Two Distinct Glutamic Acid Decarboxylase Autoantibody Specificities in IDDM Target Different Epitopes

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Although most individuals with insulin-dependent diabetes mellitus (IDDM) have autoantibodies to glutamic acid decarboxylase (GAD), antibodies to GAD are also present in some individuals with a low risk of developing diabetes. The GAD autoantibodies of IDDM are specific for the GAD_{65} isoform, do not bind denatured GAD protein, and target epitope(s) dependent on conformation of the protein. However, the IDDM epitopes have been difficult to further define because the antibodies do not bind GAD protein fragments or synthetic peptides. Since the GAD_{67} isoform is highly homologous to GAD_{65} but is usually not a target of the GAD autoantibodies in IDDM sera, we created six GAD_{65}/GAD_{67} chimeric proteins to maintain the overall GAD protein conformation and used these chimeric proteins to map conformation-dependent epitopes of GAD_{65} targeted by IDDM sera. We find that the GAD binding present in most IDDM sera (n = 11 of 12) is composed of two distinct GAD antibody specificities that target different conformation-dependent regions of the GAD_{65} protein, one that is located between amino acids 240 and 435 (termed IDDM-E1) and one that is located between amino acids 451 and 570 (termed IDDM-E2). One IDDM serum (n = 1 of 12) bound only the IDDM-E1 region. Identification of epitopes targeted by IDDM sera may allow one to distinguish between GAD antibody-positive individuals at high and low risk of developing IDDM and to determine if differences in the autoimmune repertoire directed at GAD are present. The chimeric GAD_{65}/GAD_{67} proteins may also be useful in designing GAD assays specific for IDDM. *Diabetes* 44: 216–220, 1995

A major autoantigen for both humoral and cellular autoimmunity in insulin-dependent diabetes mellitus (IDDM) is glutamic acid decarboxylase (GAD) (1–3). The possible role of GAD as the primary autoantigen in IDDM has been suggested by recent demonstrations of cellular autoimmunity directed at GAD in IDDM (4,5) and of autoimmunity directed at GAD in an animal model of IDDM (6,7). GAD autoantibodies are an important marker of the autoimmune process of IDDM because they are present in the majority of individuals with new-onset diabetes and in individuals in the prediabetic stage of the disease (8–10). Improvements and automation of the GAD antibody assay have led to the suggestion that this measurement may be a useful marker to identify individuals at high risk for developing IDDM.

However, GAD autoantibodies are also found in some individuals without IDDM, including individuals with stiff-man syndrome (SMS), polyglandular failure type I, or a restricted or β-cell-specific islet cell autoantibody (ICA) pattern (2,11–13). These individuals have a low risk of developing IDDM. Differences and heterogeneity in the GAD autoantibody profile between these nondiabetic individuals and those with IDDM suggest that disease-specific epitopes may exist (13–16). For example, SMS sera bind GAD protein fragments and denatured GAD protein used in immunoblotting (1,14,15).

In contrast, GAD antibodies in IDDM do not bind denatured GAD protein, GAD fragments, or GAD peptides, which implies that these GAD antibodies bind an epitope that is dependent on protein conformation (1,16,17). The COOH-terminal, two-thirds of the GAD_{65} protein, contains the region targeted in IDDM, but when smaller fragments of GAD_{65} protein are used, most binding by IDDM sera is lost (16,17). Hence, further localization of the IDDM epitopes is not possible with the methodology used to map GAD epitopes targeted by SMS or restricted ICA sera.

We have overcome these experimental limitations in localizing IDDM-related GAD epitopes by designing recombinant GAD proteins that maintain the conformation of the GAD protein. Our experimental approach is based on the following: *1*) GAD antibodies in IDDM bind GAD_{65} but not GAD_{67} (16,18,19); *2*) GAD_{65} and GAD_{67} are highly homologous in the middle and COOH-terminal regions of the protein (20); and *3*) GAD_{65} and GAD_{67} probably have similar protein conformations. By exchanging regions of the GAD_{65} and GAD_{67} cDNAs, we created chimeric GAD proteins and examined the binding of IDDM antibodies to different regions of the GAD_{65} protein.

RESEARCH DESIGN AND METHODS

Creation of GAD_{65}/GAD_{67} chimeric proteins. To prepare chimeric GAD_{65}/GAD_{67} cDNAs, a unique restriction enzyme site was introduced into the rat GAD_{65} cDNA or human GAD_{67} cDNA by polymerase chain reaction (PCR) or site-directed mutagenesis. Introduction of the unique restriction site in the cDNAs did not alter the predicted amino acid sequence and occurred in a stretch of amino acids that were identical in GAD_{65} and GAD_{67}. PCR amplification was performed using either the human GAD_{67} or rat GAD_{65} plasmid cDNA and primers to incorporate a 1-, 2-, or 3-base pair mutation. Site-directed mutagenesis was performed with the Transformer Mutagenesis system (Clontech, Palo Alto, CA). The presence of the mutation introduced by PCR or site-directed mutagenesis was confirmed by dideoxy sequencing. After introduction

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Received for publication 9 September 1994 and accepted 6 October 1994.

IDDM, insulin-dependent diabetes mellitus; GAD, glutamic acid decarboxylase; SMS, stiff-man syndrome; ICA, islet cell antibody; MICA, monoclonal islet cell antibody; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; TBST, Tris-buffered saline/0.05% Tween 20; PAS, protein A-sepharose; HLA, human leukocyte antigen.

of the desired restriction enzyme site into one GAD isoform, a cDNA fragment was exchanged with the corresponding cDNA fragment from the other GAD cDNA to produce a chimeric cDNA by standard subcloning techniques. For the production of GAD RNA, all wild-type and chimeric GAD cDNAs were subcloned into the plasmid, pGOV, which contains a T7 RNA polymerase promoter (16).

The following nomenclature for GAD chimera was used: GAD_{65} or GAD_{67} (amino acid number of that GAD species present in the GAD chimera)/GAD_{65} or GAD_{67} (amino acid number of that GAD species present in the GAD chimera). The GAD_{65} protein has 585 amino acids, and the GAD_{67} protein has 594 amino acids. To create the chimera GAD_{65} (1–195)/GAD_{67} (205–594), a *Hinc*II site was introduced at the appropriate location of the GAD_{67} cDNA by PCR, and the resulting GAD_{67} fragment was exchanged with the corresponding fragment of the GAD_{65} cDNA (using a native *Hinc*II site in the GAD_{65} cDNA). To create the chimera GAD_{67} (1–230)/GAD_{65} (221–585), an *Afl* II was introduced at the appropriate location of the GAD_{65} cDNA by PCR, and the resulting GAD_{65} fragment was exchanged with the corresponding fragment of the GAD_{67} cDNA (using a native *Afl* II site in the GAD_{67} cDNA). To create the chimera GAD_{65} (1–442)/GAD_{67} (452–594) and the chimera GAD_{67} (1–451)/GAD_{65} (443–585), a unique *Stu* I site was introduced by site-directed mutagenesis in the GAD_{67} cDNA. Corresponding cDNA fragments that encoded GAD_{65} (1–442) and GAD_{67} (1–451) were exchanged (using a native *Stu* I site in the GAD_{65} cDNA) to produce the chimera GAD_{65} (1–442)/GAD_{67} (452–594) and the chimera GAD_{67} (1–451)/GAD_{65} (443–585). The chimera GAD_{67} (1–230)/GAD_{65} (221–442)/GAD_{67} (452–594) was created by digesting GAD_{67} (1–230)/GAD_{65} (221–585) and GAD_{65} (1–442)/GAD_{67} (452–594) with *Stu* I and exchanging cDNA fragments. The chimera GAD_{65} (1–195)/GAD_{67} (205–441)/GAD_{65} (443–585) was created by digesting GAD_{65} (1–195)/GAD_{67} (205–594) with *Afl* II and exchanging this fragment with the corresponding fragment in GAD_{67} (1–451)/GAD_{65} (443–585). The *Afl* II and *Stu* I sites are unique for GAD_{65} and GAD_{67}, respectively.

The chimeric composition of the GAD cDNAs was confirmed by restriction enzyme digestion and dideoxy DNA sequencing. After in vitro translation, chimeric proteins were of the expected molecular weight by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. In addition, chimeric proteins containing the COOH-terminus of GAD_{65} reacted with the monoclonal antibody GAD-6 (21), which is specific for this region of GAD_{65} (16). The GAD-6 monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA; National Institute of Child Health and Human Development contract no. 1-HD-2-3144)

Serum samples. Serum samples were collected from individuals with new-onset diabetes (n = 12) and from control, nondiabetic individuals and stored at −20°C. The clinical features of the IDDM sera have been previously described (16).

Preparation and immunoprecipitation of GAD protein. Full-length rat GAD_{65}, human GAD_{67}, and the chimeric GAD_{65}/GAD_{67} cDNAs were transcribed into GAD RNA using T7 RNA polymerase in an in vitro transcription system (Megascript, Ambion) (16). Metabolically labeled recombinant GAD protein was prepared by in vitro translation of GAD RNA with reticulocyte lysate (Promega, Madison, WI) using an amino acid mixture containing [^{35}S]methionine (1,200 Ci/mmol) (16). For immunoprecipitation, 30 μl serum (1:1–1:20 dilution) was incubated with 30 μl of metabolically labeled GAD protein (50,000 Ci/min) for 12–15 h at 4°C (16). All dilutions of serum and GAD protein were made with Tris-buffered saline/0.05% Tween 20 (TBST). Protein A-sepharose (PAS, Sigma, St. Louis, MO) was washed and resuspended in TBST to a 2% solution. Two microliters of PAS (final bed volume) was added to the GAD protein/serum reaction and mixed for 1 h at 4°C by gentle rotation. The PAS pellets were then washed three times with 500 μl TBST, resuspended in 100 μl of 1% SDS, and counted by β-scintillation counting. All serum samples were assayed in duplicate. The results are presented as the means ± SE of at least three separate experiments. Our laboratory participated in the GAD antibody workshops (22) sponsored by the Immunology of Diabetes Workshop (1993, 1994). In the 1994 workshop, our GAD_{65} assay was positive in 82% of sera from individuals with new-onset diabetes, 100% of sera from individuals with prediabetes, and 3% of normal individuals.

Competitive inhibition of antibody binding to GAD protein. Unlabeled, full-length GAD_{65}, GAD_{67}, and chimeric GAD_{65}/GAD_{67} proteins were prepared in the reticulocyte lysate system as detailed above, except that the amino acid mixture contained unlabeled methionine rather than [^{35}S]methionine. For the blocking studies, unlabeled GAD protein was incubated with the test serum for 3 h at room temperature; this mixture was then added to 50,000 Ci/min of ^{35}S-labeled GAD protein and incubated for 12–15 h at 4°C (16). PAS was added, and immunoprecipitation was performed as described above. The immunoprecipitate was analyzed by scintillation counting and expressed as the percentage of GAD protein immunoprecipitated after preincubation with buffer without unlabeled GAD protein. All serum samples were assayed in duplicate. The results are presented as the means ± SE of at least three separate experiments.

RESULTS AND DISCUSSION

IDDM sera bind two regions of GAD_{65} protein. Most IDDM sera bind GAD_{65}, but not GAD_{67} (18,19) (Fig. 1*A* and *B*). Although GAD_{65} and GAD_{67} differ substantially in the NH_2-terminal one-third of the protein and the first eight amino acids of GAD_{65} are targeted by antibodies in SMS (14,15), the GAD_{65}-specific autoantibodies in IDDM sera do not bind a chimeric protein consisting of the NH_2-terminal one-third of GAD_{65} and the COOH-terminal two-thirds of GAD_{67} [GAD_{65} (1–195)/GAD_{67} (205–594)] (Fig. 1*A* and *B*). Conversely, IDDM sera bind the chimera GAD_{67} (1–230)/GAD_{65} (221–585) in equivalent amounts as full-length GAD_{65} (Fig. 1*A* and *B*). This confirms previous reports that the conformation-dependent epitope targeted in IDDM is located in the COOH-terminal two-thirds of GAD_{65} (16,17).

Our results indicate that there are two regions of the COOH-terminal two-thirds of GAD_{65} that are targeted by IDDM sera. Most IDDM sera bind equal amounts of both GAD_{65} (1–442)/GAD_{67} (452–594) and GAD_{67} (1–451)/GAD_{65} (443–585) (Fig. 1*A* and *B*). Some IDDM sera bind more GAD_{65} (1–442)/GAD_{67} (452–594) than GAD_{67} (1–451)/GAD_{65} (443–585) (Fig. 1*B*).

Both regions of GAD protein contribute to total GAD binding in IDDM sera. To further narrow the regions targeted by the autoantibodies and to assess the relative contribution of the two regions to total GAD binding in IDDM sera, two additional GAD chimera, GAD_{67} (1–230)/GAD_{65} (221–442)/GAD_{67} (452–594) and GAD_{65} (1–195)/GAD_{67} (205–441)/GAD_{65} (443–585) were created. A chimera containing only amino acids 221–442 of GAD_{65} or a chimera containing amino acids 442–585 of GAD_{65} was used to inhibit the binding of IDDM sera to full-length GAD_{65}. Two patterns of antibody binding in IDDM were observed (Fig. 2). In the first pattern, preincubation of IDDM sera with either GAD_{67} (1–230)/GAD_{65} (221–442)/GAD_{67} (452–594) or GAD_{65} (1–195)/GAD_{67} (205–441)/GAD_{65} (443–585) partially inhibited the binding to full-length GAD_{65} (Fig. 2). However, when the sera were preincubated with both chimeric GAD proteins, the binding to full-length GAD_{65} was completely inhibited (Fig. 2). These results indicate the presence of two distinct antibody specificities that target different regions of GAD_{65} and contribute to the total GAD binding in IDDM sera. In the second pattern, only the GAD_{67} (1–230)/GAD_{65} (221–442)/GAD_{67} (452–594) chimera was required to inhibit the binding to full-length GAD_{65} (Fig. 2), indicating that in this IDDM serum, an antibody specificity for amino acids 221–442 is responsible for total GAD binding. If such sera also contain a second GAD specificity that targets amino acids 443–585, this reactivity is present at a much lower titer. Our findings indicate that at least one antibody targets each region. The possibilities that more than one antibody may be responsible for the binding to that region or that more than one epitope within that region may be targeted will require further study.

Identification of amino acids targeted by IDDM sera. Since GAD_{65} and GAD_{67} are highly homologous, differences

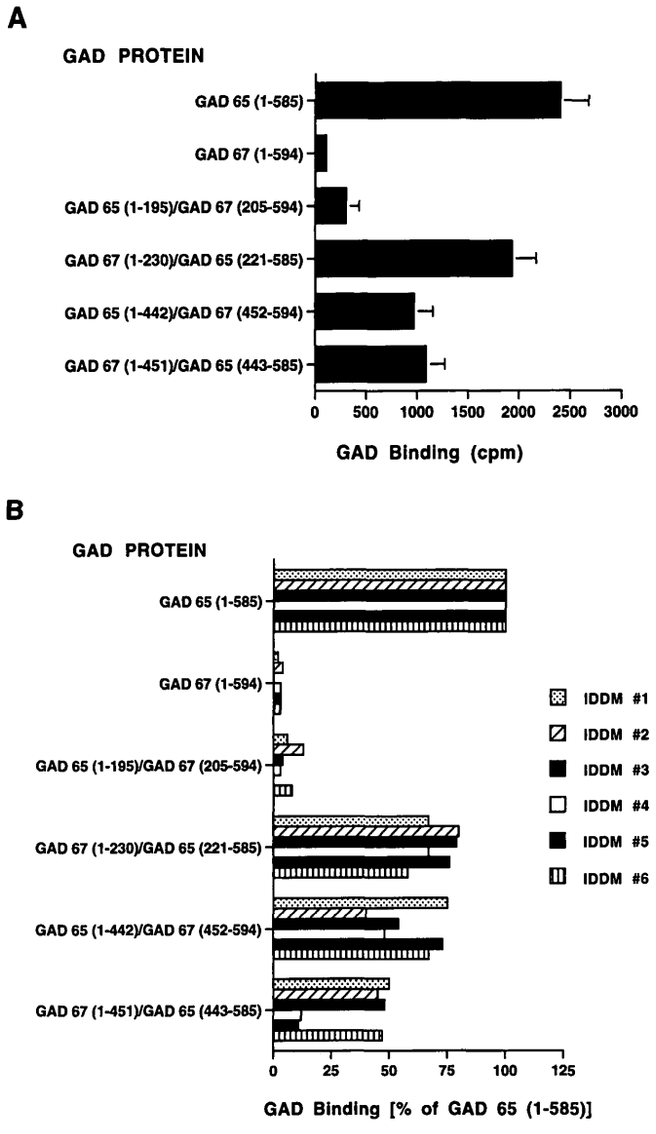

FIG. 1. Binding of GAD chimeric proteins by IDDM sera. *A*: immunoprecipitation of GAD_{65}, GAD_{67}, and chimeric GAD_{65}/GAD_{67} proteins was performed with one IDDM serum that binds GAD_{65}, but not GAD_{67}. Chimeric GAD proteins were synthesized as described in METHODS, and the amount immunoprecipitated (Ci/min) is shown on the *x*-axis. The horizontal bar shows the mean ± SE of three to four separate experiments. The following nomenclature for GAD chimera was used: GAD_{65} or GAD_{67} (amino acid number of that GAD species present in the GAD chimera)/GAD_{65} or GAD_{67} (amino acid number of that GAD species present in the GAD chimera). The binding to GAD_{67} and GAD_{65} (1–195)/GAD_{67} (205–594) by IDDM sera was not different from that of normal sera. *B*: as described in *A*, immunoprecipitations of the GAD chimera were performed with six IDDM sera. Since the amount of GAD protein immunoprecipitated by the sera varied from 2,000 to 10,000 Ci/min, the amount of GAD immunoprecipitated is expressed as a percentage of GAD_{65} immunoprecipitated (defined as 100%). Each serum is shown by a bar of different pattern. For some chimera, the bar is too small to be seen. The horizontal bar shows the mean of two to four separate experiments for each serum. The binding to GAD_{67} and GAD_{65} (1–195)/GAD_{67} (205–594) by IDDM sera was not different from that of normal sera.

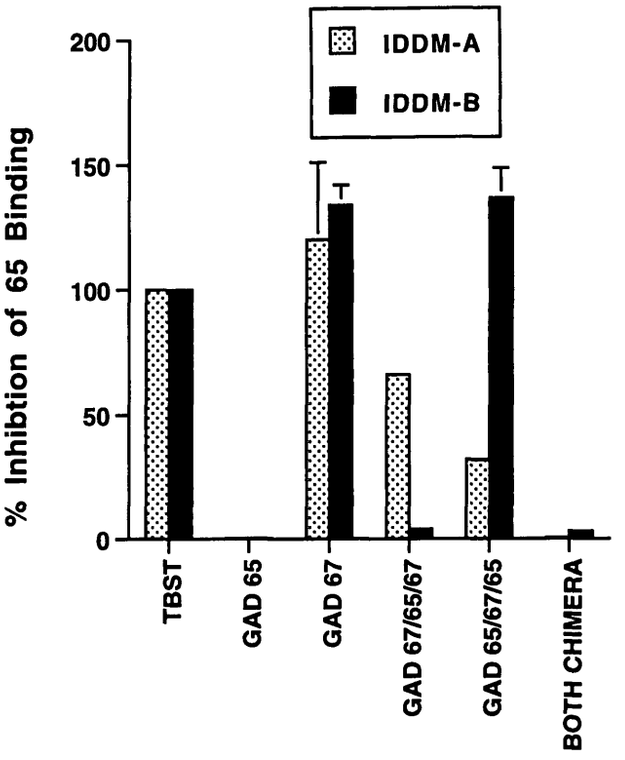

FIG. 2. Contributions of two antibody specificities to total GAD binding in IDDM sera. Two IDDM sera (IDDM-A and IDDM-B) were preincubated with either full-length GAD_{65}, full-length GAD_{67}, GAD_{67} (1–230)/GAD_{65} (221–442)/GAD_{67} (452–594) (termed GAD_{67}/65/67), GAD_{65} (1–195)/GAD_{67} (205–441)/GAD_{65} (443–585) (termed GAD_{65}/67/65), or both chimera. IDDM-A serum was used at a 1:10 dilution, and IDDM-B serum was used at a 1:20 dilution. The amount of a particular chimeric protein was the same when added alone or with the other chimeric protein. Metabolically labeled GAD_{65} was then added and immunoprecipitated as described for Fig. 1*A*. The percentage of GAD_{65} immunoprecipitated after preincubation with each GAD chimera is shown on the *y*-axis. The *x*-axis label shows the GAD protein with which the serum was preincubated. The amount of GAD_{65} immunoprecipitated after preincubation with reticulocyte lysate without GAD protein (TBST) is defined as 100%. The mean ± SE of the three sera (three measurements/serum) are shown. Some error bars are too small to be seen. Similar results were observed with two other IDDM sera.

between the two isoforms within the two regions of GAD_{65} targeted by IDDM sera may identify amino acids responsible for the binding of IDDM sera to GAD_{65}, but not GAD_{67}. After excluding amino acids that are identical in the GAD_{65} and GAD_{67} isoforms, the two areas of binding can be narrowed to amino acids 240–435 and amino acids 443–585. The GAD_{65} and GAD_{67} isoforms are 72 and 64% identical in these two regions, respectively. Since rat GAD_{65} (used for these comparisons) and human GAD_{65} are >98% identical in these regions, similar results were found with comparisons of human GAD_{65} and human GAD_{67} (data not shown). Based on nomenclature suggested by De Camilli and colleagues (14) for GAD epitopes in SMS, we propose to label the epitope contained in amino acids 240–435 IDDM-E1 and the epitope contained in amino acids 451–570 IDDM-E2. Most of the differences in protein sequence in GAD_{65} and GAD_{67} in the regions of IDDM-E1 and IDDM-E2 are single amino acids. One area of marked difference in the two GAD isoforms in the region of IDDM-E2 (between amino acids 505 and 517 where 10 of 13 amino acids are different) suggests a possible area for future study (Fig. 3). Additional studies using site-directed mutagenesis should allow for precise identification of the amino acids targeted by the GAD antibodies in IDDM sera.

To assess the frequency of GAD antibodies to these two regions, we measured the binding to IDDM-E1 and IDDM-E2 of GAD_{65} with sera from individuals with new-onset IDDM (n = 12). Most IDDM sera (n = 11 of 12) bound both IDDM-E1 and IDDM-E2 (Fig. 4). One IDDM sera bound only IDDM-E1.

The two distinct antibody specificities present in IDDM sera may be identical to monoclonal islet cell antibody

FIG. 3. Amino acid comparison of GAD_{65} and GAD_{67} in the region of IDDM-E2. The amino acid sequence, using the single letter amino acid notation system, for GAD_{65} (rat) and GAD_{67} (human) were aligned using the program MacDNASIS Pro (Hitachi, San Bruno, CA). The amino numbers for each protein are shown at the NH_2-terminal region of each protein sequence (left). Amino acids that are identical between GAD_{65} and GAD_{67} are shaded. A region of marked dissimilarity of amino acids is marked with a horizontal line.

(MICA) 1 and MICA 3 isolated from a single patient with IDDM by Richter et al. (17). Comparative studies with these monoclonal antibodies and IDDM sera are important because monoclonal antibodies derived from a single individual with IDDM may not be representative of IDDM sera, and the contribution of a particular monoclonal antibody species to the total GAD autoantibody pool cannot be estimated. The relative amount of a particular GAD autoantibody species in relation to other autoantibody species must use sera from a number of individuals with IDDM. The newly created GAD chimera will be useful in such studies.

Implications for IDDM prediction and pathogenesis. GAD antibodies are an important immunological marker for IDDM, but several groups of GAD antibody-positive individuals with a low risk of developing diabetes have now been recognized (11–13). Differences in the GAD antibody profile may allow one to distinguish between individuals at high and low risk of developing IDDM. With our identification of regions targeted by IDDM sera, a precise comparison of the GAD antibody profiles should now be possible and will be important in determining the precise role of GAD antibodies as a predictive marker for IDDM. If a specific region is targeted by IDDM sera and not by other GAD antibody-positive sera, then a GAD_{65}/GAD_{67} chimera could be used to design assays specific for IDDM. In addition, the GAD_{65}/GAD_{67} chimera will be a useful tool to determine whether differences in the two distinct GAD antibodies identify subsets of IDDM by correlating the presence or absence of a particular GAD antibody species with other aspects of IDDM,

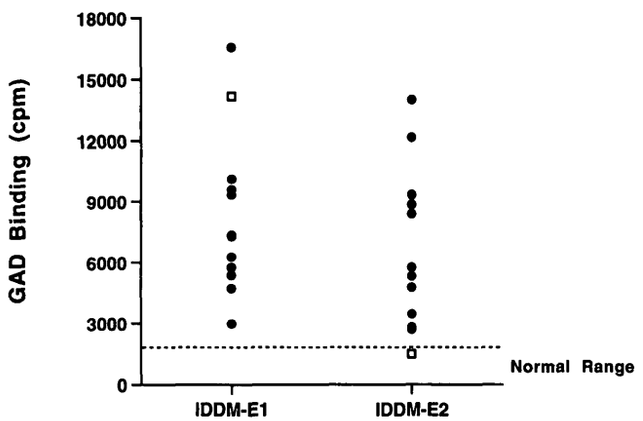

FIG. 4. Frequency of binding to different GAD epitopes in IDDM sera. Two chimeric GAD proteins, GAD_{67} (1–230)/GAD_{65} (221–442)/GAD_{67} (452–594), which contains IDDM-E1, and GAD_{65} (1–195)/GAD_{67} (205–441)/GAD_{65} (443–585), which contains IDDM-E2, were immunoprecipitated as described in Fig. 1A. A total of 12 sera (dilution 1:1–1:10) were assayed and included sera used in Figs. 1 and 2. All sera were chosen because they were known to bind GAD_{65}, but not GAD_{67} (16). ●, The Ci/min of each GAD chimeric protein bound by 11 IDDM sera. □, One serum (IDDM-B in Fig. 2) bound only IDDM-E1. Each point represents a duplicate determination for each serum. For two sera each in IDDM-E1 and IDDM-E2, the closed circles overlie each other and are difficult to distinguish. A horizontal broken line shows the range of binding by normal sera.

such as human leukocyte antigen (HLA) status, temporal relationship to other markers of autoimmunity, and β-cell function.

Possible differences in GAD epitope recognition in IDDM and in individuals with a low incidence of diabetes have led to speculation that differences in the autoimmune repertoire are important in determining whether diabetes develops (13–15). Additional studies are now possible to map precisely and compare GAD epitopes in the various GAD antibody-positive groups. If the GAD epitopes are different, this may provide insight into why IDDM develops in some individuals but not in others. If the epitopes are identical, this suggests that either GAD is not the primary antigen targeted in IDDM or that differences in another aspect of the immunity directed at GAD are responsible for the development of IDDM.

ACKNOWLEDGMENTS

These studies were supported by National Institutes of Health Grant R01-DK-43736, career development awards from the Department of Veterans Affairs Research Service and the American Diabetes Association, a merit review award from the Veterans Affairs Research Service, the Diabetes Research and Training Center at Vanderbilt University (National Institutes of Health Grant DK-20593), a University Grant from Vanderbilt University, Biomedical Research Support Group Grant RR-05424 administered by Vanderbilt University, and a grant from the Diabetes Research and Education Foundation.

We thank Drs. Tihamer Orban and Richard Jackson of Joslin Diabetes Center in Boston, Massachusetts for graciously providing some of the serum samples used for these studies. We also wish to thank Drs. James Thomas and Nancy Olsen of Vanderbilt University for reading the manuscript.

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