Autoantibodies to the 65-kDa isoform of GAD (GAD65Abs) are associated with type 1 diabetes development, but the conformational nature of the GAD65Ab epitopes complicates the evaluation of disease risk. Six GAD65-specific recombinant Fabs (rFabs) were cloned from monoclonal antibodies b96.11, DP-C, DP-A, DP-D, 144, and 221–442. The binding of GAD65Abs in 61 type 1 diabetic patients to GAD65 was analyzed by competitive radioimmunoassays with the six rFabs to ascertain disease-specific GAD65Ab binding specificities. The median binding was reduced significantly by rFab b96.11 (72% \( P < 0.0001 \)), DP-A (84%) \( P < 0.0001 \), DP-C (84%) \( P < 0.0001 \), 221–442 (79%) \( P < 0.0001 \), and DP-D (80%) \( P < 0.0001 \). The competition pattern in type 1 diabetic patients differed from that in GAD65Ab-positive late autoimmune diabetes in adults (LADA) patients (\( n = 44 \)), first-degree relatives (\( n = 38 \)), and healthy individuals (\( n = 14 \)). Whereas 87 and 72% of the type 1 diabetic sera were competed by rFab b96.11 and DP-C, respectively, only 34 and 26% of LADA patients, 18 and 25% of first-degree relatives, and 7 and 28% of healthy individuals showed competition (\( P < 0.0001 \)). These findings support the view that type 1 diabetes is associated with disease- and epitope-specific GAD65Abs and supports the notion that the middle epitope is disease associated. These GAD65-specific rFabs should prove useful in predicting type 1 diabetes and in the study of conformational GAD65Ab epitopes. Diabetes 52:2689–2695, 2003

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APS-1, autoimmune polyendocrine syndrome type 1; CDR, complementarity-determining region; GAD65Ab, autoantibody to the 65-kDa isoform of GAD; LADA, late autoimmune diabetes in adults; mAb, monoclonal antibody; rFab, recombinant Fab; RIA, radioimmunoassay.

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RESEARCH DESIGN AND METHODS

Patients and healthy subjects. Newly diagnosed GAD65Ab-positive type 1 diabetic patients (n = 61) (mean age 10 years, range 0–16, 33 female) were part of a study conducted at the St. Gorans Children Hospital, Stockholm, Sweden, and represented 60% of all children diagnosed in Stockholm during 1993–1995. The serum samples were obtained at the clinical diagnosis of diabetes.

Healthy GAD65Ab-positive first-degree relatives of type 1 diabetic patients (n = 38) (mean age 46 years, range 8–74) were identified by screening 0- to 93-year-old first degree relatives in families with at least two siblings with diabetes to a total of 1,170 probands with type 1 diabetes. The families were identified in the Diabetes Incidence Study in Sweden and the Swedish Childhood Diabetes registry. None of the GAD65Ab-positive first-degree relatives were known to have developed diabetes (in a follow-up time of minimal 2 years and maximal 12 years).

LADA patients (n = 44) (mean age 51 years, range 31–77, 16 female) were part of a screening program in the greater Seattle area. The patients were classified as having type 2 diabetes according to the 1997 American Diabetes Association criteria and were previously identified to be GAD65Ab positive (10). All patients had been diagnosed with diabetes within 12 months of blood sampling.

GAD65Ab-positive healthy individuals (n = 14) (mean age 50 years, range 30–60, 9 female) were detected in a population-based screening of 2,157 Swedish adults (11). None of the GAD65Ab-positive healthy individuals developed type 1 diabetes within 8 years after the samples were taken (12). The median GAD65Ab index for each of the four sample groups was 0.5. All subjects in this study gave informed consent. Local institutional ethics committee approval was obtained before collection of all serum samples.

Monoclonal antibodies used in this study. The epitope regions recognized by the six monoclonal antibodies (mAbs) were spread over the length of the GAD65 molecule, ensuring that different epitope specificities are represented (Fig. 1). mAb 144 and 221–442 were raised in mice and recognized epitopes representing amino acid residues 4–22 (10) and 221–442 (14), respectively. DP-A, DP-D, and DP-C were isolated from a type 1 diabetic patient (15) and recognized epitopes at amino acid residues 483–585, 96–173, and 195–412, respectively (3); b96.11 was isolated from a patient with APS-1 and recognized an epitope at amino acid residues 308–365 (16). All mAbs recognized GAD65 in its native conformation, indicating that the epitopes were located on the surface of the molecule.

Control rFab NQ22/61.1 was specific to phenyl oxazolone (17).

Purification of mAbs. The mAbs were purified from hybridoma cultures or human IgG secreting B-cells supernatant by affinity chromatography on protein A Sepharose (Pharmacia, Uppsala, Sweden) and analyzed for GAD65 binding in a radioimmunoassay (RIA).

Generation of the immunoglobulin heavy- and light-chain constructs. Gene fragments encoding entire Fab chains were amplified using the polymerase chain reaction (PCR) as described previously (18). The VLCL construct was created with primers representing the leader sequence 5–8 amino acids 5′ of the variable heavy-chain (VH) framework one region and amino acid 205–220 of the constant heavy-chain region (CHI). The VLCI construct was created with primers representing the leader sequence 5–8 amino acids 5′ of the variable light chain (VL) framework one region and amino acid 205–220 of the constant light chain region (CL). PCR was performed using standard procedures with Taq DNA polymerase (Pharmacia, Uppsala, Sweden) and amplified at an annealing temperature of 50°C.

Resulting heavy and light chain genes were cloned into the expression vector pAK19 (18). A histidine Tag was introduced at the COOH-terminus of the heavy chain to enable purification of the rFabs.

Expression and purification of the antibody rFabs. E. coli 25F2 cells containing the recombinant plasmids were grown for 16 h at 30°C in complete MOPS medium (19). Cells were then subcultured and grown in the absence of phosphate at 30°C for 4 h. The rFabs were isolated from the bacteria as described previously (18). The rFabs were purified by two subsequent affinity chromatography steps on nickel-NTA agarose (Qiagen) and protein G Sepharose (Pharmacia, Uppsala, Sweden). Fractions were examined by immunoblot for the presence of rFabs and by RIA for GAD65 binding. Active fractions were pooled, and the protein concentration was determined. The yield of functional purified rFabs was ~0.5–1 mg/l bacterial culture.

Competition studies of rFabs with mAbs and serum. Recombinant [35S]GAD antigens were produced in an in vitro–coupled transcription/translation system with SP6 RNA polymerase and nucleoside-treated rabbit reticulocyte lysate (Promega, Madison, WI) as described previously (20). Human GAD65, rat GAD67, and fusion proteins N– M– C M, and C (Fig. 1) were used in this study (5). The in vitro–translated [35S]antigen was kept at −70°C and used within 2 weeks. Binding of rFabs to radiolabeled antigen was determined as described previously (20,21) using protein G Sepharose as the precipitating agent.

The capacity of the rFabs to inhibit GAD65 binding by human serum GAD65Ab was tested in a competitive RIA using protein A Sepharose as the precipitating agent. Fab lack the C12 domain of the Fc region and do not bind protein A. Indeed we did not observe GAD65 binding of any of the rFabs when using protein A Sepharose as the precipitating agent. Serum samples were first tested at a dilution of 1/25. Samples that were competed only partially were diluted to a final serum dilution of 1/100 and reanalyzed. The rFabs were added at the maximal concentration, as determined in competition assays using the intact mAb as a competitor.

Statistical analyses. Binding of GAD65Abs to GAD65 in the presence of rFabs was expressed as follows:

\[
\text{cpm of } [35S] \text{GAD bound in the presence of rFab} \\
\times 100
\]

A reduction in binding by >10% and <50% was defined as partial inhibition. A reduction in binding of >50% of the maximal binding was defined as full inhibition of antigen binding. Human sera contain GAD65Abs of different epitope specificities, therefore a complete inhibition of GAD65 binding (100%) was not expected.

All samples were analyzed in triplicate determinations and the intra-assay average coefficient of variation was 7%, with the highest value 20 and the lowest 0.1. The significance of differences in competition between different serum groups was tested with the nonparametric Mann-Whitney U test. The significance of correlation of competition levels was analyzed using Spearman’s rank correlation test. A P value <0.05 was considered significant.

RESULTS

rFab expression and characterization. The cDNA encoding the Fab of the six mAbs were cloned. The predicted amino acid sequences were aligned (Fig. 2). A comparison of the overlapping amino acid sequence in the complementarity-determining regions (CDRs) of the heavy chain
showed that amino acid sequences derived from the human rFab DP-D and DP-A were closely related to each other (60% homology), but while they share the same CDR1 and -2 classes, they differ in the CDR3 class. rFab b96.11 heavy chain used a VH gene of the third family VH3-49 (22), from which it differed by 12 amino acids. Both mouse heavy chains used a VH gene of the VhJ558 family. 221–442 showed only four amino acid differences from J558.19 (23), whereas 144 showed seven amino acid differences from J558.42 (23). A comparison of the overlapping amino acid sequence in the CDRs of the light chain showed the highest homology when comparing b96.11 with DP-A (61% homology in the CDR sequences), but while they share the same classes for CDR1 and -2, they differ in the CDR3 class. DP-A, DP-C, and b96.11 have light chains of the lambda class. B96.11 used a VL gene of the first family V1-3 (24), from which it differed in five amino acid residues. The great variety in CDR classes and sequences between the antibodies suggests that the antibodies do not recognize the same epitopes on GAD65.

GAD65-binding. The rFab clones 144, b96.11, DP-A, DP-C, DP-D, and 221–442 were tested for their GAD65 specificity by RIA. They showed half-maximal binding at concentrations of 0.0045, 0.2, 0.007, 0.01, 0.1, and 2.4 μg/ml, respectively (Fig. 3). Control rFab NQ22/61.1 was unable to bind GAD65 (data not shown).

Epitope mapping by binding to fusion proteins. To verify the antigen region recognized by the rFabs, the rFabs were analyzed using GAD65/67 fusion proteins (Fig. 1B). The NH2-terminal fusion protein was bound only by rFab 144. The M/C fusion protein was bound by rFab b96.11, DP-D, DP-A, and 221–442. None of the rFabs bound to GAD67. The C fusion protein was bound only by rFab DP-A.

Specificity of type 1 diabetes associated GAD65Abs determined with rFabs of mAbs. Binding to GAD65 by intact mAb was competed with the respective rFabs to determine the optimal rFab concentration in the competition assays. mAbs 144, DP-A, DP-D, b96.11, DP-C, and 221–442 were used at their half-maximal binding concentration, namely 0.005, 0.06, 0.1, 0.05, 0.075, and 0.15 μg/ml, respectively. The rFab concentration determined in these experiments to achieve complete competition of its intact IgG was used in all other competition assays. The rFab concentrations found optimal in the competition assays were 0.104, 0.7, 0.36, 0.6, 1.0, and 1.3 mg/ml for rFab 144, DP-A, DP-D, b96.11, DP-C, and 221–442, respectively.

The competition between rFabs and GAD65Abs in sera of 61 type 1 diabetic patients to bind GAD65 was analyzed (Fig. 4). The median binding was reduced significantly by rFab b96.11 (72%) (P < 0.0001), 221–442 (79%) (P < 0.0001), DP-A (84%) (P < 0.0001), DP-C (84%) (P < 0.0001), and DP-D (80%) (P < 0.0001). No significant sex or age differences were observed in the competitions. HLA typing of most samples was not available; therefore, we could not...
perform statistical correlation between HLA class and rFab competition pattern. rFab b96.11 inhibited 8% of the samples fully and 79% partially. rFab DP-C inhibited 3% of the samples fully and 66% partially. rFab 221–442 inhibited 8% of the samples fully and 67% partially. rFab DP-A inhibited 2% of the samples fully and 72% partially. rFab DP-D inhibited 3% of the samples fully and 62% partially. rFab 144 inhibited 5% of the samples only partially.

The degree of competition did not correlate with the GAD65Ab titer of the sample (Fig. 4A). Incubation with higher rFab concentrations, or using a higher dilution of the serum sample, had no influence on the degree of inhibition (Fig. 4B). This indicates that optimal reaction conditions were used. The polyclonal nature of most patients’ sera is reflected in the incomplete inhibition of the majority of samples. This was further underlined by the observation that serum samples were inhibited by more than one rFab (Fig. 5).

Although samples whose GAD65 binding was competed by rFab b96.11 also showed competition by rFab 221–442, DP-C, and DP-D (Fig. 5, left panels), no significant correlation was observed between DP-A and rFab b96.11, 221–442, DP-C, and DP-D (Fig. 5, right panels). Of the 54 samples that b96.11 competed, 80% (n = 43) were competed also by 221–442 but only 39% (n = 21) were also competed by DP-A. This suggests that DP-A inhibited GAD65 binding to a different epitope.

**FIG. 4.** GAD65-specific rFabs compete with serum GAD65Abs of type 1 diabetic patients. GAD65-specific rFab 144, b96.11, DP-D, DP-A, DP-C, and 221–442 were tested for their capacity to compete with human serum GAD65Abs from type 1 diabetic patients. A: Competition with 0.6 and 1.2 µg/ml of rFab b96.11. Competed binding is presented as percentage binding related to uncompeted binding (100%). Medians for all six rFabs are indicated. B: Correlation of GAD65 binding without competitor and in the presence of rFab b96.11. GAD65 binding of samples without competitor was plotted against GAD65 binding in the presence of rFab b96.11.

**FIG. 5.** Correlation of competition. GAD65 binding of type 1 diabetic patients’ sera in the presence of rFab b96.11 (left panels) or rFab DP-A (right panels) is plotted against binding to GAD65 of the same samples in the presence of rFab 144, DP-D, DP-C, DP-A, and 221–442. P values for the correlation between the different competitors are indicated in the figure.

rFab b96.11 and DP-C specifically inhibit GAD65Abs in type 1 diabetic patients. We determined the ability of the six rFabs to compete with GAD65Abs in sera of type 1 diabetic patients (n = 61), LADA patients (n = 44), first-degree relatives of type 1 diabetic patients (n = 38),
and healthy individuals (n = 14) (Fig. 6). Most remarkable were the results observed with the rFab of b96.11 and DP-C. The percentage of serum samples that showed inhibition of GAD65 binding by rFab b96.11 or DP-C was significantly higher in sera of type 1 diabetic patients (87 and 72%, respectively) than in sera of LADA patients (17/44 [34%] and 11/44 [26%]) (P < 0.0001), first-degree relatives (7/38 [18%] and 10/38 [26%]) (P = 0.0001), or healthy individuals (1/14 [7%] and 3/14 [25%]) (P < 0.0001). Also, the median level of the competition with rFab b96.11 and DP-C was significantly higher in type 1 diabetic patients than in LADA patients (P < 0.0001), first-degree relatives (P < 0.0001), and healthy individuals (P < 0.0001 and P = 0.0002, respectively) (Fig. 6). The differences in competition using rFab 221–442, DP-D, and DP-A in the different serum groups was significant when type 1 diabetic patients were compared with first-degree relatives (P = 0.0002 and <0.0001, respectively). No significant differences were observed in the competition studies using rFab 144.

**DISCUSSION**

The identification of disease-specific GAD65Ab epitopes in type 1 diabetes has been hampered by the fact that the epitopes are conformational (25–27). Epitope identification has been attempted using GAD65/67 fusion proteins (4,5,8,25,28). Although these fusion proteins were carefully designed to preserve the conformation, they did not retain any enzymatic activity (C.S.H., unpublished observation), suggesting a loss of conformational integrity. While the analysis of antibody binding pattern using GAD65/67 fusion proteins provided valuable information about GAD65Ab binding patterns (8,9,28), information about conformational disease-specific GAD65Abs and their epitopes was limited (14). Identification of type 1 diabetes–associated GAD65Ab epitopes is, however, crucial for the understanding of the pathogenic involvement of GAD65Abs in the disease progression. Changes of GAD65Ab epitopes were observed in prediabetic children (28), indicating that the humoral response to GAD65 changes as an individual progresses to type 1 diabetes. To identify disease-associated GAD65Abs and their epitopes, we cloned rFabs of six well-characterized GAD65-specific mAbs and used them to compete GAD65Abs present in sera of type 1 diabetic patients. The use of rFabs rather than intact immunoglobulins prevented potential steric hindrance and allowed us to develop a simple RIA for the detection of specific rFab competition. While our sequence data confirmed the previously published sequences of 144 (13), DP-C, DP-D, and the heavy chain of DP-A (15), considerable differences between the DP-A light chain cloned in this study and the previously published sequence were found (15). We assume that these differences are due to a previous sequence error. Epitope mapping confirmed the previously described binding specificities (13,14,16,25). Our observation that the rFabs bound most GAD65/67 fusion proteins weaker than GAD65 confirmed our earlier finding that the epitopes’ conformations are changed in the fusion proteins. This is further supported by the finding that rFab DP-C does not bind to any of the fusion proteins, while showing strong binding to GAD65.

Although the patients’ sera present a polyclonal mixture of GAD65Abs with different epitope specificities, we were able to obtain a significant reduction in GAD65 binding using the rFabs as competitors. rFab b96.11 and DP-C, derived from human mAbs, significantly inhibited the binding of the majority of type 1 diabetic sera to GAD65. Also, rFab DP-D and DP-A, derived from mAbs isolated from a type 1 diabetic patient (15), and 221–442, derived from a murine mAb (14), inhibited the GAD65Ab binding, but to a lesser extent. These findings are in agreement with previous reports that major GAD65Ab epitopes in type 1 diabetes are located in the middle region and the COOH-terminus of the molecule (4,5,25,29). rFab 144 showed only very little competition with the patients’ sera, which is in agreement with previous findings that the NH2-terminus of GAD65 does not carry type 1 diabetes–associated epitopes (5,8,30).

Using the above described fusion proteins, we tested the epitope specificities of type 1 diabetic patients’ sera and observed type 1 diabetes–specific binding patterns as described previously (8). However, the binding of sera to the fusion proteins did not correlate to the data observed.
for the same sera in the rFab competition experiments. This indicates differences in the respective displayed GAD65Ab epitopes. As discussed above, conformations of the fusion proteins are likely to differ from that of native GAD65, whereas in the present rFab competition assay, GAD65Ab binding to native GAD65 is tested. Thus, while type 1 diabetes–specific binding pattern to GAD65 are revealed by both approaches, the rFab competition assays may be a better tool to define the native GAD65Ab epitopes.

Our hypothesis that competition with rFab will reveal disease-specific GAD65Ab binding specificities was confirmed by our observation that rFab b96.11 and DP-C specifically inhibited GAD65 binding in type 1 diabetic sera to a significantly greater degree than in sera of LADA patients, first-degree relatives, and healthy individuals, suggesting that rFab b96.11 and DP-C recognize disease-specific epitopes. The third rFab specific to the middle epitope, 221–442, competed with GAD65 binding in all four sera groups. While both rFab b96.11 and DP-C were derived from human mAbs, 221–442 was derived from a mouse mAb. It remains unclear what causes the differences in competition patterns between these three rFabs, but subtle differences in the epitopes they recognize may explain our observations. A more detailed epitope analysis will be necessary.

In conclusion, rFab b96.11, DP-C, DP-D, DP-A, and 221–442 recognize shared epitopes with GAD65Abs in type 1 diabetic patient sera and, therefore, can serve as tools for epitope mapping and detection of epitope shifts in different stages of the disease. Our data clearly suggest that rFab b96.11 and DP-C recognize epitopes that are commonly bound by GAD65Abs in type 1 diabetic patients, indicating that APS-1 and type 1 diabetic patients recognize similar epitopes.

Our results confirm previous studies showing disease-specific GAD65Ab epitopes in type 1 diabetic patients’ sera (5,8) and indicate that a disease-specific epitope is located in the middle region of GAD65 (5,8,25,29,30). In a recent report, GAD65-specific human mAbs to the COOH-terminus of GAD65 isolated from a patient with Addison’s disease partially competed GAD65 binding in 10 GAD65Ab-positive type 1 diabetic patients (31). However, it remains to be shown whether the competition is disease-specific. Our data using the COOH-terminus–specific rFab DP-A suggests that this epitope is recognized by GAD65Abs present not only in type 1 diabetic patients.

Previous data has shown that GAD65Abs can modulate GAD65 processing and presentation and thus have a pathogenic role in the development of type 1 diabetes (2,3). DP-D, DP-C, and DP-A were shown to boost the presentation of GAD65 epitopes outside their own binding sites while inhibiting the presentation of their specific epitope to T-cells (3). This is in conjunction with the observation that antibodies can suppress the presentation of certain epitopes due to their high affinity binding (32,33) and underlines the possible involvement of GAD65Abs in the modulation of T-cell responses to GAD65. rFab b96.11, DP-C, and other type 1 diabetes–specific rFabs may represent a novel tool to modulate this processing and will be of importance in elucidating the mechanisms by which GAD65Abs may influence disease progression. Future experiments will be needed to investigate whether rFab b96.11 or DP-C can block the action of pathogenic serum antibodies to reveal whether these disease-specific GAD65Abs in type 1 diabetic patients’ sera are pathogenic or the result of epitope spreading induced by the disease progression.

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