Rituximab Selectively Suppresses Specific Islet Antibodies

Liping Yu,[1] Kevan Herold,[2] Heidi Krause-Steinrauf,[3] Paula L. McGee,[3] Brian Bundy,[4] Alberto Pugliese,[5] Jeff Krischer,[6] and George S. Eisenbarth,[1] for the Type 1 Diabetes TrialNet Anti-CD20 Study Group

OBJECTIVE—The TrialNet Study Group evaluated rituximab, a B-cell–depleting monoclonal antibody, for its effect in new-onset patients with type 1A diabetes. Rituximab decreased the loss of C-peptide over the first year of follow-up and markedly depleted B lymphocytes for 6 months after administration. This article analyzes the specific effect of rituximab on multiple islet autoantibodies.

RESEARCH DESIGN AND METHODS—A total of 87 patients between the ages of 8 and 40 years received either rituximab or a placebo infusion weekly for four doses close to the onset of diabetes. Autoantibodies to insulin (IAAs), GAD65 (GADAs), insulinoma-associated protein 2 (IA2As), and ZnT8 (ZnT8As) were measured with radioimmunoassays. The primary outcome for this autoantibody analysis was the mean level of autoantibodies during follow-up.

RESULTS—Rituximab markedly suppressed IAAs compared with the placebo injection but had a much smaller effect on GADAs, IA2As, and ZnT8As. A total of 40% (19 of 48) of rituximab-treated patients who were IAA positive became IAA negative versus 0 of 29 placebo-treated patients ($P < 0.0001$). In the subgroup ($n = 6$) treated within 50 days of diabetes, IAAs were markedly suppressed by rituximab in all patients for 1 year and for four patients as long as 3 years despite continuing insulin therapy. Independent of rituximab treatment, the mean level of IAAs at study entry was markedly lower ($P = 0.035$) for patients who maintained C-peptide levels during the first year of follow-up in both rituximab-treated and placebo groups.

CONCLUSIONS—A single course of rituximab differentially suppresses IAAs, clearly blocking IAAs for >1 year in insulin-treated patients. For the patients receiving insulin for >2 weeks prior to rituximab administration, we cannot assess whether rituximab not only blocks the acquisition of insulin antibodies induced by insulin administration and/or also suppresses preformed insulin autoantibodies. Studies in prediabetic non–insulin-treated patients will likely be needed to evaluate the specific effects of rituximab on levels of IAAs. ***Diabetes*** **60:2560–2565, 2011**

From the [1]Barbara Davis Center for Childhood Diabetes, University of Colorado Denver, Aurora, Colorado; the [2]Department of Immunobiology, Yale University School of Medicine, New Haven, Connecticut; the [3]Biostatistics Center, George Washington University, Rockville, Maryland; the [4]Department of Biostatistics, Roswell Park Cancer Institute, Buffalo, New York; the [5]Diabetes Research Institute, Miller School of Medicine, University of Miami, Miami, Florida; and the [6]Division of Informatics and Biostatistics, University of South Florida, Tampa, Florida.

Corresponding author: Liping Yu, liping.yu@ucdenver.edu.

Received 18 May 2011 and accepted 7 July 2011.

DOI: 10.2337/db11-0674

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db11-0674/-/DC1.

Although the anti-CD20 monoclonal antibody rituximab (Rituxan; Genentech, Biogen-IDEC Pharmaceuticals) originally was introduced for therapy of B-cell lymphomas (1), and subsequently applied to a series of antibody-mediated autoimmune disorders, it also has a role in what were thought to be classic T-cell–mediated autoimmune disorders, such as multiple sclerosis (2,3). In several of these diseases, therapeutic responses occur without a change in levels of characteristic autoantibodies. For some immune-mediated disorders, it has been reported that only a subset of autoantibodies decreases following rituximab. For example, it has been reported that rituximab therapy does not decrease the overall thyrotropin receptor–binding antibody, whereas stimulatory thyrotropin receptor–binding antibodies were decreased (4). The etiology of this differential effect is unknown, but it has been hypothesized to relate to the site of autoantibody production, with the observation that although rituximab does not target plasma cells, it does alter tertiary lymphoid structures and short-lived plasma cells that do not reside in the bone marrow (5).

The quantitative effect of rituximab on islet autoantibodies in patients with diabetes has not been described. Type 1A, or immune-mediated, diabetes is believed to result from T-cell–mediated destruction of islet β-cells (6). Although T cells are dominant determinants of β-cell destruction in the NOD mouse, and diabetes has been reported in one child lacking B cells (7), multiple studies document a role for both B lymphocytes and autoantibodies in the NOD mouse model (8–11). For both humans and the NOD mouse, anti-CD20 antibodies suppress disease progression (11,12). The study by Pescovitz et al. (12) documented the preservation of C-peptides relative to placebo patients at the 1-year follow-up (see Supplementary Appendix). In this study, we analyzed the effects of rituximab on islet autoantibody levels in both placebo- and rituximab-treated new-onset patients from this trial, using a fluid-phase radioimmunoassay to detect autoantibodies against multiple islet antigens (autoantibodies to insulin [IAAs], GAD65 [GADAs], insulinoma-associated protein 2 [IA-2; IA2As], and ZnT8 [ZnT8As]).

RESEARCH DESIGN AND METHODS

The details of patient recruitment, protocol, and the effect on primary and secondary metabolic outcomes are published (12), and the patients in this report are identical to those in the report of Pescovitz et al. (12). For this study, participants and/or parents provided written informed consent, and consent documents were approved by independent ethics committees or institutional review boards at each participating center. In brief, a single course of rituximab (375 mg/m^2 infusions on days 1, 8, 15, and 22 of the study) versus placebo was administered in a blinded fashion to 87 patients between the ages

TABLE 1
Loss of islet autoantibodies during the first year after diabetes onset

	IAA	GADA	IA2A	ZnT8A
Rituximab patients				
Positive for autoantibodies (total)	48	38	32	37
Negative for autoantibodies at follow-up	19 (40)*	7 (18)	4 (12.5)	6 (16)
Placebo patients				
Positive for autoantibodies (total)	29	23	22	19
Negative for autoantibodies at follow-up	0 (0.0)	6 (26)	3 (13.5)	2 (11)

Data are *N* and *n* (%). *$P < 0.001$ vs. placebo.

of 8 and 40 years with type 1 diabetes who had at least one islet autoantibody (IAAs [if within 7 days of starting insulin], GADAs, IA2As, and islet cell antibodies). At follow-up visits of up to 3 years, serum samples were obtained for determination of islet autoantibodies as well as metabolic evaluation with their C-peptide levels. The subjects who had a decrease in C-peptides greater than or equal to the 50th percentile within-subject variation were defined as nonresponders. Subjects who did not meet the nonresponder criterion were classified as responders (i.e., the subjects either had an increase in C-peptides from baseline or had a decrease in C-peptides from baseline but had a within-subject variation of less than the 50th percentile).

Islet autoantibody determination. Biochemical islet autoantibodies IAAs, GADAs, IA2As, and ZnT8As were measured with radioimmunoassay using the protein A capture and 96-well filtration-plate format. All samples were analyzed in duplicate, and positive results (index higher than the 99th percentile of normal control subjects) were repeated for confirmation. The GADA and IA2A tests were done in a combined radioassay (13), and the IAA test was performed with a competition radioimmunoassay (14). Levels of autoantibodies are expressed as an index (index = $CPM_{sample} - CPM_{negative\ control}$)/($CPM_{positive\ control} - CPM_{negative\ control}$ where CPM is counts per minute, with each antibody having its own positive standard for calculations. The interassay coefficients of variation are 10.0% for GADAs, 5.7% for IA2As, 10.4% for ZnT8As, and 16% for IAAs in the low-positive range. The upper limits of normal (0.032 for GADAs, 0.049 for IA2As, 0.020 for ZnT8As, and 0.010 for IAAs) were established as the 99th percentile of 100–200 healthy control subjects. In the most recent 2010 Diabetes Autoantibody Standardization Program Workshop, assay sensitivity and specificity were 84 and 99%, respectively, for GADAs; 66 and 100%, respectively, for IA2As; 64 and 100%, respectively, for ZnT8As; and 56 and 100%, respectively, for IAAs.

Statistical analysis. The Fisher exact test was used to test for category changes in terms of the positivity of the autoantibodies. The Student *t* test was used for the comparison of mean levels of autoantibodies (Prism Software). Results were considered significant with a two-sided $\alpha < 0.05$.

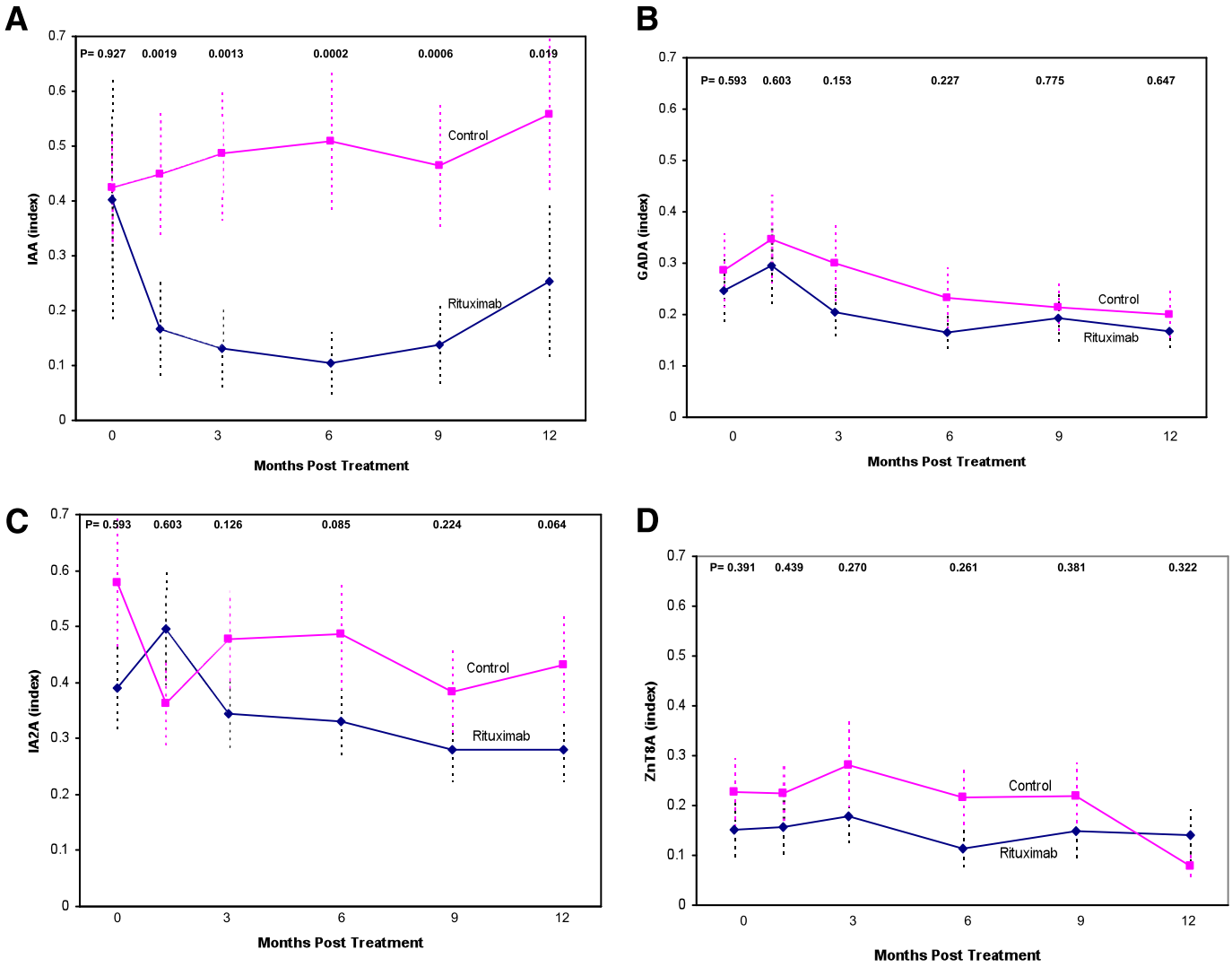

FIG. 1. The mean levels of autoantibodies among the subjects of two treated groups (the rituximab-treated group and the placebo group). *A*: IAAs. *B*: GADAs. *C*: IA2As. *D*: ZnT8As. The y-axis in all four panels represents the levels of autoantibodies expressed as index. (A high-quality color representation of this figure is available in the online issue.)

RESULTS

Of patients treated with rituximab who were initially IAA positive before the trial, 40% (19 of 48) became negative within the first 12 months after the diagnosis of diabetes (Table 1). In contrast, no placebo patient (0 of 29) became IAA negative ($P < 0.001$). Rituximab did not significantly influence the percentage of subjects who became negative for GADAs, IA2As, or ZnT8As. In the subgroup of six individuals who were treated within 50 days of diagnosis of diabetes (who received 50 days of insulin therapy), IAAs were markedly suppressed by rituximab in all patients for 1 year and in four patients for as long as 3 years, despite continuing insulin therapy.

Figure 1 illustrates the mean levels of IAAs (Fig. 1*A*), GADAs (Fig. 1*B*), IA2As (Fig. 1*C*), and ZnT8As (Fig. 1*D*) over time from trial entry. Overall, IAAs did not decrease in placebo-treated patients, whereas the IAA mean level in the rituximab-treated group was suppressed by ~75% at 6 months ($P < 0.001$), with the suppression continuing for the 12 months of follow-up. As shown in Fig. 2*A*, the IAA levels of patients treated with rituximab soon after insulin therapy remained low for >1 year (compared with most placebo-treated patients), and nearly one-half of patients did not increase to an index >0.1 with up to 3 years of follow-up. All but one of the placebo-treated patients who entered into the study within 50 days of diabetes onset developed an IAA index >0.5, and most patients rapidly achieved such levels shortly after diagnosis, as expected with a subcutaneous insulin injection. In the same patients, GADAs, IA2As, and ZnT8As were relatively stable after rituximab or placebo injections, independent of rituximab injection.

Study participants were divided into two subgroups, independent of rituximab treatment, on the basis of their C-peptide levels during the follow-up, defined as responders and nonresponders in both rituximab- and placebo-injection groups. The categorization was performed without knowledge of IAA status. Surprisingly, IAA levels were significantly lower ($P < 0.05$) in subjects defined as responders versus those who were nonresponders at time 0 for both rituximab and placebo groups (Fig. 3*A*). The level of IAAs was remarkably lower in the placebo group from baseline

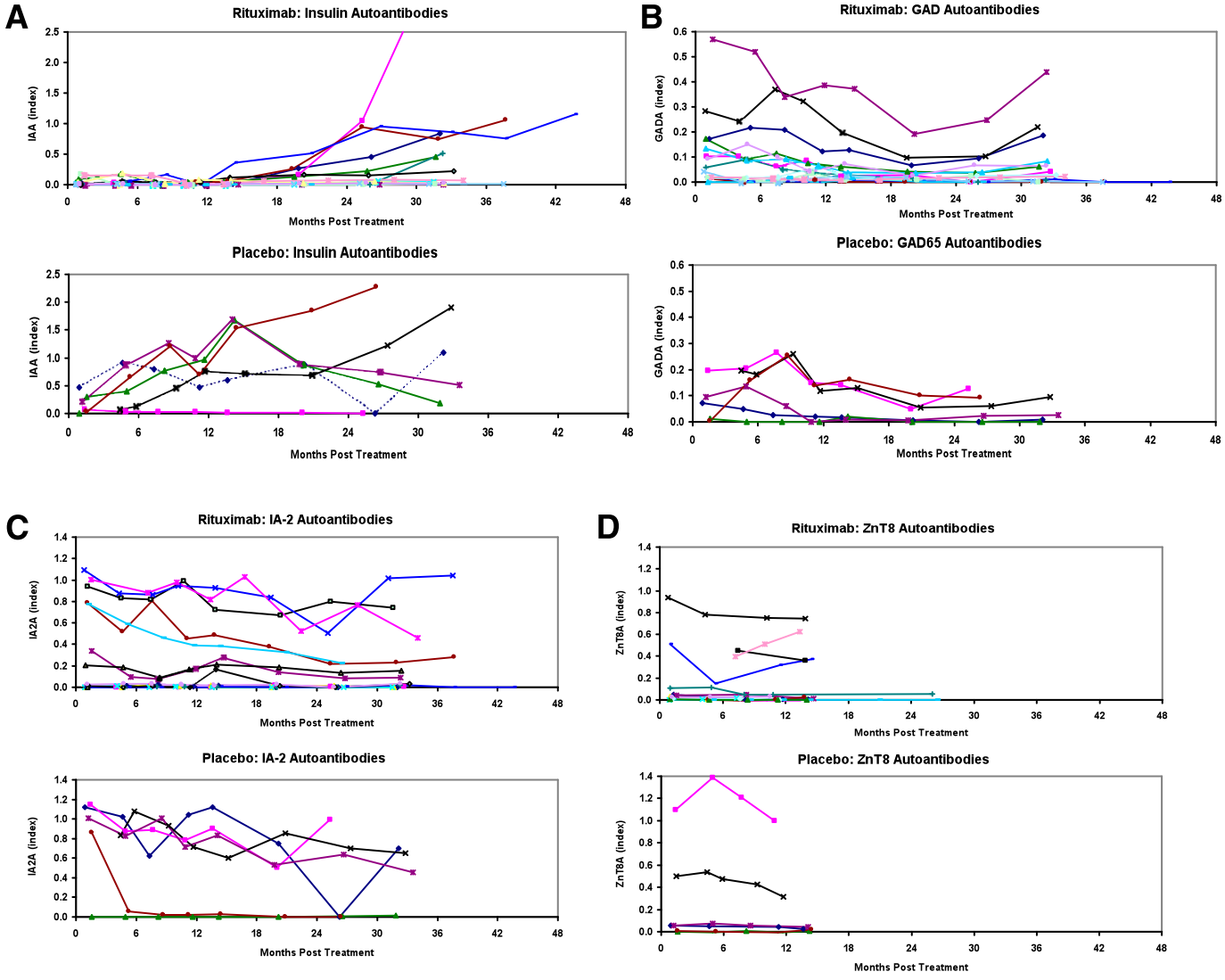

FIG. 2. Follow-up of autoantibody levels posttreatment among the subjects of two groups (rituximab-treated group and the placebo group). *A*: IAAs. *B*: GADAs. *C*: IA2As. *D*: ZnT8As. The y-axis in all four panels represents the levels of autoantibodies expressed as index. (A high-quality color representation of this figure is available in the online issue.)

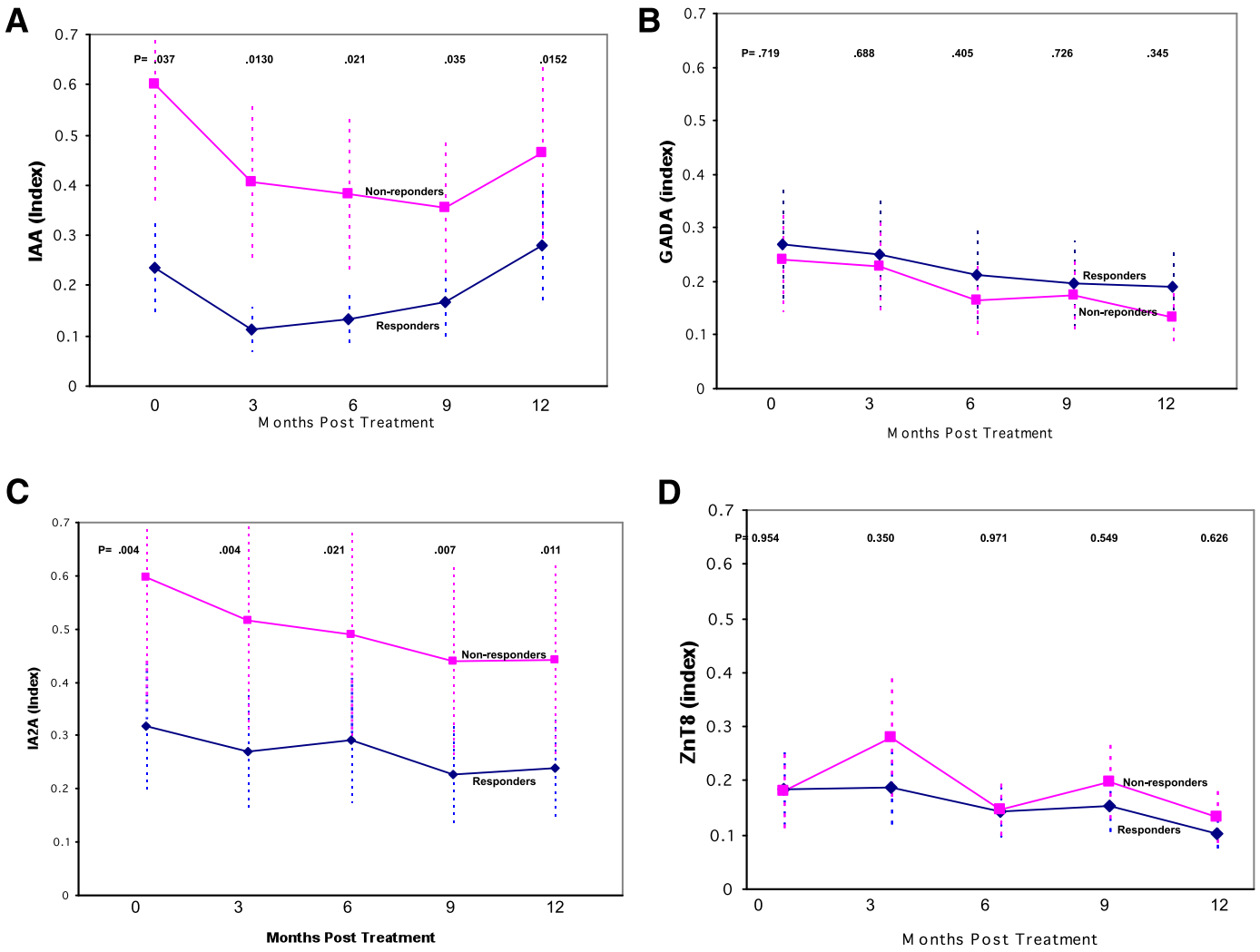

FIG. 3. The mean levels of autoantibodies in the subjects defined as responders (n = 40) vs. nonresponders (n = 38). *A*: IAAs. *B*: GADAs. *C*: IA2As. *D*: ZnT8As. The y-axis in all four panels represents the levels of autoantibodies expressed as index. (A high-quality color representation of this figure is available in the online issue.)

until the 12-month follow-up, whereas the IAAs in the rituximab-treated group were suppressed after treatment. A significantly lower level ($P < 0.05$) of IA2As also was observed in responders compared with nonresponders, as shown in Fig. 3*C*, whereas there were no differences for mean GADAs and ZnT8As between the two groups (Fig. 3*B* and *D*). At and beyond 12 months, when insulin antibodies were increasing, there was no correlation between IAA levels and C-peptide.

DISCUSSION

Type 1A, or autoimmune, diabetes is characterized by the presence of a series of autoantibodies targeting islet molecules, including insulin, GAD65, IA-2, and ZnT8 (15). The initial assay for islet autoantibodies measured cytoplasmic islet cell autoantibodies by indirect immunofluorescence, using frozen sections of human pancreata (16). IAAs are not detected with the islet cell antibody assay, whereas GADAs, IA2As, and ZnT8As can be detected. In addition to the autoantibodies, which were present before the diagnosis of diabetes, almost all patients treated with insulin (including patients with type 2 diabetes) developed high levels (relative to levels of prediabetic and new-onset patients) of insulin antibodies (17). Islet autoantibodies have a number of distinguishing characteristics. IAAs often are (but not always) the first autoantibody to appear in children followed from birth (18), and at onset of diabetes, their levels are inversely related to age of diabetes onset (19,20). GADAs change the least with age of onset. ZnT8As are rapidly lost after diabetes onset (21), and IA2As are very specific, identifying a particularly high diabetes risk of prospectively followed nondiabetic children (22). The molecules insulin and ZnT8 are, to a large extent, limited to islet cells, whereas GAD65 and IA-2 are distributed in multiple neuroendocrine tissues. Given these unique properties for each of the islet autoantibodies, it is perhaps not surprising that rituximab had a differential effect upon the measured antibodies, with the most dramatic suppression of insulin autoantibodies and antibodies.

Rituximab treatment has been reported to blunt primary and secondary antibody responses (23,24). Because patients who entered into this study were treated with insulin prior to determination of IAAs, it is not possible to isolate the effect of rituximab on pre-existing IAAs versus prevention and suppression of insulin antibodies induced by subcutaneous insulin therapy. This highlights the need for standardized assays that would distinguish the two types of

antibodies. This may be particularly important given recent findings indicating that levels of IAAs, but not GADAs, and IA2As are inversely correlated with the rate at which prediabetic children progress to diabetes (25).

A number of studies have documented the differential effects of anti-CD20 treatment on different antibodies in both humans and animal models. In general, rituximab therapy does not suppress overall IgG compared with its effect on IgM antibodies (12). IgG levels can remain unaltered, whereas specific pathogenic autoantibodies are markedly suppressed. Specific disease-associated autoantibodies that are produced in sites outside of the bone marrow may be more susceptible to anti-CD20 suppression, and this may relate to the differential effect in the current study upon IAAs (5). There is a large body of evidence in the NOD mouse model that insulin may be a primary autoantigen (26,27). In particular, mutating an antigenic insulin peptide prevents diabetes in NOD mice (27), whereas knockouts of both GAD65 and IA-2 do not influence progression to diabetes (28,29). In addition, a polymorphism of the insulin gene correlated with increased levels of thymic insulin message is associated with decreased type 1A diabetes risk (30–32).

Rituximab is remarkably effective in blocking de novo antibody responses and may be able to suppress both IAAs and insulin antibodies (the latter induced by injections of human insulin). In particular, 40% of patients treated with rituximab in the trial became negative (for variable times) for insulin antibodies/IAAs. Studies prior to, or within several weeks of, insulin therapy, given current insulin autoantibody/antibody assays, are needed to define whether rituximab can specifically suppress IAAs.

A significantly lower level of IAAs was observed, independent of rituximab treatment, in subjects defined as C-peptide responders versus nonresponders, particularly evident even in the placebo group. IA2As at entry also were found to be significantly lower (although not as dramatic a difference compared with IAAs) at entry in the placebo group for responders versus nonresponders, whereas no difference was observed for either GADAs or ZnT8As. The levels of IAAs were remarkably lower in responders compared with nonresponders at time 0 for both rituximab- and placebo-injection groups, which may indicate that the levels of IAAs, independent of rituximab treatment, are likely to be associated with disease prognosis or disease progression in patients with newly diagnosed type 1A diabetes. The relationship between the levels of IAAs and disease progression only has been investigated in the prediabetic period with the presence of anti-islet autoantibodies, but this has not been possible to study after disease onset. Among these biochemically defined islet autoantibodies, IAAs are the only ones in which the level is associated with the time to develop clinical type 1A diabetes among multiple autoantibody-positive prediabetic subjects (all followed to diabetes) in the Diabetes Autoimmunity Study in the Young (DAISY) study (33). Higher IAA levels are associated with faster progression to overt diabetes. Combining this previous study with the present observation, IAA level seems to play a role in the prediction of disease progression, and higher levels of IAAs might indicate more aggressive autoimmune destruction of pancreatic β-cells both before and after clinical onset of diabetes. The IAAs in the current study are presumably a mixed population of naturally occurring IAAs and induced insulin antibodies. It needs to be further explored whether only IAAs or both IAAs and insulin antibodies secondary to injected insulin are associated with disease progression and the potentially therapeutic effect of rituximab.

ACKNOWLEDGMENTS

The sponsor of this trial was the Type 1 Diabetes TrialNet Study Group. TrialNet is a clinical trials network funded by National Institutes of Health through the National Institute of Diabetes and Digestive and Kidney Diseases, the National Institute of Allergy and Infectious Diseases, the Eunice Kennedy Shriver National Institute for Child Health and Human Development, and the National Center for Research Resources; the Juvenile Diabetes Research Foundation International; and the American Diabetes Association.

No potential conflicts of interest relevant to this article were reported.

L.Y. researched data and wrote the manuscript. K.H., H.K.-S., P.L.M., and B.B. researched data and reviewed and edited the manuscript. A.P. contributed to discussion and reviewed and edited the manuscript. J.K. researched data and reviewed and edited the manuscript. G.S.E. researched data and wrote the manuscript.

Dr. Pescovitz, who headed the TrialNet Anti-CD20 Study, contributed greatly to the early drafts of the manuscript prior to his untimely death.

REFERENCES

1. Maloney DG, Liles TM, Czerwinski DK, et al. Phase I clinical trial using escalating single-dose infusion of chimeric anti-CD20 monoclonal antibody (IDEC-C2B8) in patients with recurrent B-cell lymphoma. Blood 1994;84:2457–2466
2. Hauser SL, Waubant E, Arnold DL, et al.; HERMES Trial Group. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. N Engl J Med 2008;358:676–688
3. El Fassi D, Nielsen CH, Bonnema SJ, Hasselbalch HC, Hegedüs L. B lymphocyte depletion with the monoclonal antibody rituximab in Graves' disease: a controlled pilot study. J Clin Endocrinol Metab 2007;92:1769–1772
4. El Fassi D, Banga JP, Gilbert JA, Padoa C, Hegedüs L, Nielsen CH. Treatment of Graves' disease with rituximab specifically reduces the production of thyroid stimulating autoantibodies. Clin Immunol 2009;130:252–258
5. Huang H, Benoist C, Mathis D. Rituximab specifically depletes short-lived autoreactive plasma cells in a mouse model of inflammatory arthritis. Proc Natl Acad Sci USA 2010;107:4658–4663
6. Bluestone JA, Herold K, Eisenbarth G. Genetics, pathogenesis and clinical interventions in type 1 diabetes. Nature 2010;464:1293–1300
7. Martin S, Wolf-Eichbaum D, Duinkerken G, et al. Development of type 1 diabetes despite severe hereditary B-lymphocyte deficiency. N Engl J Med 2001;345:1036–1040
8. Greeley SA, Katsumata M, Yu L, et al. Elimination of maternally transmitted autoantibodies prevents diabetes in nonobese diabetic mice. Nat Med 2002;8:399–402
9. Zekavat G, Rostami SY, Badkerhanian A, et al. In vivo BLyS/BAFF neutralization ameliorates islet-directed autoimmunity in nonobese diabetic mice. J Immunol 2008;181:8133–8144
10. Silveira PA, Johnson E, Chapman HD, Bui T, Tisch RM, Serreze DV. The preferential ability of B lymphocytes to act as diabetogenic APC in NOD mice depends on expression of self-antigen-specific immunoglobulin receptors. Eur J Immunol 2002;32:3657–3666
11. Hu C, Deng S, Wong FS, Wen L. Anti-CD20 treatment prolongs syngeneic islet graft survival and delays the onset of recurrent autoimmune diabetes. Ann N Y Acad Sci 2008;1150:217–219
12. Pescovitz MD, Greenbaum CJ, Krause-Steinrauf H, et al.; Type 1 Diabetes TrialNet Anti-CD20 Study Group. Rituximab, B-lymphocyte depletion, and preservation of beta-cell function. N Engl J Med 2009;361:2143–2152
13. Yu L, Rewers M, Gianani R, et al. Antiislet autoantibodies usually develop sequentially rather than simultaneously. J Clin Endocrinol Metab 1996;81:4264–4267
14. Yu L, Robles DT, Abiru N, et al. Early expression of antiinsulin autoantibodies of humans and the NOD mouse: evidence for early determination of subsequent diabetes. Proc Natl Acad Sci USA 2000;97:1701–1706

15. Bingley PJ. Clinical applications of diabetes antibody testing. J Clin Endocrinol Metab 2010;95:25–33
16. Bottazzo GF, Florin-Christensen A, Doniach D. Islet-cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies. Lancet 1974; 2:1279–1283
17. Palmer JP, Asplin CM, Clemons P, et al. Insulin antibodies in insulin-dependent diabetics before insulin treatment. Science 1983;222:1337–1339
18. Achenbach P, Koczwara K, Knopff A, Naserke H, Ziegler AG, Bonifacio E. Mature high-affinity immune responses to (pro)insulin anticipate the autoimmune cascade that leads to type 1 diabetes. J Clin Invest 2004;114: 589–597
19. Vardi P, Ziegler AG, Mathews JH, et al. Concentration of insulin autoantibodies at onset of type I diabetes: inverse log-linear correlation with age. Diabetes Care 1988;11:736–739
20. Arslanian SA, Becker DJ, Rabin B, et al. Correlates of insulin antibodies in newly diagnosed children with insulin-dependent diabetes before insulin therapy. Diabetes 1985;34:926–930
21. Wenzlau JM, Walter M, Gardner TJ, et al. Kinetics of the post-onset decline in zinc transporter 8 autoantibodies in type 1 diabetic human subjects. J Clin Endocrinol Metab 2010;95:4712–4719
22. Achenbach P, Bonifacio E, Williams AJ, Ziegler AG, Gale EA, Bingley PJ; ENDIT Group. Autoantibodies to IA-2beta improve diabetes risk assessment in high-risk relatives. Diabetologia 2008;51:488–492
23. van der Kolk LE, Baars JW, Prins MH, van Oers MH. Rituximab treatment results in impaired secondary humoral immune responsiveness. Blood 2002;100:2257–2259
24. Gonzalez-Stawinski GV, Yu PB, Love SD, Parker W, Davis RD Jr. Hapten-induced primary and memory humoral responses are inhibited by the infusion of anti-CD20 monoclonal antibody (IDEC-C2B8, Rituximab). Clin Immunol 2001;98:175–179
25. Wang J, Miao D, Babu S, et al. Prevalence of autoantibody-negative diabetes is not rare at all ages and increases with older age and obesity. J Clin Endocrinol Metab 2007;92:88–92
26. Krishnamurthy B, Dudek NL, McKenzie MD, et al. Responses against islet antigens in NOD mice are prevented by tolerance to proinsulin but not IGRP. J Clin Invest 2006;116:3258–3265
27. Nakayama M, Abiru N, Moriyama H, et al. Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice. Nature 2005;435:220–223
28. Yamamoto T, Yamato E, Tashiro F, et al. Development of autoimmune diabetes in glutamic acid decarboxylase 65 (GAD65) knockout NOD mice. Diabetologia 2004;47:221–224
29. Kubosaki A, Miura J, Notkins AL. IA-2 is not required for the development of diabetes in NOD mice. Diabetologia 2004;47:149–150
30. Bell GI, Horita S, Karam JH. A polymorphic locus near the human insulin gene is associated with insulin-dependent diabetes mellitus. Diabetes 1984;33:176–183
31. Pugliese A, Zeller M, Fernandez A Jr, et al. The insulin gene is transcribed in the human thymus and transcription levels correlated with allelic variation at the INS VNTR-IDDM2 susceptibility locus for type 1 diabetes. Nat Genet 1997;15:293–297
32. Vafiadis P, Bennett ST, Todd JA, et al. Insulin expression in human thymus is modulated by INS VNTR alleles at the IDDM2 locus. Nat Genet 1997;15: 289–292
33. Steck AJ, Johnson K, Barriga K, et al. Age of islet autoantibody appearance and mean levels of insulin, but not GAD or IA-2 autoantibodies, predict age of diagnosis of type 1 diabetes: Diabetes Autoimmunity Study in the Young. Diabetes Care 2011;34:1397–1399