Cellular Immune Responses Against Proinsulin
No Evidence for Enhanced Reactivity in Individuals With IDDM

Tamir Ellis, Eric Jodoin, Eric Ottendorfer, Patricia Salisbury, Jin-Xiong She, Desmond Schatz, and Mark A. Atkinson

Investigations of humans and nonobese diabetic mice suggest that proinsulin and/or a fragment of the region spanning C-peptide and the B-chain of insulin (i.e., proinsulin peptide) may serve as key autoantigens in IDDM. Therefore, we analyzed cellular immune reactivities against these molecules in people with or at varying risks for the disease to clarify their role in the pathogenesis of IDDM. In vitro peripheral blood mononuclear cell (PBMC) responses against these antigens, a control antigen (tetanus toxoid), and phytohemaglutinin were determined in 60 individuals with newly diagnosed IDDM (≤1 day from diagnosis) in 34 islet cell cytoplasmic autoantibody- and/or insulin autoantibody-negative first-degree relatives of the IDDM subjects, and in 28 autoantibody-negative control subjects. Unlike previous reports suggesting diabetes-associated elevations in cellular immunity to other β-cell antigens (e.g., GAD, IA-2, etc.), we observed equivalent levels of phytohemaglutinin stimulation and cellular proliferation in all groups against these antigens (all *P* values were not significant). The mean stimulation index ± SD and frequency of reactivity to proinsulin for healthy control subjects and IDDM patients, respectively, were as follows: 1 µg/ml (1.5 ± 1.0, 1 out of 17 [6%]; 1.9 ± 1.4, 4 out of 33 [12%]); 10 µg/ml (1.7 ± 1.3, 1 out of 17 [6%]; 1.2 ± 0.6, 0 out of 28 [0%]); and 50 µg/ml (1.2 ± 0.6, 1 out of 16 [6%]; 1.1 ± 0.6, 1 out of 27 [4%]). The response in healthy control subjects, autoantibody-negative relatives, and IDDM patients, respectively, against the proinsulin peptide fragment were as follows: 1 µg/ml (0.9 ± 0.4, 1 out of 12 [8%]; 1.3 ± 1.1, 4 out of 34 [11%]; 1.1 ± 0.3, 2 out of 28 [7%]); 10 µg/ml (0.9 ± 0.6, 1 out of 12 [8%]; 1.2 ± 0.6, 3 out of 34 [9%] 1.4 ± 1.7, 2 out of 28 [7%]); and 50 µg/ml (1.0 ± 0.7, 1 out of 12 [8%]; 1.2 ± 0.5, 2 out of 34 [6%]; 1.3 ± 0.5, 2 out of 28 [7%]). Taken together with previous studies reporting relatively infrequent occurrences of autoantibodies to proinsulin, the role of immunity to this molecule in the pathogenesis of IDDM in humans remains unclear. *Diabetes* 48:299–303, 1999

From the Departments of Pathology (T.E., E.J., E.O., J.X.S., M.A.A.) and Pediatrics (P.S.,D.S.), University of Florida College of Medicine, Gainesville, Florida.

Address correspondence and reprint requests to Mark A. Atkinson, PhD, Department of Pathology, Box 100275, University of Florida College of Medicine, Gainesville, FL 32610. E-mail: atkinson@ufl.edu.

Received for publication 29 April 1998 and accepted in revised form 30 September 1998.

cpm, counts per minute; HLA, human leukocyte antigen; IAA, insulin autoantibody; ICA, islet cell cytoplasmic autoantibody; PBMC, peripheral blood mononuclear cell; PHA, phytohemaglutinin; SI, stimulation index.

A considerable body of evidence suggests that the autoimmune destruction responsible for IDDM is primarily mediated through autoreactive T-cells (1–3). Because the pancreas is not accessible for immunologic investigation in humans, effector mechanisms must be studied through cells obtained from peripheral blood. Peripheral blood mononuclear cells (PBMCs) from patients with IDDM proliferate in vitro on exposure to islet cells (4,5), pancreas and β-cell membrane autoantigens (6), 38-kD islet cell autoantigen (7,8), ICA69 (9), ICA 512/IA-2 (6,10), GAD65 and GAD67 (6,11–13), and insulin/proinsulin (14–16).

When evaluating this extensive list of β-cell autoantigens for those having potential pathogenic significance, a bias toward insulin/proinsulin is often cited for a number of reasons. The first is that involving β-cell–specific expression (17,18). In addition, insulin appears to represent the target of both humoral and cellular immunity, although (apparently) at lower frequencies than other β-cell autoantigens (19). Studies of infants also suggest that autoantibodies to insulin are among the earliest humoral immune markers in people destined to develop IDDM (as compared with autoantibodies against other β-cell autoantigens, e.g., GAD and islet cell cytoplasmic autoantibodies [ICAs]) (20). Other studies have cited proinsulin autoantibodies as well as elevated serum proinsulin levels in people with or at increased risk for the disease (21–25). Finally, an elegant study of transgenic NOD mice suggested a potential primary role for proinsulin in the pathogenesis of disease in that animal model (26). For these reasons, we investigated the PBMC proliferative response to proinsulin to establish the molecule as a target of the cellular immune response.

RESEARCH DESIGN AND METHODS

Subjects. Blood samples were obtained from 122 individuals involved in our ongoing studies of the natural history of IDDM (27,28), including 60 newly diagnosed IDDM patients (age 13 ± 9.9 years). Samples were only collected from patients within 1 day of their onset of initial insulin therapy. Consistent with the experience of ourselves and others, the frequency of ICAs and insulin autoantibodies (IAAs) in this group was 68 and 42%, respectively. The ICA and IAA assays were performed as previously described (27). In addition, samples were obtained from 34 ICA^- and IAA^- first-degree relatives (age 25 ± 13.6 years; 12 men, 16 women) of the IDDM subjects used in this study. Twenty-eight autoantibody-negative healthy volunteers (age 23 ± 8.9 years) with no family history of IDDM participated to establish normal control ranges for cellular immune responses. Informed consent was obtained from each subject and/or their parents as approved by the University of Florida Institutional Review Board.

Human leukocyte antigen typing. Human leukocyte antigen (HLA)-DR types were determined using sequence-specific priming techniques as previously described (27). The respective distribution of specific HLA-DR combinations in subjects submitting to initial or available for retrospective genetic testing were as follows: newly diagnosed IDDM subjects (n = 37, 3 DR 3/4, 17 DR 4/X, 15 DR 3/X, and 2 DR X/X); autoantibody-negative first-degree relatives of an IDDM proband (n = 20, 3 DR 3/4, 7 DR 4/X, 5 DR 3/X, and 5 DR X/X); and healthy control subjects (n = 13, 0 DR 3/4, 7 DR 4/X, 3 DR 3/X, and 3 DR X/X).

Antigens. Human proinsulin was provided as a gift from Eli Lilly (Indianapolis, IN). Proinsulin peptide (amino acids 24–36; in accordance with French et al. [26]) was synthesized by the Peptide Synthesis Facility/Institutional Core for Biological Research at the University of Florida. Other stimulatory reagents were purchased from commercial sources (Tetanus toxoid [Massachusetts Public Health Biological Laboratories, Boston, MA] and phytohemaglutinin [PHA] [Sigma, St. Louis, MO]), or obtained as previously described (IA-2 antigen [10], β-casein [29]).

Analysis of PBMC proliferative response. PBMCs were isolated from heparinized whole blood by Ficoll-Hypaque density centrifugation, and 1×10^5 PBMCs per well were cultured in round-bottom 96-well tissue culture trays in RPMI-1640 (10% human AB^+ sera) for 7 days (95% air/5% CO_2) (10,11). The cells were incubated with the following antigen/mitogens in triplicate cultures: 1, 10, and 50 µg/ml of proinsulin, proinsulin peptide; 10 µg/ml (0.875 Lyons floculating U/ml) Tetanus toxoid, and 10 µg/ml PHA. Examination of cellular viability following the proinsulin addition to PBMCs in vitro revealed no evidence of acute toxicity. Responses against IA-2 and β-casein antigens were simultaneously determined in a randomly selected number of study participants under identical conditions and methods, with the definition of positive reactivity as previously described (IA-2 antigen [10], β-casein [29]). At 18 h before harvest, 1 µCi [^{3}H]thymidine was added to each well. Thymidine incorporation was assessed by Matrix 9600 beta-counting (Packard Instruments, Meriden, CT), and the mean value of each triplicate stimulation was determined. Cellular proliferation was expressed as the stimulation index (SI): mean counts per minute (cpm) incorporated in the presence of antigen divided by the mean cpm incorporated in antigen absence (medium alone). A positive cellular immune response was defined as an SI of greater than or equal to the mean plus 2 SD of the healthy control responses for proinsulin antigens, and >3.0 for responses to Tetanus toxoid. PHA responses were measured after 4 days. Analysis of differences between study groups was performed using both analysis of variance (ANOVA) and Fisher's exact testing.

RESULTS

PBMC proliferation in medium alone and in response to the various antigens was evaluated in short-term in vitro cultures established from patients with or at varying levels of risk for IDDM. These analyses were performed in two series of experiments: the first involving measurement of cellular immune responses against proinsulin and the second monitoring such activities against proinsulin peptide.

TABLE 1
Frequency and levels of cellular immune reactivities against proinsulin and Tetanus toxoid in healthy control subjects and people with IDDM

	Concentration (µg/ml)			
	Proinsulin			Tetanus
Subject group	1	10	50	10
Healthy control				
Frequency	1/17 (6)	1/17 (6)	1/16 (6)	15/17 (88)
Level	1.5 ± 1.0	1.7 ± 1.3	1.2 ± 0.6	33.5 ± 43.4
Newly diagnosed IDDM				
Frequency	4/33 (12)	0/28 (0)	1/27 (4)	28/32 (88)
Level	1.9 ± 1.4	1.2 ± 0.6	1.1 ± 0.6	25.2 ± 16.7

Frequency values within parentheses represent the percentage of positive individuals for the indicated antigen concentration. Proliferation values are presented as SIs (means ± SD).

In the first series of experiments, PHA responses of these cultures were high with no differences observed in the mean SI between control subjects (522.0 ± 331.3) and newly diagnosed IDDM patients (642.3 ± 534.8, NS). Basal responses (i.e., nonantigen or PHA-stimulated cpm) were also similar in control subjects and newly diagnosed patients (69 ± 22, 72 ± 31, respectively; NS). The proliferative responses of control and newly diagnosed patients to Tetanus toxoid stimulation were also similar in terms of their mean SI as well as their frequency of response (all NS, Table 1).

With respect to proinsulin reactivity, no differences in terms of the frequency or mean SI of responses between the two groups were observed (Table 1, Fig. 1). When the mean

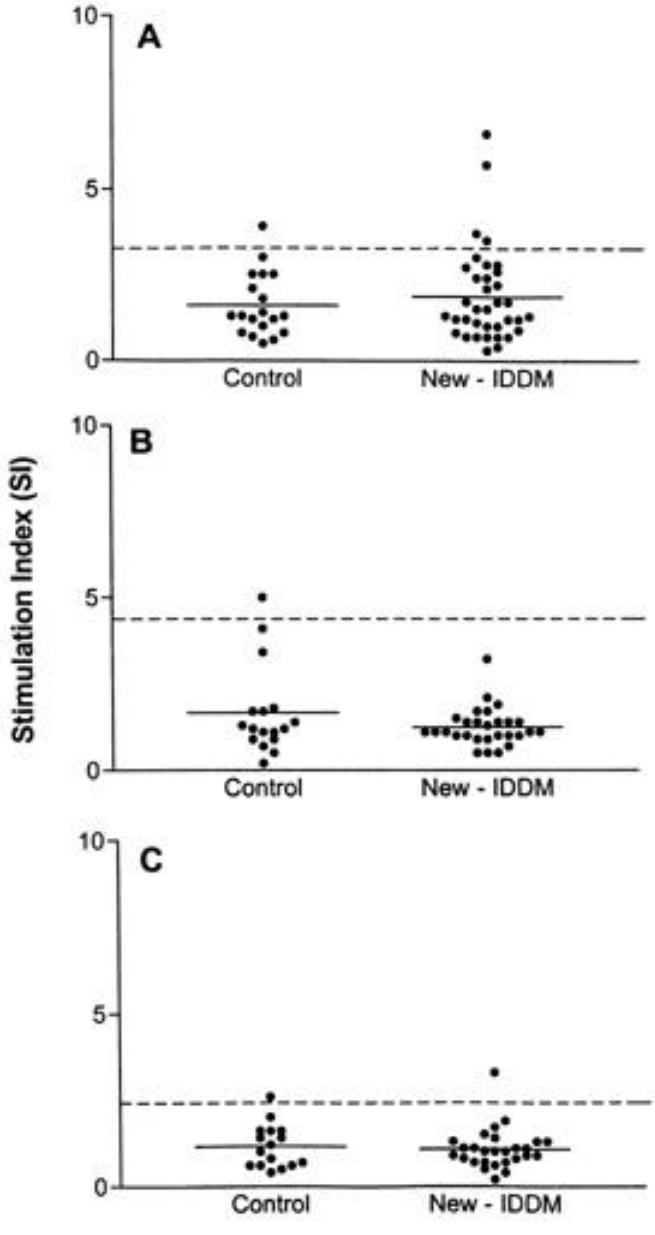

FIG. 1. Proliferation of peripheral blood mononuclear cells in response to proinsulin. Aliquots of PBMC (1×10^5) were cultures of medium containing 1 µg/ml (A), 10 µg/ml (B), or 50 µg/ml (C) of antigen. Dashed lines represent thresholds for positive reactivity. Solid bars indicate the group mean.

TABLE 2
Frequency and levels of cellular immune reactivities against proinsulin peptide and Tetanus toxoid in IDDM patients, nondiabetic siblings of IDDM probands, and healthy control subjects

	Concentration (μg/ml)			
	Proinsulin peptide			Tetanus
Subject group	1	10	50	10
Healthy control				
Frequency	1/12 (8)	1/12 (8)	1/12 (8)	10/12 (83)
Level	0.9 ± 0.4	0.9 ± 0.6	1.0 ± 0.7	18.2 ± 13.1
Autoantibody-negative relatives				
Frequency	4/34 (11)	3/34 (9)	2/34 (6)	25/34 (74)
Level	1.3 ± 1.1	1.2 ± 0.6	1.2 ± 0.5	18.8 ± 29.8
Newly diagnosed IDDM				
Frequency	2/28 (7)	2/28 (7)	2/28 (7)	27/28 (96)
Level	1.1 ± 0.3	1.4 ± 1.7	1.3 ± 0.5	28.6 ± 27.5

Frequency values within parentheses represent the percentage of positive individuals for the indicated antigen concentration. Proliferation values are presented as SIs (means ± SD).

SI to proinsulin at the 1 μg/ml concentration was compared between newly diagnosed IDDM patients and normal control subjects, somewhat higher (but not statistically significant) values were obtained in the IDDM group. Interestingly, at the higher antigen concentrations (i.e., 10 and 50 μg/ml), SI values declined and similarly, no differences between the mean SI of the newly diagnosed and control groups were observed (NS).

Because of the growing interest in the potential pathogenic significance of immune responses against a region spanning C-peptide and the B-chain of insulin, we then performed a second series of experiments monitoring PBMC reactivity against this molecule. In this set of experiments, no differences were observed in the PHA responses of cultures from control subjects (mean SI 420.1 ± 243.3), autoantibody-negative relatives (333.0 ± 359.1), and newly diagnosed IDDM patients (378.1 ± 447.4, NS). Basal responses were likewise similar in control subjects, autoantibody-negative relatives, and newly diagnosed patients (67 ± 28, 72 ± 34, 77 ± 47, respectively; NS). Proliferative responses of these subject groups to Tetanus toxoid stimulation were similar in terms of their mean SI as well as their frequency of response (all NS, Table 2). As observed with proinsulin, no differences in terms of the frequency or mean SI responses between the subject groups were observed with respect to responses to the proinsulin peptide (Table 2, Fig. 2).

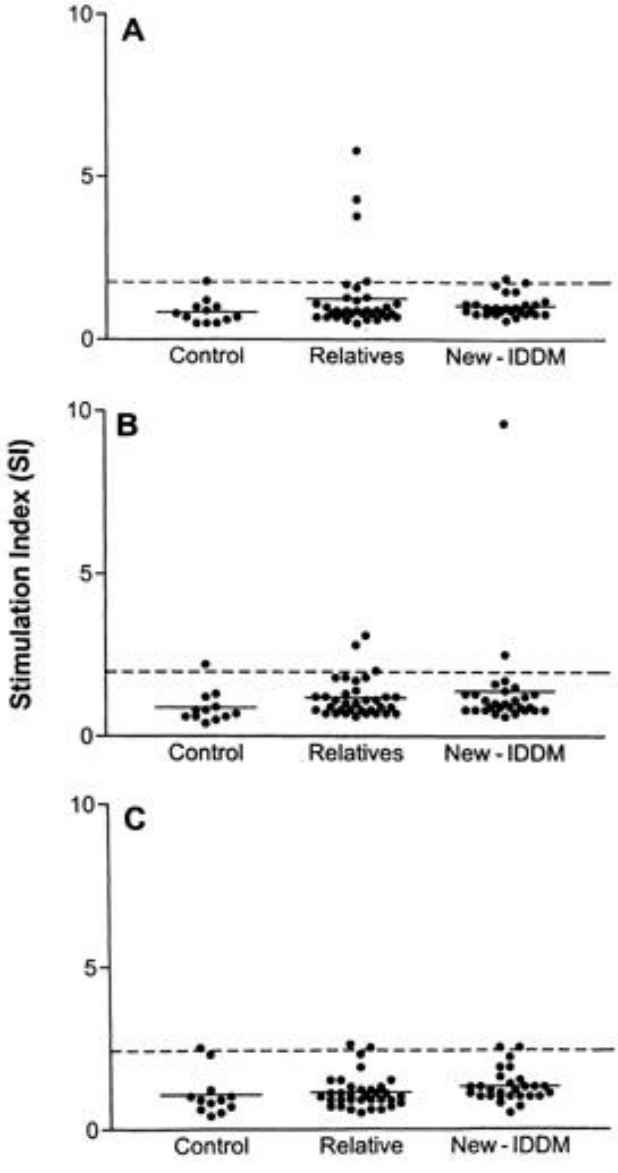

FIG. 2. Proliferation of peripheral blood mononuclear cells in response to proinsulin peptide. Dashed lines represent thresholds for positive reactivity. Solid bars indicate the group mean.

TABLE 3
Frequency of cellular immune reactivities against IA-2 and/or β-casein in IDDM patients, nondiabetic siblings of IDDM probands, and healthy control subjects tested for proinsulin/proinsulin peptide responses

Subject group	Proinsulin	Proinsulin peptide	IA-2	β-casein
Healthy control	1/10 (10)	0/10 (0)	1/10 (10)	1/9 (11)
Autoantibody-negative relatives	NT	4/21 (19)	3/19 (16)	9/19 (47)
Newly diagnosed IDDM	4/23 (17)	2/16 (13)	9/16 (56)	8/16 (50)

Frequency values within parentheses represent the percentage of positive individuals for the indicated antigen. Note the stimulation results for IA-2 and β-casein for some of these individuals have been reported elsewhere (10,29). NT, not tested.

The ability of the assay system to effectively monitor differences in the immune response against proinsulin/proinsulin peptide was determined by simultaneously monitoring PBMC stimulations against two autoantigens commonly associated with humoral and cellular immunity in IDDM, IA-2, and β-casein in a random series of the study subjects. As shown in Table 3, unlike responses against proinsulin and/or proinsulin peptide, a significantly greater proportion of individuals with IDDM displayed PBMC reactivity against IA-2 (P = 0.02) and β-casein (P = 0.04). Responses to β-casein were marginally elevated in autoantibody-negative relatives as

compared with healthy control subjects ($P = 0.08$), while reactivities to other antigens were limited (NS).

DISCUSSION

Interest in proinsulin as a molecule of pathogenic significance in human IDDM derives from studies identifying it as a limited but significant antigenic target of humoral immunity (24,25), and the demonstration of elevated proinsulin levels in increased risk and newly diagnosed IDDM subjects (21–23). When these observations were combined with a previous study indicating antiproinsulin/peptide cellular immunity in humans with IDDM (16) and the aforementioned investigations in animal models of the disease (26), it was obvious that extended investigations were required and hence formed the basis for this report.

With this background, our observation of a low frequency of proliferative T-cell responses against proinsulin in newly diagnosed IDDM patients was somewhat surprising, given the experiences of ourselves and others monitoring the cellular immune response to β-cell autoantigens. Specifically, using similar methods of detection (i.e., measurement of SI after 5–7 days of antigenic stimulation), cellular immune responses against the IDDM autoantigens GAD, ICA512/IA-2, and β-casein are observed in approximately one-quarter to two-thirds of individuals with or at increased risk for IDDM (i.e., autoantibody-positive relatives of IDDM probands). Indeed in this study (Table 3), a similar proportion of individuals with IDDM displayed PBMC reactivity against both IA-2 and β-casein. While this study did not seek to monitor such activities as its primary aim, the results obtained through simultaneous performance of such investigations do support the contention that the assay system can be effective at monitoring differences in the cellular immune response against autoantigens commonly associated with IDDM. At a minimum, our results would suggest that the precursor frequency of proinsulin reactive T-cells in peripheral blood may be much lower than other β-cell autoantigen reactive T-cells, or that the method for their identification may require a more elaborate detection system (e.g., limiting dilution assay, T-cell enrichment assays, etc.). However, such modifications may prove difficult because recent studies (30,31) have questioned the ability of T-cell separations (i.e., addition of purified T-cells to irradiated non–T-cells) or limiting dilution analysis to enhance the identification and/or more accurately predict the frequency of self-reactive T-cells in IDDM or other autoimmune diseases.

Indeed, our findings are strikingly similar to a recent carefully controlled work of Schloot et al. (15) that suggested limited to no variance of cellular immune responses against insulin in IDDM patients versus healthy control subjects. In addition, the study by Schloot et al. (15) reported limited cellular immune responses in insulin-treated IDDM subjects, suggesting that the molecule represents a weak T-cell immunogen when supplied via exogenous insulin therapy or that the administrative process induces T-cell tolerance in most subjects (15). Our results can also be considered as supporting a limited role for antiproinsulin immunity in the natural history of IDDM because the frequency of cellular immune responses in our study (i.e., optimum 12%) was nearly identical to that reported for antiproinsulin autoantibodies in people with the disease (14% [24]).

Among the many potential explanations underlying our failure to observe such reactivities would be the limitation that measurement of cellular immune responses to β-cell autoantigens is restricted to analysis with cells obtained from peripheral blood. It is possible that proinsulin (or insulin) reactive T-cells are primarily restricted through tropism to the pancreatic islets and therefore escape detection caused by extreme limitations in their number within the periphery. In addition, one recent study has demonstrated that the insulin gene is transcribed in the human thymus, its transcription level correlates with allelic variation at the IDDM2 susceptibility locus, and that proinsulin is the main protein product of the insulin gene in this organ (32). Hence, it is feasible that the low degree of immunogenicity of proinsulin (as compared with other putative β-cell autoantigens) observed in our studies might be a function of the presence of proinsulin in the thymus of individuals in early life. Finally, PBMCs contain a low percentage of cells capable of efficient antigen presentation (i.e., B-cells, monocytes, macrophages), hence imparting a restriction on the efficient detection of such immune responses. However, the unique restriction that would be afforded to proinsulin through this presentation process (in comparison to other β-cell autoantigens) is unclear.

Given the failure to observe data indicating immunity against whole proinsulin, our attention turned to measuring immunity against the proinsulin peptide. Proinsulin is processed and stored within β-cells in the secretory granules before the mature product (i.e., insulin) is secreted. In this process, proinsulin is cleaved on both sides of C-peptide (amino acids 57–87), leaving an insulin molecule of A- and B-chains connected by two interchain disulfide bonds. It has been proposed that the antigenic processing of proinsulin could result in unique antigenic peptide structures/sequences of potential pathogenic significance. In concordance with this hypothesis, Rudy et al. (16) have indicated that the proinsulin epitope subject to this investigation spans the natural convertase cleavage site. These investigators propose the hypothesis that an early event in IDDM pathogenesis may involve abnormal processing of proinsulin, a process that results in an unusual quantity or phase of proinsulin intermediate. Accordingly, individuals bearing diabetes-susceptible HLA might subsequently process and present a nontolerogenic cryptic epitope to T-cells.

Hence, our studies are also at variance with these investigations (16), suggesting that a significantly greater proportion of IDDM patients display a PBMC response against proinsulin peptide compared with healthy people with no family history of the disease. The reason(s) for this variance is unclear, and aside from the aforementioned limitations in monitoring cellular immunity, seemingly minor methodological variances (e.g., sera sources, peptide synthesis) could have led to such discrepancies. In addition, understanding the potential influence HLA could play in such responses, HLA typings were performed for these investigations. However, given the dearth of positive stimulations, a role for specific HLA associations with antiproinsulin/peptide immunity remains unclear.

An extensive body of literature continues to support the concept that insulin serves as an autoantigen of pathogenic significance in IDDM. In NOD mice, insulin-reactive T-cell lines (including those reactive to insulin peptide B9–23) are capable of disease transfer as well as prevention from IDDM (33,34). The observation that insulin administration (e.g., subcutaneous, oral, immunization, etc.) prevents disease in

animal models of IDDM (35–38) has led to the formation of clinical trials in humans aimed at disease prevention (39). For nearly 15 years, studies have identified insulin as a target of humoral immunity both at disease onset and in the asymptomatic period before the onset of overt IDDM in humans (1).

In summary, our study draws caution but not exclusion to the concept that proinsulin serves as a major autoantigen in the pathogenesis of IDDM since few individuals with the disease demonstrate significant antiproinsulin cellular immune reactivity. It is possible that identification of new methods of detection may reveal such activities and should be subject to future investigations. Clearly, further investigation is needed to identify a role for antiproinsulin immunity in the pathogenesis of this disease.

ACKNOWLEDGMENTS

This work was supported in part by grants from the National Institutes of Health (DK-45342, AI/DK-39250, AI-42288), the Juvenile Diabetes Foundation, and the American Diabetes Association.

We thank William Winter, Janet Silverstein, Andrew Muir, and Michael Clare-Salzler for their assistance in obtaining patient materials.

REFERENCES

1. Atkinson MA, Maclaren NK: The pathogenesis of insulin-dependent diabetes. *N Engl J Med* 331:1428–1436, 1994
2. Bach J-F: Insulin-dependent diabetes as an autoimmune disease. *Endocr Rev* 14:516–542, 1994
3. Roep BO: T-cell responses to autoantigens in IDDM: the search for the Holy Grail. *Diabetes* 45:1147–1156, 1996
4. Kontiainen S, James RL, Feldmann M: Human islet cell induced T-cell lines and clones from diabetic children. *Autoimmunity* 8:193–197, 1991
5. Harrison LC, De Aizpurua H, Loudovaris T, Campbell IL, Cebon JS, Tait BD, Colman PG: Reactivity to human islets and fetal pig proislets by peripheral blood mononuclear cells from subjects with preclinical and clinical insulin-dependent diabetes. *Diabetes* 40:1128–1133, 1991
6. Durinovic-Bello I, Hummel M, Ziegler AG: Cellular immune response to diverse islet cell antigens in IDDM. *Diabetes* 45:795–800, 1996
7. Roep BO, Kallan AA, Hazenbos WLW, Bruining GJ, Baileys EM, Arden SD, Hutton JC, De Vries RRP: T-cell reactivity to 38kD insulin-secretory-granule protein in patients with recent-onset type 1 diabetes. *Lancet* 337:1439–1441, 1991
8. Honeymoon MC, Cram DS, Harrison LC: Transcription factor jun-B is a target of autoreactive T-cells in IDDM. *Diabetes* 42:631–636, 1993
9. Roep BO, Duinkerken G, Schreuder GMT, Kolb H, De Vries RRP, Martin S: HLA-associated inverse correlation between T-cell and antibody responsiveness to islet autoantigen in recent-onset insulin-dependent diabetes mellitus. *Eur J Immunol* 26:1285–1289, 1996
10. Ellis TM, Schatz DA, Ottendorfer EW, Lan MS, Wasserfall C, Salisbury PJ, She JX, Notkins AL, Maclaren NK, Atkinson MA: The relationship between humoral and cellular immunity to IA-2 in IDDM. *Diabetes* 47:566–569, 1998
11. Atkinson MA, Kaufman DL, Campbell L, Gibbs KA, Shah SC, Bu D-F, Erlander MG, Tobin AJ, Maclaren NK: Response of peripheral-blood mononuclear cells to glutamate decarboxylase in insulin-dependent diabetes. *Lancet* 337:458–459, 1992
12. Honeymoon MC, Cram DS, Harrison LC: Glutamic acid decarboxylase 67-reactive T-cells: a marker of insulin-dependent diabetes. *J Exp Med* 177:535–540, 1993
13. Harrison LC, Honeymoon MC, De Aizpurua HJ, Schmidli RS, Colman PG, Tait BD, Cram DS: Inverse relations between humoral and cellular immunity to glutamic acid decarbolxylase in subjects at risk of insulin-dependent diabetes. *Lancet* 341:1365–1369, 1993
14. Keller RJ: Cellular immunity to human insulin in individuals at high risk for the development of type 1 diabetes mellitus. *J Autoimmunity* 3:321–327, 1990
15. Schloot NC, Roep BO, Wegmann D, Yu L, Chase HP, Wang T, Eisenbarth GS: Altered immune response to insulin in newly diagnosed compared to insulin-treated diabetic patients and healthy control subjects. *Diabetologia* 40:564–572, 1997
16. Rudy G, Stone N, Harrison LC, Colman PG, McNair P, Brusic V, French MB, Honeymoon MG, Tait B, Lew AM: Similar peptides from two β-cell autoantigens, proinsulin and glutamic acid decarboxylase, stimulate T-cells of individuals at risk for insulin-dependent diabetes. *Mol Med* 1:625–633, 1995
17. Hummel M, Durinovic-Bello I, Ziegler AG: Relation between cellular and humoral immunity to islet cell antigens in type 1 diabetes. *J Autoimmunity* 9:427–430, 1996
18. Harrison LC: Islet cell antigens in insulin-dependent diabetes: Pandora's box revisited. *Immunol Today* 13:348–352, 1992
19. Atkinson MA, Maclaren NK: Islet cell autoantigens in insulin-dependent diabetes. *J Clin Invest* 92:1608–1616, 1993
20. Ziegler AG, Hillebrand B, Rabl W, Mayhofer M, Hummel M, Mollenhauer U, Vordemann J, Lenz A, Standl E: On the appearance of islet associated autoimmunity in offspring of diabetic mothers: a prospective study from birth. *Diabetologia* 36:402–408, 1994
21. Heaton DA, Millward BA, Bray IP: Increased proinsulin levels as an early indicator of B-cell dysfunction in non-diabetic twins of type 1 (insulin-dependent) diabetic patients. *Diabetologia* 31:182–184, 1988
22. Spinas GA, Snorgaard O, Harrling SG, Oberholzer M, Berger W: Elevated proinsulin levels related to islet cell antibodies in first-degree relatives of IDDM patients. *Diabetes Care* 15:632–637, 1992
23. Lindgren GA, Hartling SG, Persson BE: Proinsulin levels in newborn siblings of type 1 (insulin-dependent) diabetic children and their mothers. *Diabetologia* 36:560–563, 1993
24. Kuglin B, Gries FA, Kolb H: Evidence of IgG autoantibodies against human proinsulin in patients with IDDM before insulin treatment. *Diabetes* 37:130–132, 1988
25. Kuglin V, Holder B, Bertrams J, Gruneklee D, Gries FA, Kolb H: Proinsulin autoantibodies: association with type 1 diabetes but not with islet cell antibodies, insulin autoantibodies or HLA-DR type. *J Autoimmun* 3:573–577, 1990
26. French MB, Allison J, Cram DS, Thomas HE, Cempsey-Collier M, Silva A, Georgious HM, Kay TW, Harrison LC, Lew AM: Transgenic expression of mouse proinsulin II prevents diabetes in nonobese diabetic mice. *Diabetes* 46:40–43, 1977
27. Schatz DS, Krischer J, Horne G, Riley WJ, Winter WE, Silverstein JH, Allen L, Shah S, Malone J, Maclaren NK: Islet cell antibodies predict the development of insulin-dependent diabetes in United States schoolchildren as effectively as in unaffected relatives. *J Clin Invest* 93:2403–2407, 1994
28. Atkinson MA, Bowman MA, Kao KJ, Dush PD, Simmel O, Maclaren NK: Lack of immune responsiveness to bovine serum albumin in insulin-dependent diabetes. *N Engl J Med* 329:1853–1858, 1993
29. Ellis TM, Ottendorfer E, Jodoin E, Salisbury PJ, She JX, Schatz DA, Atkinson MA: Cellular immune responses to β casein: elevated in but not specific for individuals with type I diabetes mellitus. *Diabetologia* 41:731–735, 1998
30. Bieganowska KD, Ausubel LJ, Modabber Y, Slovik E, Messersmith W, Hafler DA: Direct ex vivo analysis of activated, fas-sensitive autoreactive T-cells in human autoimmune disease. *J Exp Med* 185:1585–1594, 1997
31. Cheung R, Karjalainen J, Vandermeulen J, Singal DP, Dosch HM: T-cells from children with IDDM and sensitized to bovine serum albumin. *Scand J Immunol* 623–628, 1994
32. Pugliese A, Zeller M, Fernandez A Jr, Zalcberg LJ, Bartlett RJ, Ricordi C, Pietropaolo M, Eisenbarth GS, Bennett ST, Patel DD: The insulin gene is transcribed in the human thymus and transcription levels correlated with allelic variation at the INS VNTR-IDDM2 susceptibility locus for type 1 diabetes. *Nat Genet* 15:293–297, 1997
33. Wegmann DR, Norbury-Glasser M, Daniel D: Insulin-specific T-cells are a predominant component of islet infiltrates in pre-diabetic NOD mice. *Eur J Immunol* 24:1853–1857, 1994
34. Daniel D, Gill RG, Schloot N, Wegmann D: Epitope specificity, cytokine production profile and diabetogenic activity of insulin-specific T-cell clones isolated from NOD mice. *Eur J Immunol* 25:1056–1062, 1995
35. Atkinson MA, Maclaren NK, Luchetta R: Insulitis and diabetes in NOD mice reduced by prophylactic insulin therapy. *Diabetes* 39:933–937, 1990
36. Zhang ZJ, Davidson L, Eisenbarth G, Weiner HL: Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin. *Proc Natl Acad Sci U S A* 88:10252–10256, 1991
37. Muir A, Peck A, Clare-Salzler M, Song YH, Cornelius J, Luchetta R, Maclaren NK: Insulin immunization of nonobese diabetic mice induces a protective insulitis characterized by diminished intraislet interferon-gamma transcription. *J Clin Invest* 95:628–634, 1995
38. Karounos DG, Bryson JS, Cohen DA: Metabolically inactive insulin analog prevents type 1 diabetes in prediabetic NOD mice. *J Clin Invest* 100:1–5, 1997
39. Eisenbarth GS, Verge CF, Allen H, Rewers MJ: The design of trials for prevention of IDDM. *Diabetes* 42:941–947, 1993

Author Queries (please see Q in margin and underlined text)

Q1: Correct that ICA is defined correctly here in the Abstract?
Q2: Correct that ICA stands for islet cell cytoplasmic autoantigens? Have defined it at first mention here.
Q3: Please define DR.
Q3.5: Please expand PHA
Q4: Please check sentence carefully for your correct meaning.
Q4a: Is "or phase or proinsulin" correct?
Q5: Please provide the year for Ref. 20.

For Ref. 21 and 23, please list all authors.