Antibodies to IA-2 and GAD65 in Type 1 and Type 2 Diabetes

Isotype restriction and polyclonality

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OBJECTIVE — To determine the isotypes and clonality of antibodies to GAD (GADA) and IA-2 (IA-2A) in patients with type 1 and type 2 diabetes.

RESEARCH DESIGN AND METHODS — We studied the following consecutive series of patients who attended a diabetes center for antibodies to GADA and IA-2A: 52 newly diagnosed type 1 diabetic patients, 199 type 2 diabetic patients, 200 control patients, and a cohort of 34 nondiabetic identical twins of patients with type 1 diabetes (15 of whom developed diabetes) who were followed prospectively.

RESULTS — GADA or IA-2A were detected in 37 (71%) type 1 diabetic patients compared with only 10 (5%) type 2 diabetic patients ($P < 0.0001$). Both GAD and IA-2 antibodies, regardless of the type of diabetes, were usually subclass restricted to IgG1 and were polyclonal. IgM, IgG3, and IgE isotypes were also detected, but all isotypes of GADA and IA-2A were less prevalent than IgG1 ($P < 0.017$ for either antibody). There was no evidence of spreading or switching of isotypes before the onset of type 1 diabetes.

CONCLUSIONS — These observations suggest that the pathogenesis of antigen-specific antibodies in type 1 and type 2 diabetes is similar and probably involves a chronic nonrandom antigen-driven polyclonal B-cell activation that is consistent with a Th1-type immune response.

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I slet cell antibodies (ICAs) are a feature of type 1 diabetes that occurs in the sera of ~80% of newly diagnosed cases and prediabetic subjects (1,2). ICAs comprise several antibodies that recognize specific islet antigens, including GAD and a protein tyrosine phosphatase IA-2 (3,4). Both ICAs and GAD antibodies (GADA) can be detected in type 1 diabetic patients before and after the onset of diabetes (5), in type 2 diabetic patients (6,7), and in patients with stiff-man syndrome (SMS), a rare neurological disorder (8). In type 1 diabetic patients, ICAs are polyclonal immunoglobulins (9,10). Immunoglobulins are initially formed by peptides of different sizes (light chains associated with heavy chains). Subsequently, as the antibody response matures, the heavy chain components are altered or switched to form other antibody isotypes. In general, ICA is restricted to immunoglobulin of the IgG1 isotype belonging to the IgG subclass (9,10). The isotype profile of antigen-specific antibodies has been shown to reflect T-cell (Th1 or Th2) effector functions in animal models (11). Interferon-γ–dependent antibody isotypes in mice are mainly IgG2a and IgG3 (probable human homologs, IgG1 and IgG3) which reflects a Th1 response (11,12), whereas a Th2 response is predominantly IgE or IgG1 (probable human homologs, IgG4 and IgE). Restriction of ICAs to IgG1 in type 1 diabetes in humans may therefore represent a Th1 dominant immune response. Antigen-specific antibodies have been found in a fraction of patients who do not need insulin, although many progress to insulin dependency within a few years (5,6). These non–insulin requiring diabetic patients with autoantibodies may actually suffer from a slowly progressive form of type 1 diabetes or, alternatively, a separate form of diabetes. Neither the isotype profiles nor the clonality of antigen-specific antibodies in type 1 and type 2 diabetes have been characterized. The aim of this study was to determine whether the isotype and the clonality of GADA and IA-2A in type 2 diabetic patients differed from those found in type 1 diabetic patients.

RESEARCH DESIGN AND METHODS

Subjects

Patients of European origin with either type 1 or type 2 diabetes were studied, and their type of diabetes was determined by standard guidelines (13); cases in which this definition was uncertain were excluded. Classification of type 1 diabetes was based on 1) control of diabetes with insulin from time of diagnosis, 2) diabetic ketoacidosis or marked ketonuria at time of clinical onset, 3) lack of obesity, and 4) age at diagnosis (<40 years). Classification of type 2 diabetes was based on 1) adequate control of diabetes on diet alone or oral hypoglycemic agents at time of sampling, 2) no history of diabetic ketoacidosis or ketonuria, and 3) age at time of diagnosis (>30 years). Nondiabetic twins were administered oral glucose tolerance tests in doses of 75 g or 1.75 g/kg, whichever dose was less, to confirm that they were neither initially diabetic nor diabetic at intervals thereafter. We performed a cross-sectional study of antibodies to GAD65 and IA-2 in a consecutive series of selected

patients who attended a diabetes center and in a prospective study of a cohort of nondiabetic identical twins of type 1 diabetic patients. The patients included 52 newly-diagnosed type 1 diabetic patients (mean age 20 years, range 5–36; 18 females) who were all tested within 1 month of diagnosis; 199 type 2 diabetic patients (mean age 57 years, range 30–75; disease duration 4 years, range 0–28; 101 female); and 200 normal healthy control subjects (mean age 29 years, range 14–71; 95 female) who were selected to have a similar distribution for age and sex to the combined groups of 251 diabetic patients. None of the control subjects had a family history of diabetes, were taking drugs, or had clinical signs of illness. The subjects gave informed consent, and the study was approved by the ethical committees at St. Bartholomew's Hospital and the University of Rome, La Sapienza.

Because isotype assays are expensive, our strategy was to test a sample of subjects to identify dominant isotypes and then to screen all type 1 and type 2 diabetic patients for the dominant isotypes. Initially, we tested antibody isotypes IgG1, IgG2, IgG3, IgG4, IgM, and IgE to both GAD and IA-2 in 1) 10 type 2 diabetic patients (mean age 55 years, range 34–75; five female) who were all GADA positive, and 2) a cohort of 34 nondiabetic identical twins of patients with type 1 diabetes. The latter cohort was referred as a consecutive series to the British Diabetic Identical Twin Study; of these 34 twins, 15 subsequently developed type 1 diabetes, and samples were tested both before and at diagnosis. The mean period of follow-up was 47 months (range 4–156), the mean age at time of the first sample before diagnosis of type 1 diabetes was 14 years (range 7–31), and the mean age at time of the second sample at diagnosis was 17 years (range 7–44; eight females). The 19 other twins remained nondiabetic (age at entry 17 years, range 4–36; nine females) having been followed for a median of 107 months (range 55–189); the earliest available samples on them were tested, and these twins had an estimated disease risk of <2% (1). To establish a cutoff for positivity for this initial analysis, we also tested 37 control subjects (mean age 26 years, range 9–47; 24 males). Subsequently, we tested selected dominant isotypes in 52 type 1 diabetic patients and 199 type 2 diabetic patients, and we established the cutoff for positivity for dominant isotypes in 200 normal healthy control subjects. Sera were stored

at −20°C. Analysis was performed on batched samples by an observer blinded to the clinical status of the subjects.

Laboratory methods

Standard antigen-specific antibody assays. The radioimmunoprecipitation assays for IA-2 (amino acids 603–979), the intracellular fragment of IA-2 containing the immunodominant epitope, and GAD65 use in vitro transcription and translation systems as previously described (1). All samples, including positive and negative control standard sera, were tested in duplicate. Each assay for GADA and IA-2A included serially diluted sera from an SMS patient and a prediabetic individual, respectively. The prediabetic sera (from a twin who subsequently developed type 1 diabetes but was not part of this study) allowed consistency in detecting positivity at values >1:8,000 for the respective assays, although the results were the same when the cutoff for positivity was >3 SD above the control population. In the fourth GAD and third IA-2 proficiency testing surveys (unpublished) and in a GAD proficiency workshop, our assays had a sensitivity, specificity, validity, and consistency of 100% (14).

Antibody isotypes

In vitro transcribed and translated GAD or IA-2 labeled with [35S]methionine were used as the antigens and were prepared as described above. A dose of 50 µl of the antigen containing 50,000 counts/min (CPM) was incubated with 2 µl of test serum samples overnight at 4°C in 96-well millipore filter plates. Monoclonal biotinylated anti-human antibodies (IgG1 clone HP6069, IgG2 clone HP6002, IgG3 clone HP6047, IgG4 clone HP 6046, IgM clone HP6083 [Zymed, Camarillo, CA], and IgE clone G7-26 [Beckton Dickinson U.K., PharMingen, Oxford, U.K.]) at concentrations of 4, 4.8, 2.4, 1.6, 2, and 5 µg, respectively, were added and incubated at 4°C on a shaker for 1.5 h. The reactivity and the specificity of these subclass specific monoclonal antibodies have been established in a large World Health Organization study (15). Antigen-antibody complexes bound to biotinylated monoclonal anti-human antibodies were separated for 1.5 h at 4°C with 50 µl of immobilized streptavidin agarose (Pierce & Warriner, Chester, U.K.), which binds biotin on the antibody molecules (16). Bound immune complexes were washed 12 times with Tris buffer saline and Tween on

the millipore plate washer, were allowed to dry at room temperature before the addition of meltilex solid scintillant (Wallac, Turku, Finland), and were counted on the Wallac 1260 Microbeta scintillation counter. Each assay for IA-2 and GAD65 isotypes included a serum sample from an SMS patient who showed reactivity to all the tested isotypes, a prediabetic individual (a twin who subsequently developed type 1 diabetes but was not part of this study) who was positive for GAD and IA-2 antibodies, and an antibody-negative normal control subject to confirm specificity and reactivity of the antibody isotypes. To establish a cutoff for positivity, the results were calculated in control subjects as the mean CPM for each antibody isotype minus the blank CPM. Positive results in patients were defined as CPM >3 SD above control mean levels.

Clonality

To determine clonality, we tested type 1 and type 2 diabetic patients positive for the dominant GAD and IA-2 isotype and control subjects. Sera were tested after immunoprecipitation as described above, except instead of using biotinylated monoclonal anti-human antibodies, we used biotin conjugated mouse anti-human κ–light chain (clone G20-193) and λ–light chain (clone JDC-12) monoclonal antibodies at a concentration of 10 µg per well.

Statistical analysis

The proportion of subjects in each group positive for each antibody or combination of antibodies was compared using two-sided analysis or one-sided where indicated with Fisher's exact test and Yate's correction. Changes in the study were considered significant at P < 0.05.

RESULTS

Standard antigen-specific antibody assays

GADA were detected in 29 (56%) newly diagnosed type 1 diabetic patients and 10 (5%) type 2 diabetic patients and IA-2A in 23 (44%) newly diagnosed type 1 diabetic patients and 5 (3%) type 2 diabetic patients. In 37 (71%) newly diagnosed type 1 diabetic patients compared with only 10 (5%) type 2 diabetic patients, one or more GADA and IA-2A was detected (P < 0.0001). In antibody-positive patients, combinations of IA-2A and GADA were as frequent in newly diagnosed type 1 diabetic patients (15 of 37) as type 2 dia-

Table 1—Presence of GADA, IA-2A, and corresponding IgG1, IgG2, IgG3, IgG4, IgE, and IgM isotypes in nondiabetic twins, type 1 diabetic twins both before and at diagnosis, and type 2 diabetic patients

Antibodies	Nondiabetic twins	Type 1 diabetic twins before diagnosis	Type 1 diabetic twins at diagnosis	Type 2 diabetic patients
n	19	15	15	10
GAD	1	12	10	10
GAD IgG1	1	8	7	5
GAD IgG2	0	0	3	0
GAD IgG3	0	3	0	0
GAD IgG4	0	1	0	0
GAD IgE	0	0	0	0
GAD IgM	0	1	0	0
IA2	0	13	12	4
IA2 IgG1	0	8	10	0
IA2 IgG2	0	3	3	0
IA2 IgG3	0	0	0	0
IA2 IgG4	0	0	0	0
IA2 IgE	0	0	2	0
IA2 IgM	0	0	0	0

Data show numbers scoring greater than mean $\pm$ 3 SD of control subjects (n = 37). None of the control subjects was positive for antibodies or antibody isotypes.

betic patients (5 of 10). The presence of either a single antibody or combinations of antibodies in newly diagnosed type 1 and type 2 diabetic patients was not related to age or BMI (data not shown).

Screening for dominant isotypes
Selected groups were screened for six isotypes. These groups included 34 twins followed prospectively, of whom 15 developed diabetes, and 10 type 2 diabetic patients with GADA. At the time of diagnosis of type 1 diabetes in the 15 twins, IgG1 isotype of GAD and IA-2 were dominant (Table 1), occurring in 7 of 15 and 10 of 15 twins, respectively. IgG1 isotypes were also dominant before the onset of diabetes in these twins, and the isotypes did not change significantly between the prediabetic sample and the sample at diagnosis (Table 1). Of five isotypes other than IgG1 tested at diagnosis, we detected GAD IgG4 (1 of 15), IA-2 IgG2 (3 of 15), and IA-2 IgE (2 of 15). Of five isotypes other than IgG1 tested in the prediabetic sample, we detected GAD IgG3 (3 of 15), GAD IgM (1 of 15), and IA-2 IgG2 (3 of 15). There was no statistically significant spreading or switching of the isotype response from the prediabetic sample to the sample at diagnosis (Table 1).

There was no significant difference for isotype-antibody pattern in GADA[+] patients with type 1 or type 2 diabetes

(Table 1). Of 10 type 2 diabetic GADA[+] patients, 5 had GAD IgG1 but no other GAD or IA-2 isotype. In this initial screen of twins and type 2 diabetic patients, 21 serum samples were GAD IgG1^{+}, but only 4 had another isotype (P < 0.0001); moreover, 18 samples were IA-2 IgG1-positive, but only 8 had another IA-2 isotype (P = 0.0167).

Of the 19 twins who have not developed diabetes to date, none had IA-2 anti-

bodies or IA-2 isotypes; only 1 had GADA, and this patient had the GAD IgG1 isotype (Table 1).

Screening with selected isotypes
The dominant isotype for both GAD and IA-2 was IgG1. Because IgG1 and IgE could reflect a Th1- and Th2-type immune response, respectively, we elected to screen all 251 patients for these two isotypes. Of 52 type 1 diabetic patients, IgG1 GADA were detected in 15 patients (29%), and IgG1 IA-2A were detected in 22 patients (42%) (Figs. 1 and 2). More IA-2A^{+} type 1 diabetic patients had IgG1 isotypes than GADA[+] patients (P = 0.04) (Figs. 1 and 2). Of 199 type 2 diabetic patients, IgG1 GADA were detected in nine (5%), but none had IgG1 IA-2A (Fig. 3). IgE isotypes in type 1 diabetic patients were infrequent and much less prevalent than IgG1 isotypes for both GADA and IA-2A (P < 0.0001) (Figs. 1 and 2). GADA in type 2 diabetic patients, when using either the standard assay or IgG1 isotype assay, were more frequent than IA-2A (7 vs. 3%, respectively) (one-sided P = 0.03).

Clonality
The IgG1 antibodies to both GAD or IA-2 usually comprised both κ– and λ–light chains, consistent with a polyclonal antibody response. Thus, in type 1 diabetes, both κ- and λ-chains were detected in 83% of IgG1 GADA and in 68% of IgG1 IA-2A. Moreover, in type 2 diabetic patients, 70% of IgG1 GADA were positive for both
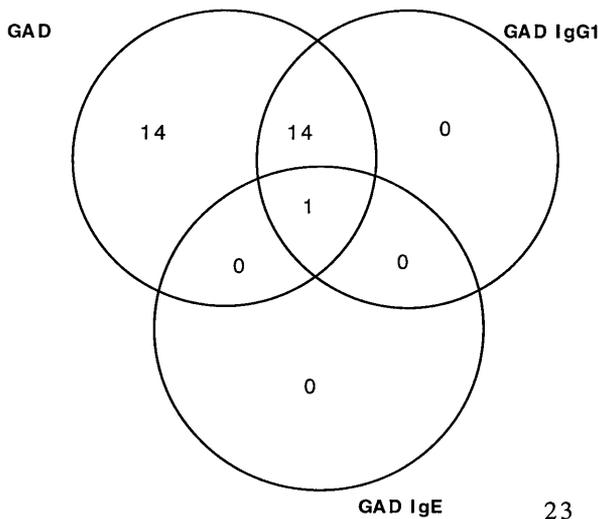

Figure 1—The numbers of 52 newly diagnosed type 1 diabetic patients with antibodies to GAD, GAD IgG1, and GAD IgE isotypes. Intersecting regions indicate number of patients positive for combinations of antibodies.
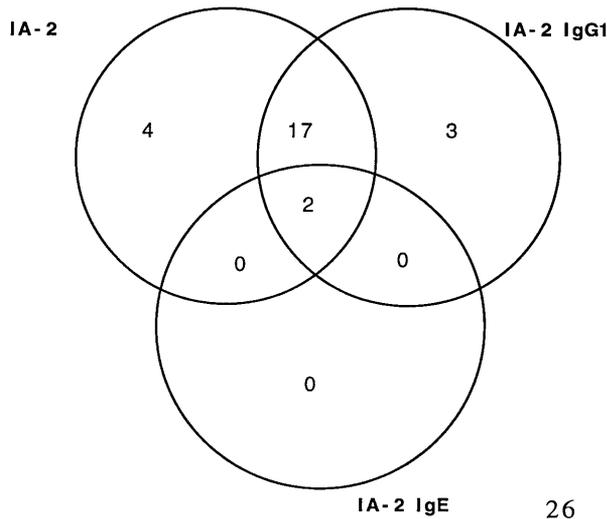
Figure 2—The numbers of 52 newly diagnosed type 1 diabetic patients with antibodies to IA-2, IA-2 IgG1, and IA-2 IgE isotypes. Intersecting regions indicate number of patients positive for different com- binations of antibodies.

chains; none of these patients had IgG1 IA-2A. IgG1 GADA in the remainder of type 1 and type 2 diabetic patients expressed λ– but not κ–light chains. IgG1 IA-2A in the remaining type 1 diabetic patients, on the other hand, had either κ– (16%) or λ (16%)–light chains.

CONCLUSIONS — By using antigen-specific assays, we found that the majority of GADA and IA-2A were restricted to the IgG1 subclass, regardless of whether they were detected in type 1 or type 2 diabetic patients. GAD and IA-2 IgG1 isotypes were also identified as dominant in type 1 diabetic patients before they developed diabetes, and there was no evidence that the isotype switched or spread in the few years before the onset of the disease. None of the other isotypes were detected in more than a fraction of the patients. These results are consistent with a chronic antigen-driven B-cell activation in both type 1 and type 2 diabetes.

We have identified a broad range of isotypes in patients with SMS, but, in antibodies from patients with type 1 and type 2 diabetes, we have identified isotype restriction (17). In our initial screening of patients, we found that isotypes other than IgG1 were uncommon in both type 1 and type 2 diabetes. Because the standard GADA and IA-2A assays use protein A Sepharose, which precipitates only IgG but not IgM, IgA, IgD, or IgE isotypes, it is possible that the standard assay underestimates the prevalence of antigen-specific antibodies in diabetic patients. Our results

do not support this proposal. Moreover, we found no evidence for a Th2-like isotype profile, including IgE antibodies, in the twins who did not develop diabetes, although a separate study of GADA+ non-diabetic siblings detected IgG2 and IgG4 GADA in 10 of 15 (18). The assays for standard antibodies and antibody isotypes are distinct, involving different immuno-precipitation procedures. The isotype assays, particularly the GAD assays, were less sensitive than the standard antibody assays, as reflected by the lower counts in the immunoprecipitate (data not shown). Even though this reduced sensitivity of the

isotype assays may have decreased the chances of detection of some isotypes, it should not have altered our observation that IgG1 was predominant.

Subclass restriction has been demonstrated in several autoimmune diseases, and autoantibody isotypes may be related to the clinical outcome (19). Subclass restriction results from the preferential switching to a particular isotype by proliferating B-cells. Restriction of the isotype may be genetically determined, dependent on the antigen that maintains antibody production, or the result of immunoregulatory factors. The restriction of both GADA and IA-2A to IgG1 in diabetes is consistent with an antigen-driven dominant Th1 immune response that operates in the disease both before and at clinical diagnosis. Our results are in line with studies of ICA, GADA in newly diagnosed type 1 diabetic patients, and monoclonal GADA derived from type 1 diabetic patients, all of which show a predominant GAD IgG1 isotype (9,10,20–24). Disappointingly, we were unable to detect a class switch in prediabetic twins along the lines described for insulin antibodies in the nonobese diabetic (NOD) mouse (25). It is possible that sampling at an earlier stage may show antibody switching, although antibodies detected in babies who subsequently developed type 1 diabetes were already IgG1 immunodominant (23).

GADA or IA-2A were detected in 5% of type 2 diabetic patients. An additional 2% of patients were positive for the GAD IgG1 isotype but negative on the standard GADA assay possibly because of decreased speci-
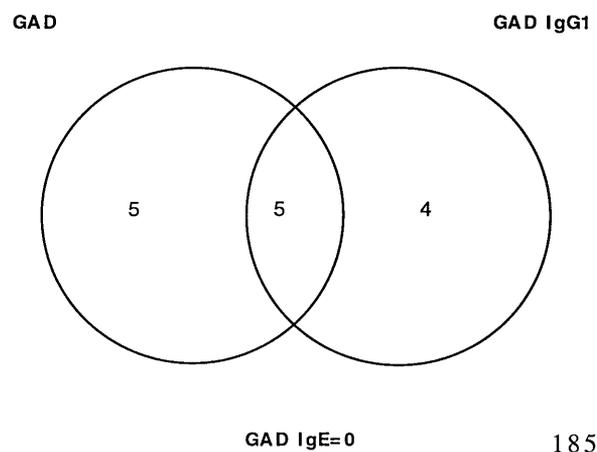

Figure 3—The numbers of 199 type 2 diabetic patients with antibodies to GAD and GAD IgG1 iso- type. Intersecting regions indicate number of patients positive for different combinations of antibodies. In four type 2 diabetic patients, GAD IgG1 antibodies were detected in the absence of GAD antibodies in the standard immunoprecipitation assay. GAD IgE isotypes were not detected.

ficity of the monoclonal anti-human IgG1 antibodies used in the assay. None of the type 1 diabetic patients or control patients had GAD IgG1 in the absence of GADA. Type 2 diabetic patients who have GADA or ICAs have many features of type 1 diabetes, including low or normal body weight, high rates of HLA-DR3 and -DR4, and a propensity to become insulin-dependent (6,7,26,27). In our study, as in other reports, IA-2A, as compared with GADA, were less common in type 2 diabetic patients (6,26). Such a low frequency of IA-2A in type 2 diabetic patients could be because subjects with IA-2A pass rapidly into insulin dependence and are not ascertained or because the disease process in type 2 diabetic patients with antibodies is distinct from the disease process in type 1 diabetic patients. Our present observations suggest this latter explanation is unlikely. We found that autoimmune diabetes, regardless of whether the patient is designated insulin-dependent or non–insulin-dependent, is associated with antigen-specific IgG1 isotype–restricted antibodies. Furthermore, patients with adult-onset type 1 diabetes have a low frequency of IA-2A (28). These results support the hypothesis that autoimmune diabetic patients who do not receive insulin therapy have a disease process similar to that of patients with type 1 diabetes. If true, then these patients are potential candidates for trials of therapy that modulate the type 1 diabetes-associated disease process, thus avoiding some of the concerns regarding the use of drugs in nondiabetic healthy children (26).

Subclass restriction of antibodies could reflect monoclonal B-cell responses. However, the majority of patients we tested with GADA and IA-2A, regardless of their type of diabetes, had both κ– and λ–light chains consistent with a polyclonal antibody response. Subclass restriction and polyclonality of autoantibodies to specific antigens do not support the proposal that autoimmune diabetes results from a random genetic event, such as a somatic mutation. Our observations confirm similar results for ICA, which comprises antigen-specific antibodies, including GADA and IA-2A (9,10). We conclude that the nature of antigen-specific antibodies in type 1 and type 2 diabetes is similar, because it probably involves a chronic nonrandom antigen-driven polyclonal B-cell activation, and is consistent with a Th1-type immune response.

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