Cellular Immunity to a Determinant Common to Glutamate Decarboxylase and Coxsackie Virus in Insulin-dependent Diabetes

Mark A. Atkinson,* Mark A. Bowman,* Lalita Campbell,* Bethany L. Darrow,* Daniel L. Kaufman,‡ and Noel K. Maclaren*
** Department of Pathology and Laboratory Medicine, College of Medicine, University of Florida, Gainesville, Florida 32610; and*
‡ Department of Molecular and Medical Pharmacology, University of California–Los Angeles, Los Angeles, California 90024

Abstract

Insulin-dependent diabetes (IDD) results from the autoimmune destruction of the insulin-producing pancreatic β cells. Autoreactive T-lymphocytes are thought to play a pivotal role in the pathogenesis of IDD; however, the target antigens of these cells, as well as the inductive events in the disease, are unclear. PBMC in persons with or at increased risk for IDD show elevated reactivity to the β cell enzyme glutamate decarboxylase (GAD). To identify the T-lymphocyte–reactive determinants of GAD, an overlapping set of synthetic peptides was used to stimulate the PBMC from these individuals, PBMC responsiveness to GAD peptides was not restricted to those with IDD, and a number of peptides elicited responses in PBMC. However, the major determinant of GAD recognized by persons at increased risk for IDD was amino acids 247–279, a region which has significant sequence similarity to the P2-C protein of Coxsackie B virus (47% of 15 increased risk [islet cell autoantibody–positive relatives]; 25% of 16 newly diagnosed IDD patients; and 0% of 13 healthy control subjects). Responses to tetanus and insulin antigens were not different between the study groups. In addition, PBMC from individuals responding to GAD peptides within 247–279 also responded to a Coxsackie viral peptide (i.e., P2-C amino acids 32–47), an observation supporting potential molecular mimicry in this immune response. Although the role of environmental agents in the pathogenesis of the disease remains unclear, these cellular immunological findings support the epidemiological evidence suggesting an inductive role for enteroviruses like Coxsackie B in the autoimmunity underlying IDD. (*J. Clin. Invest.* 1994. 94:2125–2129.) Key words: insulin • T lymphocyte • autoimmunity • islet cell autoantibodies

Address correspondence to Mark A. Atkinson, Ph.D., Department of Pathology, University of Florida, Box 100275 JHMHC, Gainesville, FL 32610.

Received for publication 22 April 1994 and in revised form 7 July 1994.

1. *Abbreviations used in this paper:* CDC, Centers for Disease Control; GAD, glutamate decarboxylase; IAA, insulin autoantibody; ICA, islet cell autoantimbody; IDD, insulin-dependent diabetes; JDF, Juvenile Diabetes Foundation; NOD, nonobese diabetic; SI, stimulation index.

J. Clin. Invest.

0021-9738/94/11/2125/05 $2.00
Volume 94, November 1994, 2125–2129

Introduction

Insulin-dependent diabetes (IDD),[1] or type 1 diabetes, results from the autoimmune destruction of the insulin-producing pancreatic β cells. A chronic mononuclear cell infiltration of the pancreatic islet cells is the pathological hallmark of the disease. The lesion is composed predominantly of T-lymphocytes, cells which are thought to play a pivotal role in the pathogenesis of the disorder (1, 2). Islet- or islet-cell protein–reactive PBMC can be identified in persons with or at increased risk for IDD (3–8). One antigenic target of both PBMC (6–8) and autoantibodies (9, 10) from persons with or at risk for IDD is the enzyme glutamate decarboxylase (GAD).

At least two molecular forms of GAD exist (i.e., GAD 65 and GAD 67), with both forms expressed in brain and islets (11, 12), and the former isoform being the principle target of immunity in IDD, as well as the predominant form in human islet cells (11–13). GAD catalyzes the formation of the inhibitory neurotransmitter gamma–aminobutyric acid from glutamine. Within the islet, GAD may have a role in the inhibition of somatostatin and glucagon secretions, as well as in regulating insulin secretion and proinsulin synthesis.

Because of the presumed role of lymphocytes in the pathogesis of the IDD, their reactivities in diagnostic assays may provide a superior marker for the natural history of the disease. Both we and others have shown that PBMC from approximately one half of new-onset IDD patients respond to GAD (6–8). In addition, we and others have also observed that the T-lymphocyte response to GAD in nonobese diabetic (NOD) mice was a key event in the induction and propagation of β cell autoimmunity (14, 15). Furthermore, whereas the cellular immune response in these animals was confined initially to a limited region of GAD, this was followed by intramolecular spreading of T-lymphocyte antigenicity to additional GAD peptide determinants, as well as to other autoantigens associated with IDD (14). To further our understanding regarding the role of anti-GAD immunity in the pathogenesis of human IDD, we examined the natural history of GAD determinant recognition by PBMC from patients with or at increased risk for the disease.

Methods

Human subjects. Blood samples were obtained from 44 caucasian individuals participating in studies of the natural history of IDD (16, 17). These included 16 newly diagnosed IDD patients (age: 14.2±6.8 yr [range: 6–33]; 9 males, 7 females); 15 islet cell autoantibody (ICA)– or insulin autoantibody (IAA)–positive first degree relatives of IDD patients (age: 23.4±16.1 yr [range: 8–60]; 6 males, 9 females; ≥ 10 Juvenile Diabetes Foundation (JDF) units); and 13 healthy control

subjects (age: 29.2±10.4 yr [range: 22–51]; 4 males, 9 females) with no family history of IDD and no detectable ICA or IAA. Increased-risk subjects were defined as first-degree relatives of an IDD patient who were positive for ICA and/or IAA. Natural history studies of IDD would indicate that ~ 50–75% of such relatives will progress to IDD within 5 yr, compared with < 0.3% of healthy control subjects (17, 18). Informed consent was obtained from the subjects and/or their parents according to the subject's age, as approved by the University of Florida Institutional Review Board. For these studies, newly diagnosed IDD was considered as samples within 3 mo of IDD onset in patients diagnosed by World Health Organization criteria (19).

Antibody analyses and HLA-typing. ICA were determined by indirect immunofluorescence using unfixed, snap frozen human pancreas as previously described (17). A test was considered to be positive for ICA when the intensity of the fluorescence and pattern of staining of the undiluted serum was the same or greater than a laboratory standard serum that had been calibrated to approximate 10 JDF units (20). All positive serum samples were expressed in JDF units by comparing the end point dilution of each positive serum to a standard calibration dilution curve using the international JDF reference serum accepted by the Immunology of Diabetes Workshops. IAA were determined by a modified radiobinding assay (17). The assay used human insulin ligand monoiodinated at amino acid 14 of the A chain, which was generously provided by Eli Lily Co. (Indianapolis, IN). Any serum with binding levels > 3 SD over the mean of 83 controls submitted for the Third International Insulin Antibody Workshop of the International Diabetes Workshops was defined as positive. Viral antibody titers to Coxsackie B variants were determined by capture ELISA at the Centers for Disease Control (CDC) (Atlanta, GA). Samples were reported as positive or negative for viral antibodies by comparison with a series of CDC standard control sera. HLA-DR typing was performed using denaturing gradient gel electrophoresis of PCR products of specific oligonucleotide priming, as previously described (17).

Cellular immunity. PBMC proliferation was determined as previously described (6, 21). Briefly, PBMC were isolated from heparinized whole blood by Ficoll-Hypaque density centrifugation, and 1×10^5 PBMC per well were cultured in round-bottom 96-well tissue culture trays in RPMI-1640 (10% human AB+ sera) for 7 d (95% air/5% CO_2). For these studies, PBMC were incubated with the following antigens in triplicate cultures: 1 μg/ml of PHA; 1, 5, and 10 μg/ml of GAD peptide; 1 and 10 μg/ml of human insulin; and 10 μg/ml of tetanus toxoid. Synthetic GAD peptides (Table I, overlapping 20–23mers) corresponding to the sequence of human GAD 65 (GenBank M81882), were produced by Advanced Chemtech (Lexington, KY). 18 h before harvest, 1 mCi [^{3}H]thymidine was added to each well. [^{3}H]thymidine incorporation was assessed by beta-particle counting (Matrix 96; Packard Instruments, Meriden, CT) and the mean value of each triplicate stimulation determined. Cellular proliferation was expressed as the stimulation index (SI=mean cpm incorporated in the presence of antigen divided by the mean cpm incorporated in medium alone). An SI of ≥ 3 was defined as positive. Numerical values are reported as the mean±SEM. PHA responses were measured at 4 d. Analysis of differences between study groups was performed using ANOVA and two-tailed Fisher's testing.

Results

Cellular immunity to GAD in IDD. PBMC responsiveness to GAD peptides was observed in all three study groups (Fig. 1). The frequency of immune responsiveness to a given GAD peptide within the three study groups was compared by statistical analysis, and revealed an elevated frequency of immune responsiveness in both increased-risk and newly diagnosed IDD subjects to GAD peptides 17 and/or 18 (i.e., amino acids 247–266 and 260–279). This finding was especially interesting, since we have shown previously that this region (i.e., amino acids 250–273, Fig. 2) has significant sequence similarity with the P2-C protein of Coxsackie B virus (10). When we analyzed these data as the ability of an individual's PBMC to respond against either GAD peptide 17 and/or 18, 47% (7/15) of persons at increased risk for IDD, 25% (4/16) of newly diagnosed IDD patients, and none of 13 healthy control subjects were positive (Fig. 1). The mean SI (cpm) to GAD 247–266/GAD 260–279 were 1.0±0.1 (136±87)/0.9±0.1 (140±154) in healthy controls; 3.1±1.3 (150±182)/1.6±0.3 (218±471) in increased-risk subjects; and 1.5±0.5 (130±106)/1.5±0.3 (178±135) in newly diagnosed IDD patients.

In contrast to the GAD peptide responses, PBMC reactivity to tetanus toxoid was equal in frequency between the study groups, being present in 92% (12/13) of healthy control subjects, 85% (11/13) of newly diagnosed IDD, and 87% (13/15) of increased-risk subjects ($P = 0.82$). The mean cpm for medium alone/medium plus tetanus toxoid were: healthy control subjects 142±28/3245±657; increased-risk subjects 88±28/2029±681; and newly diagnosed IDD patients 130±22/2004±367. The mean SI to tetanus toxoid were 35±9.4 for healthy control subjects, 49±16 for increased-risk subjects, and 31±12 for newly diagnosed IDD patients. The mean SI to tetanus toxoid was not significantly different between study groups ($P = 0.59$), but tetanus responses for all groups were elevated in comparison SI responses to GAD peptides. These findings may be due to a marked difference in the frequency of reactive cell populations to these antigens in PBMC, differences in the degree of immunological regulation of responses to endogenous versus exogenous (i.e., tetanus) antigens, or the stimulations provided by multiple peptide determinants within whole tetanus antigen versus a single peptide response associated with the GAD peptides. The mean cpm/mean SI to insulin in the healthy control and increased-risk groups were 151±57/1.7±0.5 and 289±113/2.2±0.7, respectively. 20% (3/15) of increased-risk subjects were responsive to insulin (Fig. 1), a figure in accordance with previous studies analyzing insulin specific T-lymphocyte responses (5), but not significantly different from the 8% (1/12) of healthy control subjects who responded (SI = 7) to insulin ($P = 0.61$).

No correlations between reactivity to GAD peptide 17 and/or 18 and the subject's age, sex, or the ICA titer were observed. No individuals responded to both GAD peptides 17 and 18 (Fig. 3). One explanation for this difference in reactivity to the two GAD peptides would involve HLA association with PBMC reactivity. HLA information was obtained on 10 of the 11 persons responding to GAD peptides 17 or 18. Reactivity to GAD peptide 17 was observed in persons with HLA-DR 3,4 ($n = 2$); DR 3,X ($n = 1$, where X is non-DR3 or DR4); DR 4,X ($n = 2$); and DR X,X ($n = 1$). However, reactivity to GAD peptide 18 was only observed in persons positive for the HLA-DR4 antigen (DR 3,4 [$n = 1$] and DR 4,X [$n = 3$]). However, further analysis of a large number of responding individuals will be required to determine the genetic control of the antipeptide response. Alternatively, since GAD peptides 17 and 18 both share mimicry to the Coxsackie P2-C viral sequence, PBMC from responding individuals may be reacting to sequences outside the region of overlap. A series of overlapping synthetic peptides for the region of GAD 247–279 are currently being produced in order to address this possibility.

Critical information to support the mimicry hypothesis would involve the demonstration that peptides from the Coxsackie viral sequence similarity region would stimulate cellular

Table I. Overlapping Synthetic Peptides for Human GAD 65

1) MASPGSGFWSFGSEDGSGDS	2) GSGDSENPGTARAWCQVAQKFTG
3) QKFTGGIGIGNKLCALLYGD	4) LLYGDAEKPAESGGSQPPRA
5) QPPRAAARKAACACDQKPCSC	6) KPCSCSKVDVNYAFLHATDL
7) HATDLLPACDGERPTLAFLQ	8) LAFLQDVMNILLQYVVKSFDRS
9) SFDRSTKVIDFHYPNELLQE	10) ELLQEYNWELADQPQNLEEILM
11) EEILMHCQTTLKYAIKTGHP	12) KTGHPRYFNQLSTGLDMVGL
13) DMVGLAADWLTSTANTNMFT	14) TNMFTYEIAPVFVLLEYVTL
15) EYVTLKKMREIIGWPGGSGD	16) GGSGDGIFSPGGAISNMYAM
17) NMYAMMIARFKMFPEVKEKG	18) PEVKEKGMAALPRLIAFTSE
19) AFTSEHSHFSLKKGAAALGI	20) AALGIGTDSVILIKCDERGK
21) DERGKMIPSDLERRILEAKQ	22) LEAKQKGFVPFLVSATAGTT
23) TAGTTVYGAFDPLLAVADICKK	24) DICKKYKIWMHVDAAWGGGLLMS
25) GLLMSRKHKWKLSGVERANS	26) ERANSVTWNPHKMMGVPLQC
27) VPLQCSALLVREEGLMQNCNQ	28) QNCNQMHASYLFQQDKHYDL
29) KHYDLSYDTGDKALQCGRHV	30) CGRHVDVFKLWLMWRAKGTTG
31) KGTTGFEAHVDKCLELAEYLYN	32) EYLYNIIKNREGYEMVFDGK
33) VFDGKPQHTMVCKWYIPPSL	34) IPPSLRTLEDNEERMSRLSK
35) SRLSKVAPVIKARMMEYGTT	36) EYGTTMVSYQPLGDKVNFFR
37) VNFFRMVISMPAATHQDIDF	38) ATHQDIDFLIEEIERLGQDL

immune responses of IDD patients or those at increased risk for the disease. Towards this, we measured the PBMC response to a Coxsackie viral peptide (i.e., P2-C amino acids 32–47) in three individuals who were previously identified as reactive to GAD peptide 18. Strikingly, all three individuals were responsive to this Coxsackie viral peptide in addition to their continued reactivity to GAD peptide 18 (SI of 10.0/10.1, 2.9/7.0, and 4.3/3.6 for Coxsackie viral/GAD peptide 18 responses, respectively). In contrast, none of the eight healthy controls tested to date (Atkinson, M., unpublished observations) have provided a positive response to the Coxsackie viral peptide (1.1±0.2). Furthermore, we have observed that immunization of mice with Coxsackie virus P2-C protein or the Coxsackie viral peptide containing the region of sequence similarity with GAD 65, can induce T cell immune responses which crossreact with GAD 65 or GAD 65 peptides corresponding to the region of sequence similarity (21a).

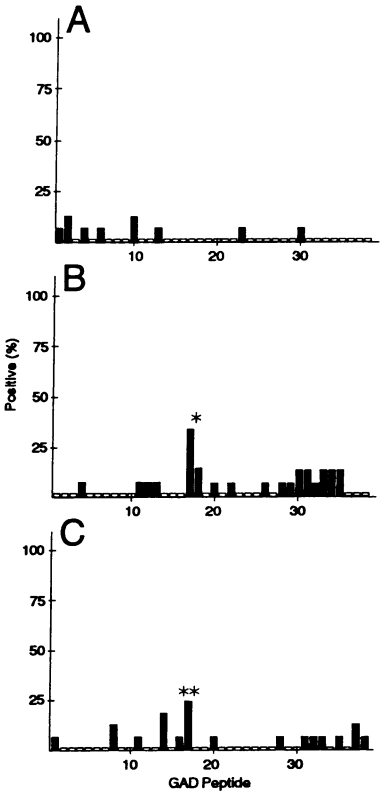

Figure 1. Antigen-included proliferation of PBMC from control (*A*), increased-risk IDD (*B*), and newly diagnosed IDD subjects (*C*). Y-axis indicates percentage of subjects who elicited a positive response (SI≥3) to GAD peptides 1–38 (*x-axis*). Values at an antigen concentration of 10 μg/ml are shown. Statistical significance for frequency of peptide responses for GAD peptide 17 and/or 18 (*P = 0.005; **P = 0.07), in comparison to healthy control subjects.

Humoral immunity to Coxsackie virus in IDD. Anti-Coxsackie virus antibodies were analyzed in all subjects to indicate previous viral exposure. Antibody responses to at least one Coxsackie viral strain were present in all persons tested (n = 9) whose PBMCs were reactive to GAD peptides 17 and/or 18, with two-thirds (6/9) positive against Coxsackie virus B4. Anti-Coxsackie viral antibodies against any variant were not observed significantly more often in a specific subject group (frequency of 57, 69, and 81% of control, new-onset, and increased-risk subjects, respectively). It must be emphasized that given the extended natural history of IDD, our lack of knowledge regarding the profile of anti-Coxsackie viral antibodies over time, as well as the relative sequence homology in the P2-C between various strains of Coxsackie virus, the direct correlation between anti-Coxsackie viral responses and cellular immune activities to a determinant of GAD are unclear and must be the subject of further investigations.

Figure 2. Sequence homology between Coxsackie virus and human GAD 65. Solid lines enclose identical amino acid residues. Dashed lines enclose amino acid residues with similar charge, polarity, or hydrophobicity. Numbers refer to the number of amino acid residues from the amino terminus of each protein.

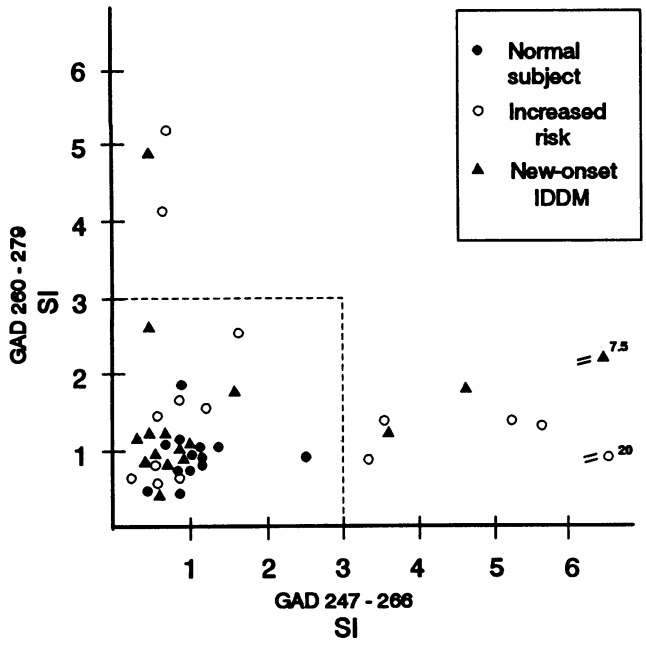

Figure 3. Scatter plot of responses to GAD peptides 247–266 (*x-axis*) and 260–279 (*y-axis*). Subject populations are identified as •, normal; ○, increased-risk; and ▲, newly diagnosed IDD subjects. Dashed line indicates minimal-positive response (SI≥3).

Discussion

The identification of cellular reactive epitopes within target antigens in autoimmune disorders has been investigated for a number of reasons, including an enhanced understanding of the pathogenesis of the disorder, the development of diagnostic tests for predicting disease, and the development of peptide-specific therapies for the prevention of disease. The most characterized T-lymphocyte reactive determinants analyzed to date in human autoimmune disease include the acetylcholine receptor and myelin basic protein, which respectively serve as target autoantigens in myasthenia gravis and multiple sclerosis (23–25). Our observations in IDD are consistent with these studies in that synthetic peptides could be used to identify autoreactive determinants, responses to autoantigenic peptides were observed in autoimmune as well as in healthy control subjects, specific peptide recognition was in some but not all cases HLA-DR–restricted, and many T-lymphocyte reactive determinants were observed within a single antigen (23–25).

The absence of a uniform PBMC reactive determinant in IDD subjects was predictable, given the known differences in an individual's MHC, as well as through our studies of immune responses to GAD in NOD mice (14), where an intramolecular spreading of cellular determinants was observed in the natural history of the disease in these animals. Therefore, the lack of uniformity of human PBMC responsiveness to the Coxsackie region with sequence similarity to GAD, or against peptides from another region of GAD, as well as the lower frequency of reactivity in newly diagnosed IDD patients versus increased-risk subjects, may be explained in part by the complex genetics of this disorder and/or by the molecular diversification of cellular immunity to autoantigens in the natural history of the disease. Consistent with our observations in NOD mice (14) was our finding that the frequency of detecting an anti-GAD cellular immune response appears higher in the period before the onset of disease (i.e., increased-risk subjects) in comparison to the onset of IDD (Fig. 1), although these frequences do not reach statistical significance, most likely because of the small number of subjects within the study groups. In mice, we hypothesized that this difference occurs due to the decline of anti-β cell immunity as one approaches the onset of IDD (1, 14). It was also interesting that the middle and latter one-third of GAD provided much of the protein antigenicity (Fig. 1), an observation consistent with previous studies identifying these areas as the predominant regions of GAD recognized by autoantibodies in sera of IDD patients (22).

Although a small number of IDD cases are known to occur years (i.e., 5–20 yr) after in utero viral infection (e.g., congenital rubella syndrome [26]), no other data conclusively implicates a virus as the environmental trigger of the autoimmune reactions that result in β cell destruction. However, it has been postulated that IDD may result from a misdirected immunological attack upon pancreatic β cells by lymphocytes responding to an acute or chronic viral infection. In this process, termed molecular cross-reactivity, sequence homologies between host and microbial antigens result in the generation of an anti host immune response. Molecular mimicry may account in part for the pathogenesis of rheumatic fever (27). Other associations between viral infections and IDD have been established through epidemiological studies examining immunity to members of the enterovirus family, and particularly to Coxsackie B virus. Data on the appearance of Coxsackie viral antibodies are controversial (28, 29); however, this association is strengthened by the report describing the isolation of a strain of Coxsackie virus from the pancreas of a newly diagnosed IDD patient (30). Evidence for molecular mimicry as a potential pathogenic mechanism underlying IDD was enhanced by our finding of a sequence homology between human GAD 65 and a Coxsackie B virus protein (10). The identification of determinant(s) within this region of GAD is consistent with the recent identification of cellular immunity directed against the middle one-third of GAD in humans with IDD (8), as well as with our finding of this region as an early target of cellular immunity in NOD mice (14).

Our identification in this report of a cellular immune response against this region in persons with, or at risk for, IDD supports the epidemiological evidence suggesting an inductive role for Coxsackie B virus in IDD. In addition, these observations provide an important link between an environmental agent and an immunological effector system associated with the formation of IDD. Future studies will address the potential role of GAD peptide reactive cells as predictive markers for progression to IDD, as well as their role in the pathogenesis of the disorder.

Acknowledgments

We thank Barbara Zorovich and Debbie Derovanesian for their assistance with patient samples; Dr. Mark Pallansch of the CDC for determination of Coxsackie viral antibodies; and Drs. Paul Lacy, Michael Clare-Salzler, Paul Lehmann, and Ivan Gerling for their critical review of this manuscript.

This study was supported in part by the National Institutes of Health (R29DK-45342), The Clinical Research Center at The University of Florida, Diabetes Research and Education Foundation, Juvenile Diabetes Foundation, and the American Diabetes Association.

References

1. Atkinson, M. A., and N. K. Maclaren. 1990. What causes Diabetes? *Sci. Am.* 7:62–67.

2. Castano, L., and G. S. Eisenbarth. 1991. Type-I diabetes: a chronic autoimmune disease of human, mouse, and rat. *Annu. Rev. Immunol.* 8:647–679.

3. Roep, B. O., S. D. Arden, R. R. De Vries, and J. C. Hutton. 1990. T-cell clones from a type 1 diabetes patient respond to insulin secretory granule proteins. *Nature (Lond.).* 345:632–634.

4. Roep, B. O., A. A. Kallan, W. L. W. Hazenbos, E. M. Bruning, E. M. Bailyes, S. D. Arden, J. C. Hutton, R. R. De Vries. 1991. T-cell reactivity to a 38kD insulin-secretory-granule protein in patients with recent-onset type 1 diabetes. *Lancet.* 337:1439–1441.

5. Harrison, L. C., S. X. Chu, H. J. DeAizpurua, M. Graham, M. C. Honeyman, and P. G. Colman. 1992. Islet reactive T cells are a marker of preclinical insulin-dependent diabetes. *J. Clin. Invest.* 89:1161–1165.

6. Atkinson, M. A., D. L. Kaufman, L. Campbell, K. A. Gibbs, S. C. Shah, D. F. Bu, M. G. Erlander, A. J. Tobin, and N. K. Maclaren. 1992. Response of peripheral-blood mononuclear cells to glutamate decarboxylase in insulin-dependent diabetes. *Lancet.* 339:458–459.

7. Honeymoon, M. C., D. S. Cram, and L. C. Harrison. 1992. Glutamic acid decarboxylase 67-reactive T cells: a marker of insulin-dependent diabetes. *J. Exp. Med.* 177:535–540.

8. Harrison, L. C., M. G. Honeymoon, H. J. DeAizpurua, R. S. Schmidli, P. G. Colman, B. D. Tait, and D. S. Cram. 1993. Inverse relation between humoral and cellular immunity to glutamic acid decarboxylase in subjects at risk of insulin-dependent diabetes. *Lancet.* 341:1365–1369.

9. Baekkeskov, S., H. Jan-Aanstoot, S. Christgau, A. Reetz, M. Solimena, M. Cascalho, F. Folli, H. Richter-Olesen, and P. De-Camilli. 1990. Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA synthesizing enzyme glutamate decarboxylase. *Nature (Lond.).* 347:151–156.

10. Kaufman, D. L., M. G. Erlander, M. Clare-Salzler, M. A. Atkinson, N. K. Maclaren, and A. J. Tobin. 1992. Autoimmunity to two forms of glutamate decarboxylase in insulin-dependent diabetes mellitus. *J. Clin. Invest.* 89:283–292.

11. Karlsen, A. E., W. A. Hagopian, C. E. Grubin, S. Dube, C. M. Disteche, D. A. Adler, H. Barmeier, S. Mathewes, F. J. Grant, D. Foster, and A. Lernmark. 1991. Cloning and primary structure of a human isoform of glutamic acid decarboxylase. *Proc. Nat. Acad. Sci. USA.* 88:8337–8341.

12. Michelsen, B. K., J. S. Petersen, E. Boel, A. Moldrup, T. Dyrberg, and O. D. Madsen. 1991. Cloning, characterization and autoimmune recognition of rat islet glutamic acid decarboxylase in insulin dependent diabetes mellitus. *Proc. Nat. Acad. Sci. USA.* 88:8754–8759.

13. Kim, J. W., W. Richter, H. J. Aanstoot, Y. Shi, Q. Fu, R. Rajotte, G. Warnock, and S. Baekkeskov. 1993. Differential expression of GAD65 and GAD67 in human, rat, and mouse pancreatic islets. *Diabetes.* 42:1799–1808.

14. Kaufman, D. L., M. Clare-Salzler, J. Tian, T. Forsthuber, G. S. P. Ting, P. Robinson, M. A. Atkinson, E. E. Sercarz, A. J. Tobin, and P. V. Lehmann. 1993. Spontaneous loss of T cell self tolerance to glutamate decarboxylase is a key event in the pathogenesis of murine insulin-dependent diabetes. *Nature (Lond.).* 366:69–72.

15. Tisch, R., X. D. Yang, S. M. Singer, R. S. Liblau, L. Fugger, and H. McDevitt. 1993. Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice. *Nature (Lond.).* 366:72–75.

16. Atkinson, M. A., N. K. Maclaren, D. W. Scharp, P. E. Lacy, and W. J. Riley. 1990. 64,000 M_r autoantibodies as predictors of insulin-dependent diabetes. *Lancet.* 335:1357–1360.

17. Riley, W., N. Maclaren, J. Krischer, R. Spillar, J. Silverstein, D. Schatz, S. Schwartz, J. Malone, S. Shaw, C. Valdheim, and J. Rotter. 1990. A prospective study of the development of diabetes in relatives of patients with insulin-dependent diabetes. *N. Engl. J. Med.* 323:1167–1172.

18. Bruining, G. J., J. L. Molenaar, D. E. Grobbee, A. Hofman, G. F. Scheffer, H. A. Bruining, A. M. deBruyn, and H. A. Valkenburg. 1989. Ten year follow-up study of islet-cell antibodies and childhood diabetes mellitus. *Lancet.* 8647:1100–1103.

19. Michaelis, D., I. Rjasanowski, W. Hildmann, K. D. Kohnert, and K. V. Richter. 1985. Validity of WHO criteria for classification of newly diagnosed diabetes. *Exp. Clin. Endocrinol.* 85:61–69.

20. Bonifacio, E., A. Lernmark, and R. L. Dawkins. 1988. Serum exchange and use of dilutions have improved precision of measurement of islet cell antibodies. *J. Immunol. Methods.* 106:83–88.

21. James, S. P. 1990. Measurement of basic immunologic characteristics of human mononuclear cells. *In* Current Protocols in Immunology. J. Coligan, A. Kruisbeek, D. Margulies, E. M. Shevach, and W. Strober, editors. John Wiley & Sons Inc., New York. 1–10.

21a. Tian, J., P. V. Lehmann, and D. L. Kaufmann. 1994. T cell cross-reactivity between Coxsackievirus and glutamate decarboxylase is assoicated with a murine diabetes susceptibility allele. *J. Exp. Med.* In press.

22. Richter, W., J. Endl, T. H. Eiermann, M. Brandt, R. Kientsch-Engel, R. Thivolet, H. Jungfer, and W. A. Scherbaum. 1992. Human monoclonal islet cell antibodies from a patient with insulin-dependent diabetes mellitus reveal glutamate decarboxylase as the target antigen. *Proc. Nat. Acad. Sci. USA.* 89:8467–8471.

23. Melms, A., S. Chrestel, B. C. G. Schalke, H. Wekerle, A. Mauron, M. Ballivet, and T. Barkas. 1989. Autoimmune T lymphocytes in myasthenia gravis. Determination of target epitopes using T lines and recombinant products of the mouse nicotinic acetylcholine receptor gene. *J. Clin. Invest.* 83:785–790.

24. Jingwu, Z., R. Medaer, G. A. Hashim, Y. Chin, E. VandenBerg-Loonen, and J. C. Raus. 1992. Myelin basic protein-specific T lymphocytes in multiple sclerosis and controls: precursor frequency, fine specificity, and cytotoxicity. *Ann. Neurol.* 32:330–338.

25. Manfredi, A. A., M. P. Protti, M. W. M. Dalton, J. F. Howard, Jr., and B. M. Conti-Tronconi. 1993. T helper cell recognition of muscle acetylcholine receptor in myasthenia gravis. Epitopes on the gamma and delta subunits. *J. Clin. Invest.* 92:1055–1067.

26. Menser, M. A., J. M. Forrest, and R. D. Bransby. 1978. Rubella infection and diabetes mellitus. *Lancet.* i:57–60.

27. Bisno, A. L. 1990. The resurgence of acute rheumatic fever in the United States. *Ann. Rev. Med.* 41:319–329.

28. Barrett-Connor, E. 1985. Is insulin-dependent diabetes mellitus caused by coxsackivirus B infection? A review of the epidemiologic evidence. *Rev. Infect. Dis.* 7:207–215.

29. Szopa, T. M., P. A. Titchener, N. D. Portwood, and K. W. Taylor. 1993. Diabetes mellitus due to viruses—some recent developments. *Diabetologia.* 36:687–695.

30. Yoon, J. W., M. Austin, T. Onodera, and A. L. Notkins. 1979. Isolation of a virus from the pancreas of a child with diabetic ketoacidosis. *N. Engl. J. Med.* 300:1173–1179.