Exposure to Exogenous Insulin Promotes IgG1 and the T-Helper 2–Associated IgG4 Responses to Insulin but Not to Other Islet Autoantigens

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Insulin immunization in animal models induces T-helper (Th) 2–like antibody subclass responses to insulin and other β-cell antigens. The aim of this study was to determine whether exposure to insulin in humans resulted in a similar subclass bias of the humoral immune response. Levels of IgG subclass antibodies to insulin (IAs), GAD, and IA-2 were measured before and after treatment with insulin in the following groups of patients: 29 patients with newly diagnosed type 1 diabetes treated with intravenous and/or subcutaneous insulin; 10 newly diagnosed patients randomized to cyclosporin A (CsA) or placebo plus subcutaneous insulin for 12 months; and 14 islet cell antibody–positive relatives receiving either intravenous and subcutaneous insulin prophylaxis or no treatment. At the onset of diabetes, the major subclass distributions of insulin autoantibodies (IAAs) were IgG1 and, to a lesser extent, IgG4. After insulin treatment in the 29 new-onset patients, IAs were initially of the IgG1 subclass. IgG4-IAs appeared later, but at 12 months, they were at higher levels than IgG1-IAs in 11 patients. Responses were higher in children compared with adults and were higher in subjects with IAAs ($P < 0.001$). Insulin prophylaxis in relatives showed a similar profile, with a decline in levels of IgG1-IAs after cessation of daily subcutaneous insulin. Patients treated with CsA took longer to develop IAs and showed suppressed levels of IgG4-IAs; however, their levels of high-titer IgG1-IAs persistently rebounded after completion of CsA therapy. Despite the presence of IgG4-IAs in most insulin-treated patients and relatives, a shift to IgG4–anti-GAD or IgG4–IA-2 was not found for up to 3 years after the initiation of insulin therapy. While our findings need to be correlated with T-cell cytokine responses, we suggest that the strong IgG4-IA response in insulin-treated patients is consistent with an enhancement of Th2 immunity, but there is no evidence of subsequent spreading of potentially Th2-associated IgG4 responses to other autoantigens.
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CsA, cyclosporin A; GADA, GAD antibody; IA, insulin antibody; IA-2A, IA-2 antibody; IAA, insulin autoantibody; ICA, islet cell antibody; IL, interleukin; IVGTT, intravenous glucose tolerance test; PBS, phosphate-buffered saline; SIP, Schwabing Insulin Prophylaxis; TBST, Tris-buffered saline with Tween; TBT, Tris-buffered Tween; Th, T-helper.

Type 1 diabetes is considered to be a consequence of T-helper (Th) 1 lymphocyte–dominated responses to islet antigens, which leads to the destruction of pancreatic β-cells (1). In animal models of type 1 diabetes, modulation of this response can be effected by administration of islet antigens (2–5). Immunization with insulin, GAD, or specific autoantigen peptides at an early age can delay and even prevent the onset of diabetes in NOD mice (2–5). Immunization appears to alter the phenotype of T-cells found in islet infiltrates to one that is Th2-dominated and induces an antibody response typical of Th2 immunity (6). Therefore, the protection offered by immunization with antigens has been suggested to be due to a spreading immune deviation away from Th1 toward Th2, as a result of selective Th2 priming by antigen (6,7).

In humans, attempts to modulate β-cell autoimmunity by administering the β-cell–specific antigen insulin to subjects either during the prediabetic stage (8,9) or at the manifestation of the disease (10,11) have also been suggested to be effective, as the onset of diabetes could be delayed or clinical remission could be improved. The issue of whether protection in humans is also through induction of protective Th2 immunity has not been examined. In mice, Th2 immune responses are characterized by antibodies of the IgG1 subclass (12,13); accordingly, IgG1 antibodies are found after administration of insulin (6). Although distinct antibody subclass associations with Th1 or Th2 immunity are not as clear in humans as they are in mice, the Th2 cytokine interleukin (IL)-4 can induce both IgE and IgG4 antibody production, and these antibodies are characteristic of the Th2-dominated response of atopy (14). Human IgG4 antibodies, therefore, likely represent the equivalent of a mouse IgG1 response.

Insulin treatment in humans is associated with the production of insulin antibodies (IAs) (15). In this study, we measured IgG subclass responses to insulin to test the hypothesis that immunization with antigens will induce Th2 immunity to specific antigens and induce spreading to Th2 immunity to other islet autoantigens. We investigated the course of the humoral immune response to insulin by determining total and specific IgG antibody subclass titers in the following groups: ***1***) patients treated with intravenous and subcutaneous insulin or with subcutaneous insulin alone from diabetes onset; ***2***) a group of newly diagnosed patients who were treated with cyclosporin A (CsA) or placebo and subcutaneous insulin for 12 months in the European-Canadian Cyclosporin Trial (16,17); and ***3***) prediabetic first-degree rel-

atives treated with intravenous and subcutaneous insulin in the Schwabing Insulin Prophylaxis (SIP) pilot intervention trial (9). Changes in the subclass of antibodies to GAD and IA-2 after insulin therapy were also investigated.

RESEARCH DESIGN AND METHODS

Subjects

Insulin-treated patients with newly diagnosed type 1 diabetes. Samples were analyzed from 29 patients with type 1 diabetes treated with either high-dose intravenous insulin infusion for 1–2 weeks followed by subcutaneous insulin therapy (***n*** = 14) or subcutaneous insulin therapy alone (***n*** = 15) (11). Those patients who were treated with intravenous plus subcutaneous insulin included 6 children (median age 7 years, range 2–12) and 8 adults (median age 29 years, range 19–36). Of the patients treated with subcutaneous insulin alone, 6 were children (median age 11 years, range 3–17) and 9 were adults (median age 31 years, range 24–37). In all of the patients, samples were measured for IA and IgG-IA subclasses at diabetes onset (insulin autoantibodies [IAAs]), after 12 months of insulin therapy, and, in the majority of the patients, at intervals of ~3 months or less after the onset of diabetes. GAD antibody (GADA) and IA-2 antibody (IA-2A) subclasses were measured at onset and after 1 and 2–3 years of treatment in 14 and 10 patients, respectively.

Patients with newly diagnosed type 1 diabetes treated with CsA plus insulin. Ten patients with newly diagnosed type 1 diabetes who were randomized into the Canadian-European Cyclosporin Trial by our participating center in Munich were analyzed for this study (17). Five patients (median age 18 years, range 17–28) were randomized into the CsA arm and treated with an initial daily dose of 10 mg CsA/kg plus subcutaneous insulin given orally in divided doses at 12-h intervals. Patients were on CsA for 12 months at the dose adjusted to maintain whole-blood concentrations of 400–800 ng/ml. The other 5 patients (median age 25.6 years, range 23–29.8) were treated with subcutaneous insulin alone and placebo. IA and IgG-IA subclasses were analyzed at diabetes onset (IAA) and at time intervals up to 34 months of follow-up (median 20 months, range 17–34).

First-degree relatives treated with insulin prophylaxis. A total of 14 islet cell antibody (ICA)-positive first-degree relatives were randomized to either the insulin prophylaxis (***n*** = 7) or control arm (***n*** = 7) in the SIP trial (9). All had ICA values >20 Juvenile Diabetes Foundation units, a first-phase insulin response to an intravenous glucose tolerance test (IVGTT) less than the 5th percentile of control subjects (<65 μU/ml), and a normal oral glucose tolerance test before entry into the study. Subjects randomized to the prophylaxis arm were treated with human insulin by continuous insulin infusion for 7 days, followed by daily subcutaneous injections for 6 months and repeated 7-day intravenous treatments every 12 months for a maximal period of 5 years (range 0.6–5); these subjects were followed for up to 8.6 years. Subjects randomized to the control arm had no treatment and were followed with blood samples and IVGTTs at the same time intervals as those patients randomized to the prophylaxis arm. Five subjects in the prophylaxis arm (aged 4, 4, 6, 13, and 17 years) developed antibodies to insulin, and 6 of the subjects in the control arm (aged 4, 8, 13, 13, 24, and 55 years) had IAAs during the study. IA/IAA subclasses were measured in these 11 subjects at various time points up to 36 months after the start of intervention. GADAs were detected in all 5 of the subjects in the prophylaxis arm, in whom an insulin antibody response was seen, and IA-2As were detected in 4 of the 5 patients. The subclasses of GADA and IA-2A were measured in these subjects at the start, during the first year, and 3 years after the start of intervention.

All intervention studies were approved by the respective local ethics boards.

Autoantibody assays. IAs detected before known exogenous insulin treatment were defined as IAAs, and those measured after exogenous insulin treatment were defined as IAs. IAAs and IAs were determined by binding to ^{125}I-labeled insulin in a protein A/G radiobinding assay, as described previously (18). The upper limit of normal values, which was defined by the 99th percentile of antibody levels in nondiabetic control children, was 4 U.

IgG1 subclasses of IAAs and IAs were measured using the protein A/G radiobinding assay, during which the addition of the protein A/G sepharose was substituted with IgG subclass–specific antibody-bound sepharose beads as previously described (19). Serum (5 μl) was incubated with 1,159 nU ^{125}I-insulin (Hoechst, Frankfurt, Germany) (specific activity 360 μCi/μg) in 25 μl 50 mmol/l Tris 1%, Tween 20, pH 8 (Tris-buffered Tween [TBT]) at 4°C for 72 h before addition of 50 μl IgG subclass-specific antibody-coated sepharose bead suspension for 1 h at room temperature.

Biotin-labeled mouse monoclonal antibodies against human IgG1, IgG2, IgG3, and IgG4 and, as a control for nonspecific binding, against rat IgM were obtained commercially (Pharmingen, San Diego, CA). Sepharose 4B streptavidin beads (Zymed, San Francisco, CA) were prepared by washing once with ice-cold phosphate-buffered saline (PBS) (50 mmol/l phosphate buffer, 150 mmol/l NaCl, pH 7.4), incubating beads with biotinylated monoclonal antibody with rotation at 4°C for 18 h, washing twice in PBS and once in assay buffer, and resuspending in assay buffer. Beads were then washed 5 times in cold TBT and counted for 10 min. For each serum, nonspecific binding was also measured using beads coated with anti-rat IgM monoclonal antibody. Results were expressed as milliunits of insulin bound per milliliter of serum and calculated as follows:

$$\frac{\text{IgG subclass specific counts} - \text{anti-rat IgM counts}}{\text{total counts per tube}} \times 1.159 \times 200$$

The threshold for positivity for each subclass was calculated as 0.25 mU/ml.

IgG1-GADA and –IA-2A and IgG4-GADA and –IA-2A were measured using a radiobinding assay as previously described (19). Serum (0.5 μl) was incubated overnight at 4°C with 25,000 cpm of ^{35}S-labeled methionine in vitro translated antigen in 25 μl of 50 mmol/l Tris, 150 mmol/l NaCl, 1% Tween 20, pH 7.4 Tris-buffered saline with Tween (TBST). A 50-μl suspension of sepharose beads coated with IgG subclass-specific antibody (Pharmingen) was added, and plates were incubated for 1 h at room temperature before washing in cold TBST and counting. Results were expressed as the change in the difference between IgG subclass-specific cpm and anti-rat IgM cpm, and they were converted to a standard deviation score (SDS) calculated from the mean and standard deviation of results obtained after subtraction of nonspecific binding to the anti-rat IgM for 44 control subjects. Mean + 3 SD was used as the threshold for detection (19).

Statistical analysis. Antibody levels between study groups and the relationship to IAAs were analyzed using the Mann-Whitney ***U*** test. Linear regression analysis was used to determine associations of total and IgG subclass antibody titers with age. For all statistical methods, the Statistical Package for Social Sciences (SPSS, Chicago) was used.

RESULTS

IgG1 and IgG4 are the predominant subclass responses of IAA and IA in patients with type 1 diabetes. IAAs were detected before insulin treatment in 12 of the 29 patients with type 1 diabetes. These patients included 10 of the 12 children. IgG1 and/or IgG4 were the most common subclasses of IAA in these 12 patients. Of these 12 patients, 5 had IgG1-IAA plus IgG4-IAA, 3 had IgG1-IAA only, and 2 had IgG1-IAA plus IgG2-IAA. In 2 patients, subclass IAA was not measurable.

After 12 months of treatment with insulin, IAs were detected in 26 of the 29 patients; once again, IgG1 and IgG4 were the highest subclass responses (Fig. 1). IgG1-IA was detected in 25 patients, and IgG4-IA was detected in 23 patients. Relatively

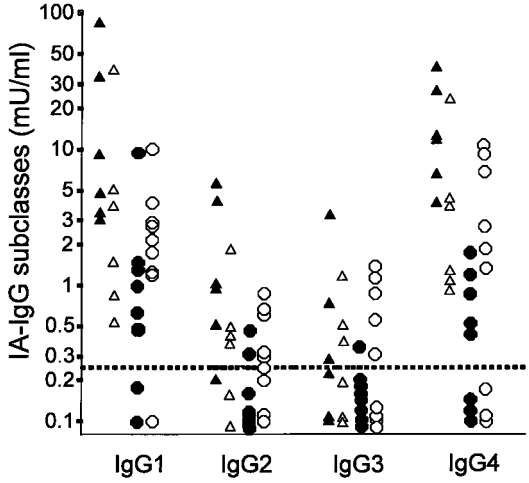

FIG. 1. Levels of IgG-IA subclasses after 12 months of insulin therapy in children with newly diagnosed type 1 diabetes treated with intravenous and subcutaneous insulin (▲) (*n* = 6) or with subcutaneous insulin only (△) (*n* = 6) and in adult patients treated with intravenous and subcutaneous insulin (●) (*n* = 8) or with subcutaneous insulin only (○) (*n* = 9). The dotted horizontal line denotes the threshold for positivity. IgG-IAs are shown on a logarithmic scale.

weak IgG2-IAs and IgG3-IAs were found in 16 and 12 patients, respectively. In children, compared with adults, higher levels of total IAs (median 296 vs. 80 U), IgG1-IAs (median 15.4 vs. 2.5 mU/ml), IgG2-IAs (median 1.0 vs. 0.2 mU/ml), and IgG4-IAs (median 9.9 vs. 2.3 mU/ml) were found ($P < 0.01$). When patients treated with intravenous plus subcutaneous insulin and subcutaneous insulin only were analyzed separately, differences between children and adults were found only in subjects treated with intravenous plus subcutaneous insulin ($P < 0.01$). Children had high levels of IAs, regardless of whether they received intravenous insulin, whereas adults treated with intravenous plus subcutaneous insulin had marginally lower 12-month levels of total IAs ($P = 0.06$) and IgG1-IAs ($P = 0.09$) than adults treated with subcutaneous insulin only. Overall, total IA levels and IgG1-IA, IgG2-IA, and IgG4-IA levels at 12 months were indirectly correlated with age ($P < 0.01$; $P < 0.03$ for IgG4-IA) and were higher in patients with IAAs at diabetes onset ($P < 0.002$) (Fig. 2). Multivariate analysis could not distinguish whether both age and IAAs were independently associated with IA responses.

In all of the patients, IgG1-IA levels paralleled the total IA response (Fig. 3). After 3 months of treatment, IgG1-IAs were already present at levels above those at diabetes onset in 24 patients, whereas IgG4-IAs were low or undetectable in most of the patients (Fig. 3). The IgG1-IA level peaked between 3 and 12 months after the start of insulin treatment (median 6 months) and reached a plateau or declined thereafter. In contrast, IgG4-IA levels increased markedly after 6 months of treatment, and by 12 months, they were still rising in 20 patients and were above IgG1-IA levels in 11 patients, with no significant difference between IgG1-IA and IgG4-IA levels. When detected, both IgG2-IA and IgG3-IA paralleled IgG1-IA levels (data not shown).

The IgG4 response appeared earlier in patients with IAAs. Of 12 patients with IAAs at the onset of diabetes, 10 showed increases in IgG4-IA levels after only 3 months of treatment, whereas only 3 of 17 patients without IAAs showed increases in IgG4-IA levels at the same time ($P < 0.001$). More than half of the IAA-negative patients still did not have IgG4-IAs after 6 months of insulin treatment (Fig. 3). In adult patients, treatment with intravenous insulin appeared to inhibit the early IgG1-IA and IgG4-IA responses. Compared with subjects treated with subcutaneous insulin alone, both responses were significantly diminished until 6 months of treatment ($P < 0.05$).

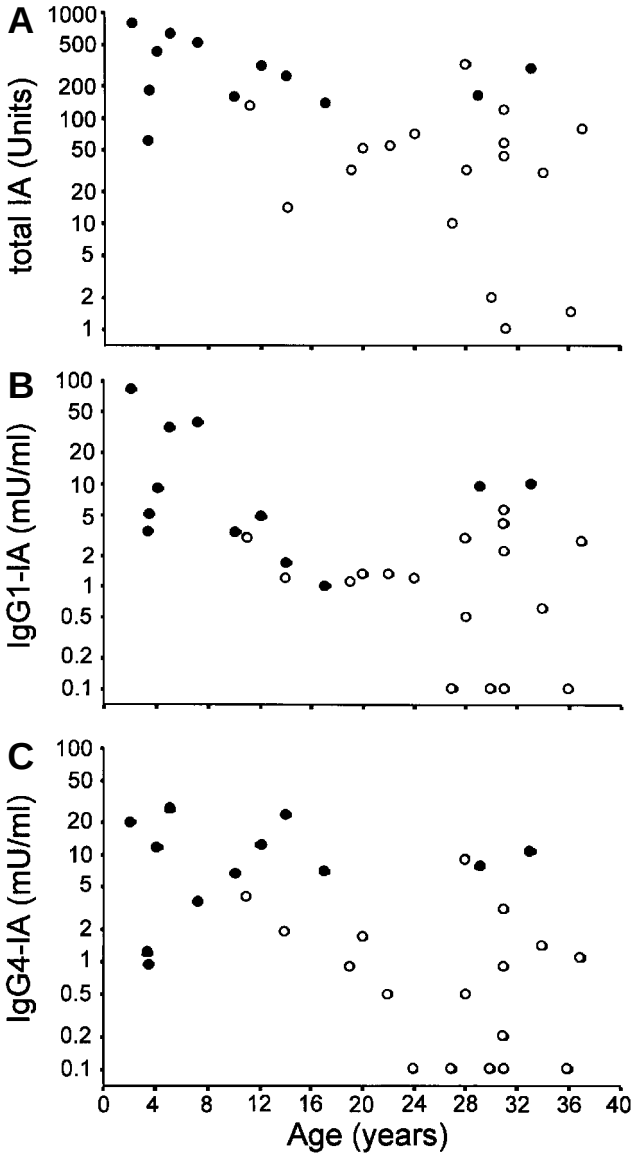

FIG. 2. Correlation of total IA (*A*), IgG1-IA (*B*), and IgG4-IA levels (*C*) after 12 months of insulin treatment with age in 29 patients with newly diagnosed type 1 diabetes. Total and subclass antibodies are shown on a logarithmic scale. ●, Individuals who had IAAs before start of insulin therapy; ○, subjects without IAAs.

CsA treatment leads to enhanced IgG1 responses to insulin. In contrast to adult patients treated with subcutaneous insulin (Fig. 3) and to patients treated with placebo plus subcutaneous insulin (data not shown), none of the 5 patients treated with CsA showed increases in total IA or IgG1-IA titers in the first 6 months of insulin treatment (Fig. 4). At 12 months, however, this suppression was no longer evident. Total IAs were detected in all 5 CsA-treated patients, and IgG1-IAs were detected in 4 of the 5 patients at levels (median 22 U and 3 mU/ml, respectively) similar to those found in both the 5 placebo-treated control patients (median 54 U and 1.5 mU/ml) and the adult patients treated with subcutaneous insulin only (median 113 U and 3 mU/ml). In addition, 1 patient also had IgG2-IAs, 1 had IgG3-IAs, and remarkably, none of the CsA-treated patients had IgG4-IAs within the first 12 months of treatment, whereas 4 of 5 placebo-treated patients had IgG4-IAs within this period of time (median 3.1 mU/ml, $P < 0.02$). IA responses increased markedly after cessation of CsA treatment. In the 5 patients, IA levels ranged from 74 to 852 U 15 months after diabetes onset (3 months after removal of CsA) and continued to rise thereafter. The marked rise in IA was predominantly due to very high levels of IgG1-IAs (median at 15 months 11 mU/ml, range 6–65), which remained higher than other IgG subclasses of IA in all CsA-treated patients for up to 1 year after CsA treatment was stopped. IgG4-IAs also became detectable in 4 of the patients at 15 months (median 0.6 mU/ml, range 0.1–2.3), but not at levels higher than those found in the adult patient cohorts. IgG2-IA and IgG3-IA levels were low and did not increase significantly after cessation of CsA therapy.

Humoral response to exogenous insulin in prediabetes is also predominantly IgG1 and IgG4. Of the 7 insulin-treated islet antibody–positive relatives in the SIP trial, 5 developed IAs during intervention. IA levels in these 5 subjects

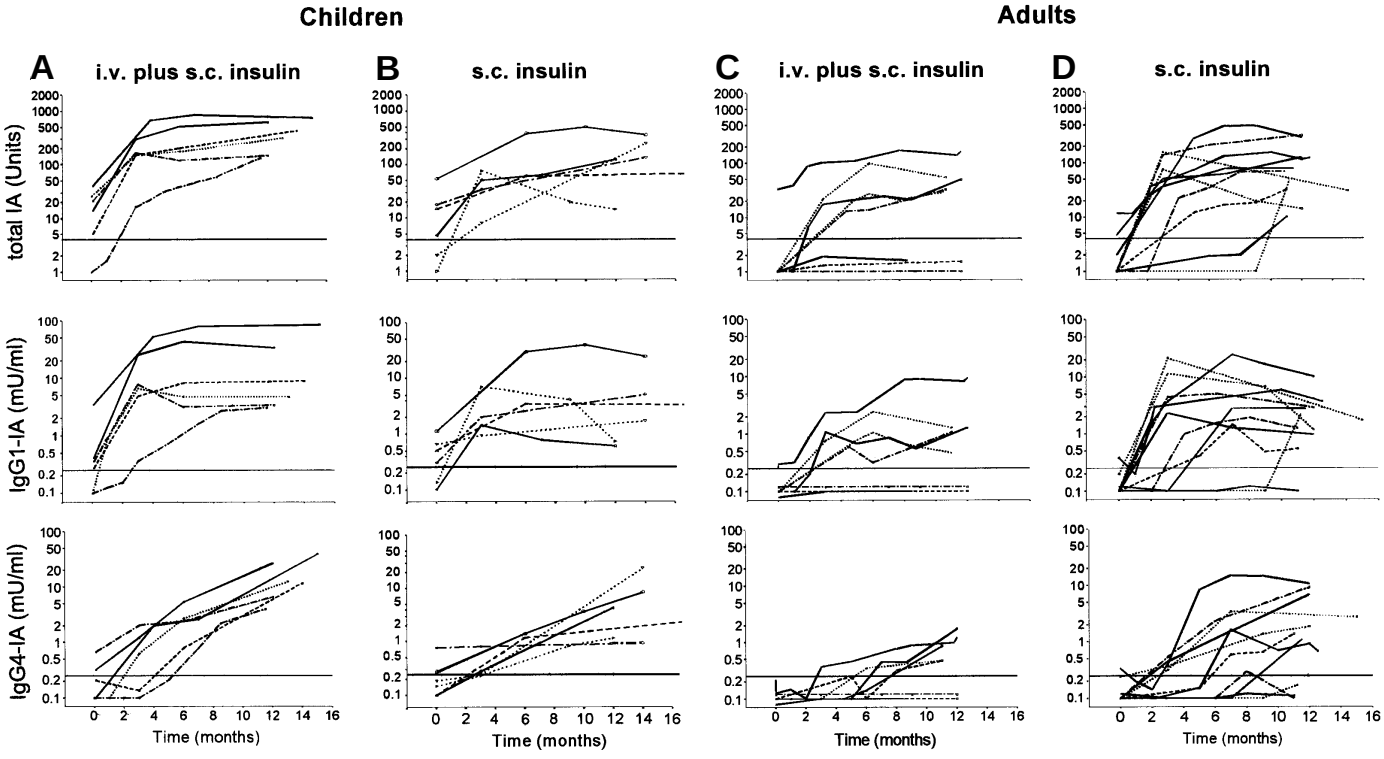

FIG. 3. Individual time course of total IAs (upper panel), IgG1-IAs (middle panel), and IgG4-IA (lower panel) in children with newly diagnosed type 1 diabetes treated with intravenous and subcutaneous insulin (*A*) or with subcutaneous insulin only (*B*) and in adult patients treated with intravenous and subcutaneous insulin (*C*) or with subcutaneous insulin only (*D*). Antibody levels are shown on a logarithmic scale.

were high and reached peak levels by the end of the 6-month treatment with daily subcutaneous insulin (range 55–726 U) (Fig. 5). IAs were predominantly IgG1, which were detected at very high levels (median 38 mU/ml, range 3.8–63). Lower levels of IgG2-, IgG3-, and IgG4-IAs were seen in all 5 subjects at the end of the 6-month subcutaneous insulin treatment period. Whereas IgG1-IA levels decreased within a few months after cessation of the subcutaneous treatment, IgG4-IAs continued to increase to levels above those of IgG1-IAs in 3 relatives (Fig. 5***B***–***D***). Of the 7 subjects, 4 did not develop type 1 diabetes 5.3, 6, 6, and 8.6 years after the start of insulin prophylaxis. Two of these subjects had no IA response, 1 had high IA levels of all subclasses (and now has low levels of IgG1-IAs only) (Fig. 5***A***), and 1 continues to have IAs of all IgG subclasses (Fig. 5***B***). The 3 subjects who developed diabetes 0.6, 4.1, and 4.2 years after the start of prophylaxis also varied in their IA responses: 1 developed diabetes immediately after the 6-month subcutaneous insulin treatment, when IAs of all subclasses were high (data not shown); 1 lost all subclasses 3 years after the start of prophylaxis (Fig. 5***C***); and 1 retained IAs of all subclasses until diabetes onset (Fig. 5***D***). All of the 7 nontreated islet antibody–positive control first-degree relatives developed type 1 diabetes, 0.4, 0.4, 0.7, 2.6, 3.7, 4.2, and 6.5 years after randomization. Of these subjects, 4 had IAAs at entry into the study, 2 developed weak IAAs at diabetes onset, and 1 remained IAA^- (9). IAA subclasses were heterogeneous in these subjects. Two relatives had IgG1 and IgG4, the levels of which declined steadily until diabetes onset; 1 subject had relatively stable IgG1-IAAs only; and, in 1 subject, only IgG3-IAAs could be detected throughout follow-up until diabetes development at 4.2 years. Of the 2 subjects who developed IAAs, 1 had IgG1-IAAs and the other had IgG4-IAAs immediately before diabetes onset (data not shown).

Insulin treatment did not induce GADA-IgG or IA-2A–IgG subclass changes. IgG subclasses of GADAs and IA-2As were measured at diabetes onset and up to 41 months after insulin therapy in 14 patients with GADAs (6 children and 8 adults) and in 10 patients with IA-2As (7 children and 3 adults) to determine whether insulin therapy may induce changes in autoantibody subclass. No consistent changes were observed. Three GADA-positive patients had IgG4-GADA at diabetes onset. In all 3 patients, IgG4-GADAs became undetectable, and total and IgG1-GADAs declined markedly after diabetes onset. In 2 additional patients, IgG4-GADAs became weakly detectable (Fig. 6), and total and IgG1-GADAs remained stable after diabetes onset. In the remaining 9 patients, GADAs were predominantly IgG1, declining markedly after diabetes onset in 4 patients (data not shown). IA-2As were predominantly IgG1 in all 10 $IA\text{-}2A^+$ patients. Total and IgG1–IA-2A titers declined in 5 of these patients, remained stable in 4 patients, and increased in 1 patient. Only 1 patient had IgG4–IA-2A at diabetes onset, which became undetectable along with a marked decline in total and IgG1–IA-2A levels 12 months later. No patients developed IgG4–IA-2As. Similarly, only 1 of the first-degree relatives receiving prophylactic insulin in the SIP trial had IgG4–IA-2As, which were detected at similar levels throughout follow-up, and IgG4-GADAs were not found for up to 3 years after the start of insulin prophylaxis in the first-degree relatives.

DISCUSSION

Intensive insulin therapy at onset of type 1 diabetes as well as prophylactic insulin treatment in antibody-positive relatives of

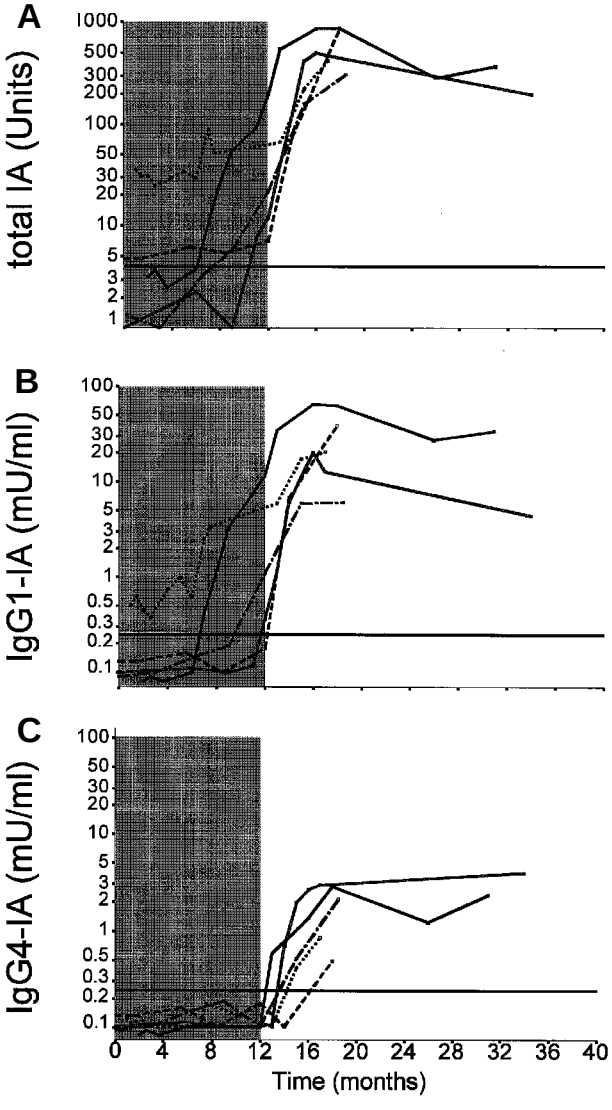

FIG. 4. Individual time course of total IAs (*A*), IgG1-IA (*B*), and IgG4-IA (*C*) in 5 newly diagnosed diabetic patients treated with CsA for 12 months (gray area) and subcutaneous insulin. The horizontal line denotes the threshold for positivity. Total and subclass antibodies are shown on a logarithmic scale.

patients with type 1 diabetes have been suggested to improve β-cell function and slow down the rate of β-cell destruction (10,11). This is likely to act in part through effecting a period of relative β-cell rest, in which β-cells experience reduced stress (20) and express fewer autoantigens (21). Exogenous insulin treatment in animal models also results in both changes within the islet infiltrate with more IL-4–producing and less interferon-γ–producing cells and a marked reduction in diabetes development (3). The diabetes protection after insulin treatment in mice can be transferred by CD4 spleen cells, and it is thought that the major protective effect by insulin therapy is through the induction of regulatory Th2 CD4 cells (22–25). Supporting this view is the observation that the antibody response to insulin in these models is predominantly of the Th2-associated mouse IgG1 and IgG2b subclasses (6). Therefore, we studied the IgG subclass of insulin antibodies in humans to see whether there is evidence of a Th2-biased response to exogenous insulin therapy, which would further support its prophylactic use for disease suppression. We found that, regardless of whether insulin was administered before or after diabetes onset, subcutaneous insulin therapy induced a strong IgG1-IA and IgG4-IA response. Although these findings need to be correlated with insulin-stimulated cytokine secretion, human IgG4 antibodies can be stimulated by the Th2 cytokines IL-4 and IL-13 in vitro and, together with the IgE response in allergy, are usually indicative of Th2 immunity (14,26,27). The findings could, therefore, be consistent with the hypothesis that exogenous insulin also promotes Th2 immunity in humans.

Interestingly, the autoimmune humoral response to insulin can also contain IgG4 antibodies, which, in some individuals, are predominant over other subclasses. In this and a previous study (19), we found IgG4-IAAs in ~50% of subjects who developed IAA before insulin therapy. Other investigators have also reported the presence of IgG4-IAAs (28). The autoimmune response is clearly of lower magnitude and the IgG4 antibodies less prevalent than that against exogenous subcutaneous insulin, but, nevertheless, differs from those against other autoantigens, such as GAD and IA-2, which are IgG1 and rarely include IgG4 antibodies (19). Interestingly, many patients develop an early IgG1 peak insulin autoantibody response that subsides to levels closer to those of other subclasses. This occurrence is similar to what we found in this study after the commencement of insulin therapy. Therefore, we suggest that the endogenous immunization against insulin in autoimmune diabetes has features of chronic or repeated antigen exposure. We further suggest that the autoimmune response to insulin may contain both a Th1 and Th2 component and that exogenous insulin is mainly effective in promoting the Th2 component. Insulin is not unique as a self-protein that drives an IgG4 response when given subcutaneously, as it has been shown that patients receiving factor VIII coagulant protein develop IgG1 and IgG4 factor VIII inhibitors (29). Whether this type of antibody response is typical of self-proteins is unclear. The influence of IL-4 on IgG4 production (14) is particularly interesting in view of the low T-cell IL-4 production in response to mitogens previously described in patients with type 1 diabetes (30–32), and it will be important to determine whether treatment with exogenous insulin can correct this.

Several factors were found to affect the immune response to exogenous insulin. First, the humoral response appeared augmented in patients who were young and/or had IAAs at the commencement of treatment, suggesting either a greater capacity for inducing potential modulatory effects if treatment is given early in life, as previously shown in mice (7), or if the immune system is already actively primed against insulin. Second, overall IA responses, including IgG4-IAs, showed a tendency to be suppressed in adult patients who received intravenous insulin before subcutaneous insulin. Interestingly, the complete absence of a humoral response to insulin was only seen in adults treated with intravenous insulin (both in insulin-treated patients and in prediabetic subjects), suggesting that intravenous treatment could induce tolerance. Moreover, the yearly intravenous treatment in the SIP trial did not appear to further stimulate the humoral IA response. These findings are consistent with data showing that intravenous antigen inactivates T-cells (33). However, such an effect was not seen in children in our study. Because almost all children had IAAs at the start of insulin therapy, inactivation via intravenous antigen

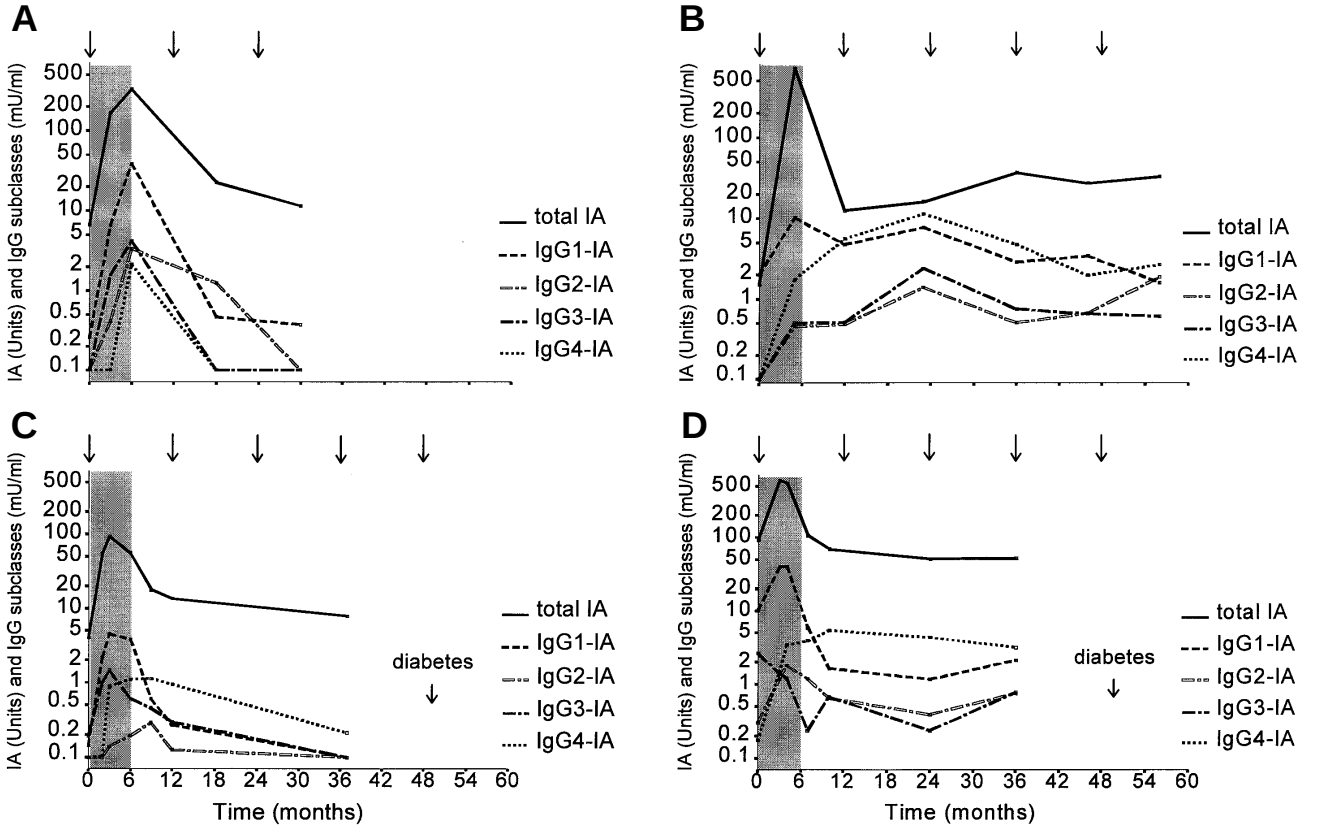

FIG. 5. Time course of IA and IgG subclasses in 4 first-degree relatives treated with intravenous insulin for 7 days (*A–D*), followed by daily subcutaneous injections for 6 months (gray area) in the SIP trial. Courses of 7-day intravenous insulin treatments (indicated by the arrows) were repeated every 12 months. Two individuals developed type 1 diabetes while under insulin prophylaxis (*C* and *D*), as indicated by an arrow. Total and subclass antibodies are shown on a logarithmic scale.

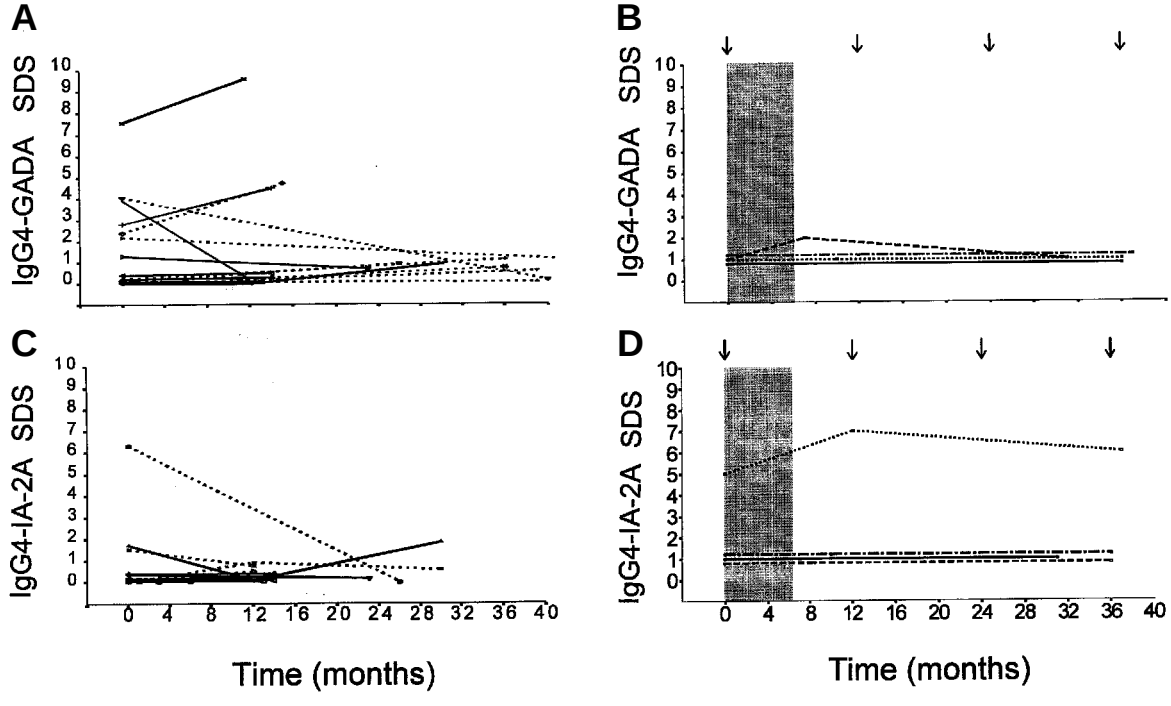

FIG. 6. Time course of IgG4-GADA (*A* and *B*) and IgG4–IA-2A (*C* and *D*) in patients with newly diagnosed type 1 diabetes (*A* and *C*) treated with either intravenous plus subcutaneous insulin or with subcutaneous insulin only and in islet antibody–positive first-degree relatives (*B* and *D*) treated with a 7-day course of intravenous insulin every 12 months (indicated by the arrows) and with a course of subcutaneous insulin for the initial 6 months (gray area).

therapy may be ineffective in the presence of ongoing autoimmunity. Consistent with this is the observation that IAA, 12-month IA, IgG1-IA, and IgG4-IA responses were 3–7 times higher in the 1 adult patient who was treated with intravenous insulin and had IAAs than in those in adult intravenous insulin–treated patients without IAAs. Furthermore, all intravenous insulin–treated subjects with no IA response did not have IAAs at the start of treatment.

CsA treatment also affected the IA response as previously reported for total IA titers (34). IgG1-IA was delayed and IgG4-IA was absent during CsA treatment. Cessation of treatment, however, resulted in large and enhanced IgG1-IA levels with a subsequent development of IgG4-IA. Remarkably, and in contrast to the CsA placebo control subjects, IgG4-IA levels did not reach those of IgG1-IA in any of the CsA-treated patients for up to 1 year after CsA treatment was stopped. Discontinuation of CsA was previously shown to result in a marked increase in insulin requirement and deterioration of metabolic control (35).

Although both the IAA and IA responses can have a strong IgG4 component, its presence was not particularly associated with protection from diabetes. We previously found no significant delay in diabetes onset if IAA was of the IgG4 subclass (19), which suggested that a potential Th2 autoimmune response to insulin is insufficient to prevent spreading of autoimmunity to other islet autoantigens and diabetes development. Although numbers are small, we now find that, whereas insulin treatment delayed diabetes onset in subjects with prediabetes (9), diabetes outcome in the insulin-treated relatives was not associated with an IgG4 humoral response to insulin treatment. This indicates, therefore, that the IgG4-IA response is unlikely to be an effective marker to predict successful treatment in individual subjects.

Finally, it was shown that insulin treatment in the NOD mouse was associated with Th2 spreading to other antigens (6). Whereas we found that IgG4-IAs were detected in some prediabetic subjects long after the cessation of subcutaneous insulin therapy, we found no changes in the IgG subclass of either GADA or IA-2A, and in particular, we found no induction of IgG4 autoantibodies for up to 3 years of daily exposure to either intravenous plus subcutaneous or subcutaneous insulin in humans. Several reasons may explain a potential lack of spreading in these subjects. First, it is quite conceivable that modulation is not expressed at the β-cell level and that without studying the relevant T-cell responses, we cannot exclude the induction of GAD- or IA-2–reactive regulatory Th2 cells. Second, as previously found in mice, the ability to induce Th2 spreading may be attenuated at a late stage in the disease process (7). Third, because Th2 spreading in mice was observed when antigen was given together with potent Th2-inducing adjuvants, intravenous and subcutaneous insulin therapy may not be sufficiently immunogenic to result in shifts in the T-cell pool large enough to effect spreading. Fourth, even though insulin may be able to effect a Th2 response against itself, it may be ineffective at inducing Th2 spreading.

In conclusion, the findings of this study show that exposure to exogenous insulin promotes an IgG1 and a Th2-associated IgG4 antibody response. While a Th2 response needs to be shown at the T-cell level, the findings are encouraging in view of ongoing and proposed intervention trials using exogenous insulin administration. However, the Th2 spreading associated with diabetes protection after immunization with autoantigen in the NOD mouse was not evident at the humoral level in insulin-treated subjects, and it may be necessary to use a stronger Th2-inducing regimen (36) to be fully effective in diabetes delay or prevention in humans.

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