chi^2 = 65.56, P < 0.0001$) was observed upon analysis of the D6Mit14 locus in the telomeric region (Table 1). We then performed a more comprehensive study of this chromosome, analyzing DNA from all 105 mice with a total of eight microsatellite markers. Table 1 shows the results from this screening. Most striking were the results found with microsatellites D6Mit58 and D6Mit60. These telomeric microsatellites, which have been mapped to the same location on chromosome 6 (refs. 20 and 21, supplemented by additional markers), showed a 100% correlation with inheritance of the antigen phenotype (Fig. 1B and Table 1). Analysis with markers on either side of D6Mit58/60 showed that there was decreased correlation with the phenotype as distance from the D6Mit58/60 locus increased. Therefore, the gene associated with the expression of the BDC-6.9 antigen lies within the interval between D6Mit25 and D6Mit57, and as estimated from the recombination frequencies, maps closest to D6Mit58/60.

Table 1. Marker loci at the telomeric end of chromosome 6 show significant linkage with the BDC-6.9 antigen phenotype

<table>
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<tr>
<th>Marker locus</th>
<th>RF</th>
<th>Antigen+</th>
<th>Antigen−</th>
<th>$\chi^2$</th>
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<tr>
<td>D6Mit1</td>
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<td>22/55</td>
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<tr>
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<td>43/50</td>
<td>44/55</td>
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<td>52/55</td>
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<tr>
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<td>50/50</td>
<td>55/55</td>
<td>101.03*</td>
</tr>
<tr>
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</tr>
<tr>
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<td>52/55</td>
<td>78.81*</td>
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<td>45/50</td>
<td>51/55</td>
<td>65.56*</td>
</tr>
</tbody>
</table>

A total of 105 BC1 mice were genotyped by PCR amplification of microsatellite DNA regions on chromosome 6; the chromosome 6 microsatellite primers tested are indicated. Based on T-cell proliferation results, mice were grouped as either positive for the stimulating islet antigen (antigen+) or negative (antigen−). Concordance is defined as the number of mice within each antigen grouping whose genotype at the given microsatellite locus matches, or is concordant with, the phenotype. The $\chi^2$ value is listed for each primer tested. Pairwise recombination frequencies are given (RF).

*P < 0.0001.

BDC-6.9 mouse, Testing to date indicates that our panel of T-cell clones responds to an islet-specific antigen, so genes that are expressed in the islet are of primary interest. As the insulin 1 gene (Ins1), formerly thought to be located on chromosome 6 (24, 25), has now been mapped to mouse chromosome 19 by Rowe et al. (26) and Ko et al. (27), and as none of our islet-specific T-cell clones responds to insulin in T-cell proliferation assays (18), we have excluded insulin and its precursors as possible candidates. There are two genes encoding islet proteins that have been mapped to the telomeric region of chromosome 6: BDC-6.9 islet-expressed candidate genes located near the region of interest (refs. 22, 23). Due to its distance from the other markers tested, the map position for D6Mit61 is taken from the current mouse map from the *Encyclopedia of the Mouse Genome III*, as published (22). D6Mit52, a centromeric marker in the linkage group, was chosen as the anchor for the rest of the map. This microsatellite has been linked to the known gene Prp, which is an anchor locus for the *Encyclopedia of the Mouse Genome III* map (22, 23). Since this map is only intended to be schematic, the antigen gene is shown to be located in the D6Mit25–D6Mit57 interval (open bars), with the greatest probability of being located within 0.96 centimorgan (cM) (calculated distance based on a recombination frequency of <0.0095) of either side of D6Mit58/60 (solid bar). Id6 (hatched bar) is a diabetes susceptibility locus that is currently linked most closely to D6Mit14 but also shows minor linkage to a marker associated with the Prp locus, proximal to D6Mit58/60 (14). We have thus pictured Id6 as being located somewhere within this large interval and, therefore, being a potential candidate locus.
chromosome 6, in the range of 59–65 cM from the centromere. These are the genes that encode lactate dehydrogenase β chain (Ldh2) (28–31) and islet amyloid polypeptide (iAPP) (32). Although lactate dehydrogenase may be an unlikely candidate as it is found in many tissues other than the pancreas, islet amyloid polypeptide (IAPP), a secretory protein that is specific to the β cells, could be a possibility. IAPP has generally been dismissed as a possible autoimmune target in human IDDM because although the presence of anti-IAPP autoantibodies has been documented, they do not correlate with expression of diabetes (33). It has recently been shown that human IAPP-derived amyloid deposits, frequently observed in type 2 diabetic tissues, may form intracellular aggregates with degenerating secretory vesicles and membranous organelles (34). Biochemical investigation of the β-cell proteins to which our T-cell clones respond has indicated that antigenicity is associated with the membrane portion of the β secretory granules, but not with cytosolic or soluble granule fractions (18). Although the mouse form of the protein is lacking the amino acid sequence that confers amyloid formation in humans (35), it may be that other IAPP–granule membrane associations can exist. It will be of interest to obtain and test secreted and prepropeptide forms of IAPP with the BDC-6.9 clone.

Although the definitive test of IAPP as an antigen for the T-cell clones will have to await assay of the protein with BDC-6.9, it is interesting to note that the IAPP structural genes of the NOD differ from normal (i.e., BALB/c) mice. We have begun to investigate differences between the NOD and BALB/c forms of IAPP by looking for mutations in the NOD cDNA sequence. Primers were designed, based on the published BALB/c cDNA sequence (36), to span the region containing the secreted form of IAPP, its signal peptide, and its C- and N-terminal propeptides. In a preliminary experiment, by using RNA isolated from NOD-islet β tumor cells of the NOD/Lt-Tg(RIPTag)1Llt mouse (37), we determined the cDNA sequence and found a single nucleotide substitution between the NOD type cDNA and the published BALB/c sequence in each of the propeptide regions (data not shown). These differences would result in a Gly → Arg amino acid substitution in the C-terminal propeptide and a Ser → Thr substitution in the N-terminal peptide. Sequences from the SWR mouse should also be investigated since IAPP from this strain should show the NOD amino acid substitutions if IAPP is indeed of antigenic significance.

Another locus of interest is Idd6, a diabetes susceptibility locus that Ghosh et al. (14) have associated with overt disease and, to a much lesser extent, insulitis. Idd6 was linked with marginal significance to two markers on either side of D6Mit58/60 (D6Mit14 and D6Nds8), with the best association being to the telomeric marker D6Mit14. This leaves open the possibility that Idd6 could map more closely to D6Mit58/60. D6Mit58/60 was not tested by Ghosh et al. (14) however, and since the initial identification of Idd6 was based on an entirely different phenotypic analysis and mouse backcross, we cannot determine whether there is an association between Idd6 and the BDC-6.9 “antigen gene.” If the gene encoding the BDC-6.9 antigen phenotype is associated with Idd6, it would imply that the ability to respond to this islet antigen is a factor in the development of overt diabetes.

**The Antigen Reactivity of BDC-6.9 Is Not Unique to That Clone**

We have recently established that a second islet-specific diabeticogenic T-cell clone, BDC-9.25, appears to have the same NOD-restricted antigen response pattern as clone BDC-6.9. There is no question that these are two distinct clones since BDC-9.25 was independently derived from a separate NOD mouse and its T-cell receptor uses Vß6, whereas the BDC-6.9 T-cell receptor is Vß4. The demonstration of two distinct autoreactive T cells with the same apparent antigen specificity could suggest that the target autoantigen is a major component of the T-cell response in diabetes pathogenesis.

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