The Relationship Between Humoral and Cellular Immunity to IA-2 in IDDM

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Autoantibodies to the neuroendocrine protein insulinoma-associated protein 2 (IA-2), a member of the tyrosine phosphatase family, have been observed in individuals with or at increased risk for IDDM. Because this disease is thought to result from a T-cell–mediated autoimmune destruction of the insulin-producing pancreatic β-cells, we analyzed humoral and cellular immune reactivity to this autoantigen to further define its role in the pathogenesis of IDDM. Peripheral blood mononuclear cells (PBMC) from individuals with newly diagnosed IDDM or at varying levels of risk for the disease were stimulated in vitro with the entire 42-kDa internal domain of IA-2 (amino acids 603–979), a series of control antigens (glutathionine-S-transferase, tetanus toxoid, *Candida albicans*, mumps, bovine serum albumin), and a mitogen (phytohemagglutinin). The frequency and mean stimulation index of PBMC proliferation against IA-2 was significantly higher in newly diagnosed IDDM subjects (14 of 33 [42%]; 3.8 ± 4.5 at 10 μg/ml) and autoantibody-positive relatives at increased risk for IDDM (6 of 9 [66%]; 3.9 ± 3.2) compared with autoantibody-negative relatives (1 of 15 [7%]; 1.8 ± 1.0) or healthy control subjects (1 of 12 [8%]; 1.5 ± 1.0). The frequencies of cellular immune reactivities to all other antigens were remarkably similar between each subject group. Sera from 58% of the newly diagnosed IDDM patients tested were IA-2 autoantibody positive. Despite investigations suggesting an inverse association between humoral and cellular immune reactivities against islet-cell–associated autoantigens, no such relationship was observed (r_s = 0.18, P = 0.39) with respect to IA-2. These studies support the autoantigenic nature of IA-2 in IDDM and suggest the inclusion of cellular immune responses as an adjunct marker for the disease. *Diabetes* 47:566–569, 1998

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IA-2, insulinoma-associated protein 2; IAA, insulin autoantibody; JDF, Juvenile Diabetes Foundation; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; 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 membranes/autoantigens (6), 38-kDa islet-cell autoantigens (7,8), insulin (6,9), ICA69 (10), ICA512 (6), GAD_{65}, and GAD_{67} (11,12). One report has suggested that measurement of cellular immune reactivity against a β-cell autoantigen (i.e., GAD_{67}) may be superior to determining the level of autoantibodies to the same antigen in terms of disease prediction (13). In addition, this study reported an inverse relationship between humoral and cellular immunity to GAD. When taken together with reports indicating similar inverse relationships in immunity to ICA69 and insulin (6,10), such information is often cited as supportive evidence for a T-helper-1–like nature to the pathogenic mechanism underlying the formation of IDDM.

Recently, we described a new member of the protein tyrosine phosphatase family, insulinoma-associated protein 2 (IA-2; 14). IA-2 cDNA predicts a transmembrane protein of 979 amino acids in length and a molecular mass of 105,847 Da. The gene is expressed in islet, insulinoma (human, mouse, and rat), brain, and neuroendocrine cells (14). We and others have demonstrated the presence of autoantibodies to IA-2 or its fragments in newly diagnosed IDDM patients, as well as in individuals at increased risk for the disease (15–18). Because autoantibodies to IA-2 and GAD_{65} appear to occur at the highest frequency in people with IDDM (as compared with other autoantibodies to recombinant β-cell autoantigens) and because cellular immune mechanisms are most likely the principal mediators of β-cell destruction, we investigated the PBMC proliferative response to IA-2.

RESEARCH DESIGN AND METHODS

Subjects. Blood samples were obtained from 69 individuals involved in our ongoing studies of the natural history of IDDM (20), including 33 newly diagnosed IDDM subjects (age 12.5 ± 9.9 years [range 2–54]). Samples were collected only from patients within 3 days of the onset of initial insulin therapy. In addition, samples were obtained from nine ICA and/or insulin autoantibody (IAA) positive relatives of an IDDM proband, as well as 15 autoantibody-negative first-degree relatives of the aforementioned IDDM subjects of this study. For the autoantibody-positive relative group, only those with ICA values ≥20 Juvenile Diabetes Foundation (JDF)

TABLE 1
Cellular immune reactivities in people with or at varying risks for IDDM

Subject	*n*	PHA	IA-2 (1)	IA-2 (2)	Tetanus	*Candida*	Mumps	BSA	GST
Healthy control	12	472 ± 418	1.6 ± 1.1	1.5 ± 1.0	12.8 ± 14.3	3.1 ± 5.2	2.4 ± 1.9	1.0 ± 0.7	1.5 ± 1.3
Autoantibody-negative relatives	15	318 ± 286	1.8 ± 0.7	1.8 ± 1.0	5.1 ± 6.5	1.7 ± 0.8	5.0 ± 9.1	1.2 ± 0.5	NT
Autoantibody-positive relatives	9	623 ± 383	3.4 ± 3.0	3.9 ± 3.2	13.6 ± 21.0	4.4 ± 3.8	9.1 ± 14.1	2.0 ± 0.7	NT
Newly diagnosed IDDM	33	702 ± 561	2.7 ± 2.5	3.8 ± 4.5	10.2 ± 8.5	2.4 ± 2.3	2.8 ± 2.2	1.1 ± 0.4	1.9 ± 1.9

Proliferation values are presented as stimulation indexes (means ± SD). IA-2 (1) represents antigen at a 1 μg/ml concentration; IA-2 (2) represents antigen at a 10 μg/ml concentration. GST, glutathione S-transferase; NT, not tested.

units or IAA levels ≥109 units (a level for positivity previously described) (15,19,20) were included in this investigation. With this criteria, two of these nine subjects were ICA and IAA positive; four were ICA positive and IAA negative; and three were ICA negative and IAA positive. Twelve healthy volunteers 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.

Autoantibody analyses. Islet cell cytoplasmic autoantibodies were determined by indirect immunofluorescence using unfixed, snap-frozen human pancreas as previously described (20). All positive serum samples were expressed in JDF units by comparing the endpoint dilution of each positive serum to a standard calibration dilution curve using the international JDF reference serum accepted by the Immunology of Diabetes Workshops. Insulin autoantibodies, GAD_{65} autoantibodies (GADA), and IA-2 autoantibodies (IA-2A) were determined and reference ranges for positivity established as previously described (15,19,20). Our laboratory is a regular participant in international autoantibody workshops designed for assay standardization and proficiency.

HLA typing. HLA-DR types were determined using sequence-specific priming techniques as previously described (20). The respective distribution of specific HLA-DR combinations in subjects submitting to genetic testing were as follows: newly diagnosed IDDM subjects (n = 25, 5 DR 3/4, 6 DR 4/X, 11 DR 3/X, and 3 DR X/X); autoantibody-positive relatives of an IDDM proband (n = 7, 2 DR 3/4, 1 DR 4/X, 1 DR 3/X, and 3 DR X/X); autoantibody-negative first-degree relatives of the IDDM subject (n = 13, 2 DR 3/4, 3 DR 4/X, 6 DR 3/X, and 2 DR X/X); and healthy control subjects (n = 10, 1 DR 3/4, 2 DR 4/X, 2 DR 3/X, and 5 DR X/X).

Antigens. Full-length IA-2 cDNA was prepared as previously described for studies of humoral immunity (15). Because of marked proteolytic instability of the extracellular domain of the IA-2 molecule, the internal domain of IA-2 (amino acids 603–979) was prepared as previously described (19) for studies analyzing cellular immunity. IA-2 preparations were free of endotoxin (i.e., below detection limits of 0.06 EU/ml) as determined in the limulus amoebocyte lysate assay (BioWhittaker, Walkersville, MD) and were homogeneous by SDS-PAGE. Glutathionine-S-transferase was prepared as previously described (15). Other antigens were purchased from commercial sources: tetanus toxoid (Massachusetts Public Health Biological Laboratories, Boston, MA); *Candida albicans* extract (Berkeley Biologicals, Berkeley CA); mumps extract (Merck, Sharp, and Dohme, Westpoint, PA); bovine serum albumin (BSA) (Sigma, St. Louis, MO); and phytohemagglutinin (PHA) (Sigma).

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 positive sera) for 7 days (95% air/5% CO_2) (11,21). The cells were incubated with the following antigen/mitogens in triplicate cultures: 1 and 10 μg/ml IA-2; 1 μg/ml glutathionine-S-transferase; 10 μg/ml BSA; 10 μg/ml (0.875 Lyons floculating units/ml) tetanus toxoid; 20 μg/ml *Candida albicans* extract; 80 tissue culture infectious doses $(TCID)_{50}$/ml mumps extract; and 10 μg/ml PHA. Added to each well 18 h before harvest was 1 μCi [^{3}H]thymidine. Thymidine incorporation was assessed by Matrix 9600 β-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 incorporated in the presence of antigen divided by the mean counts per minute incorporated in antigen absence (medium alone). A positive cellular immune response was defined as an SI of ≥mean plus 2 SD of the healthy control responses for test antigens or an SI of ≥3 for reactivities against recall antigens. Because two concentrations (10-fold in difference) of IA-2 were utilized, we applied a previously reported method (6) wherein the SI of the antigenic concentration with the highest reactivity per individual was included in these analyses for determination of the frequency of response. PHA responses were measured after 4 days. Analysis of differences between study groups was performed using both ANOVA and Fisher's exact test. Relationships between humoral and cellular immunity were evaluated by Spearman's rank-sum correlation.

RESULTS

PBMC proliferation in medium alone and in response to recall antigen (tetanus toxoid, mumps, *Candida*), control antigen (BSA), mitogen (PHA), and IA-2 was evaluated in short-term in vitro cultures established from the newly diagnosed IDDM patients, autoantibody-positive and -negative relatives, and healthy control subjects. With respect to the level (i.e., mean SI) of proliferation to recall or control antigens, no significant differences were observed between subject groups in their response to tetanus toxoid or BSA (Table 1). Elevated cellular immune reactivities were observed (Table 1) in the autoantibody-positive relatives in response to *Candida albicans* (P = 0.05 vs. healthy control subjects) and mumps (P = 0.02 and 0.04 for newly diagnosed IDDM patients and healthy control subjects, respectively). However, the mean response of autoantibody-positive relatives to these antigens was strongly influenced by the inclusion of a single subject with marked (SI > 40) immune responses to these recall agents. Furthermore, the frequency (% positive) of responses to the recall and control antigens were similar (NS) in all subject groups. Mitogenic responses were high in all study groups (Table 1), with no significant differences observed in the mean SI between the study groups.

With respect to immune responses to IA-2, modest differences were observed in both the frequency and mean SI of responses between the study groups. Comparison of the mean SI with IA-2 (1 μg/ml concentration) in autoantibody-positive relatives versus normal control subjects revealed somewhat higher values in the group at increased risk for IDDM (P = 0.06; Table 1). At the higher antigen concentration (i.e., 10 μg/ml), further differences between the mean SI of the newly diagnosed IDDM subjects and autoantibody-positive relatives versus the control group were observed (P = 0.06 and 0.05, respectively; Table 1). Additional support for the specificity of the IA-2–stimulated response in the newly diagnosed IDDM subjects was observed through analysis of the PBMC reactivity to glutathionine-S-transferase (i.e., a component of the recombinant expression system). Neither the mean SI (NS) (Table 1) nor the frequency of a positive anti–glutathione S-transferase response (1 of 26 [4%]; 1 of 12 [8%]; P = 1.0) was different between the IDDM and control groups, respectively.

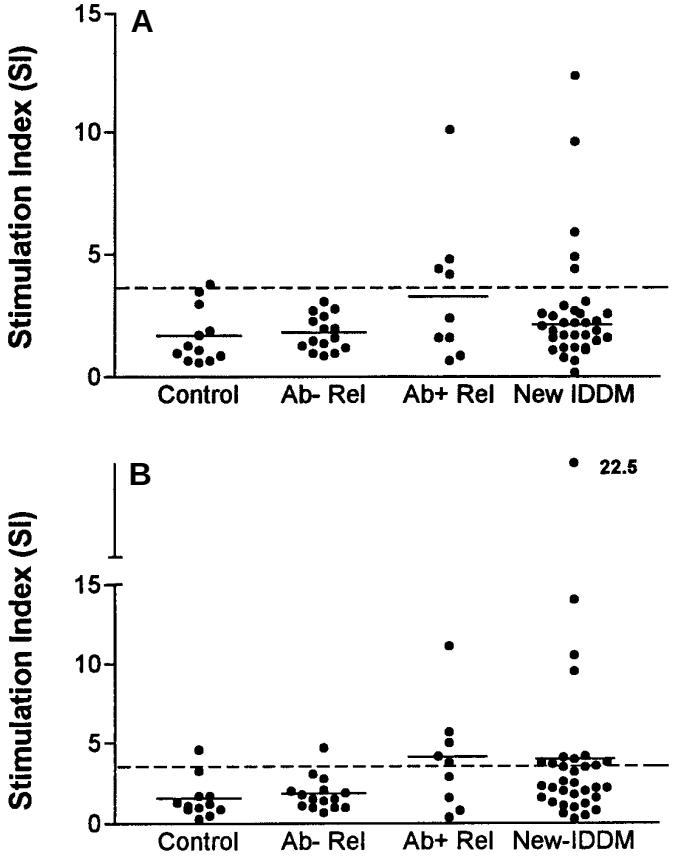

FIG. 1. Proliferation of PBMCs in response to recombinant human IA-2. Aliquots of PBMC (1×10^6) were cultures of medium containing (*A*) 1 µg/ml or (*B*) 10 µg/ml recombinant IA-2. Dashed lines represent thresholds for positive reactivity. Solid bars indicate the group means.

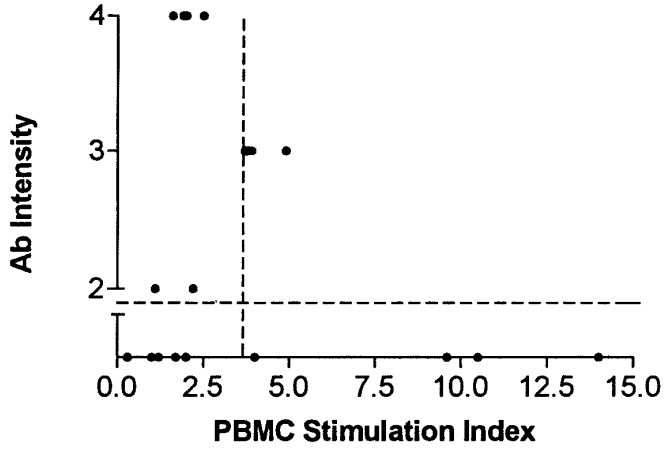

FIG. 2. Relationship between IA-2 autoantibodies and IA-2 reactive PBMCs in patients with newly diagnosed IDDM. Dashed lines represent thresholds for positive reactivity.

Using the formula (see METHODS) wherein the concentration of IA-2 providing the maximal SI was used to define the response in an individual, PBMCs from 14 of 33 (42%) newly diagnosed IDDM patients were IA-2 reactive compared with only 1 of 15 (7%) autoantibody-negative relatives and 1 of 12 (8%) healthy control subjects (P = 0.02 and 0.07, respectively). A similar increase in frequency of cellular immune reactivity against IA-2 was observed (Fig. 1) through comparison of autoantibody-positive relatives (6 of 9 [66%]) with autoantibody-negative relatives (P = 0.01) and healthy control subjects (P = 0.004).

Previous reports indicate that IA-2 reactive autoantibodies are present in 50–60% of recent-onset IDDM patients (15–19). Consistent with those reports, sera from 11 of 19 (58%) newly diagnosed IDDM patients whose samples were available for such study were IA-2 positive. Other IDDM-associated autoantibodies were present in this group at the following frequencies: ICA 68% (17/25); IAA 30% (7/23); and GADA 64% (16/25). These frequencies are indicative of our previous experiences with these markers of IDDM. Statistical analysis revealed no association between humoral and cellular immune reactivities to IA-2 (r_s = 0.17, P = 0.39, Fig. 2). However, it is interesting to note that the four individuals with the highest levels of antibody reactivity against IA-2 lacked an identifiable PBMC response to this antigen. Conversely, the three individuals having the highest levels of cellular immune reactivity to IA-2 lacked measurable IA-2 autoantibodies.

DISCUSSION

Our studies demonstrate that a significant yet modestly greater proportion of IDDM patients or those at increased risk for the disease display a PBMC response to IA-2 compared with individuals at low risk for IDDM. Rather than an issue of the presence or absence of IA-2 reactive cells, we view these results as supporting the concept of increased precursor frequency in the peripheral blood of those prone to or with IDDM. Our work confirms and expands on the previous reports by Durinovic-Bello and co-workers (6,22) who demonstrated specific immunity to ICA512, also a fragment of IA-2, in people with IDDM. Those reports and our studies reported herein indicate similarities in terms of the frequency of an autoantigenic response, but those authors did not investigate cellular immune responses in autoantibody-negative relatives—an important population when examining factors related to genetic susceptibility and determining the ability to accurately identify individuals at increased risk for the disease.

Whereas the purity of the recombinant IA-2 proteins used in this study was extremely high, we cannot exclude the possibility that some of the PBMC proliferative responses observed resulted from trace bacterial contaminants in the cultures. However, proliferative responses to bacterial contaminants cannot explain why PBMC responses were observed preferentially in IDDM patients and autoantibody-positive relatives than in autoantibody-negative relatives or healthy control subjects.

The measurement of cellular immune responses against antigens is subject to multiple methods and restrictions in experimental design: monitoring cellular proliferation in short-term cell lines stimulated in vitro with antigen and interleukin-2; analyzing the precursor frequency of reactive cells; determining the specific cytokine(s) produced in response to antigen challenge; fine matching of subject groups for HLA; and using specific peptides for binding and/or stimulation. While not utilizing these methods, we did investigate rela-

tives with high and low risk for the disease to control in the most feasible manner the issues of HLA-matching and risk for IDDM in determining the level of immune responsiveness. The results of these efforts support the notion of enhanced cellular immune responses against IA-2 in those at the highest risk for the disease. Studies are currently in progress to examine whether specific major histocompatibility complex (MHC) class II allele/IA-2 peptide combinations can be identified in IDDM patients with B- and T-cell responses to IA-2, as well as to determine the aforementioned issue of precursor frequencies in peripheral blood.

These findings do not support previous reports (10,13,22) identifying an inverse relationship between humoral and cellular immunity to a β-cell autoantigen in IDDM. The reasons for these contrasting results are unclear and may reflect, among many possible explanations, a situation where some β-cell antigens elicit inverse immune responses whereas others do not. In addition, although an inverse relationship was not identified by analysis of this population, anecdotal cases displaying this effect were clearly identified. Clearly, the controversy surrounding the relationship between humoral and cellular immunity to β-cell autoantigens will continue.

In conclusion, we have documented an autoantigen-specific PBMC proliferative response in a high percentage of patients with or at increased risk for IDDM. Further characterization of cytokines elicited in response to this antigen, as well as the MHC binding epitopes of IA-2, should lead to considerable insight into the mechanisms of autoimmunity underlying IDDM.

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