The incidence of type 1 diabetes has increased rapidly over recent decades, particularly in young children. We aimed to determine whether this rise was associated with changes in patterns of humoral islet autoimmunity at diagnosis. Autoantibodies to insulin (IAA), GAD (GADA), islet antigen-2 (IA-2A), and zinc transporter 8 (ZnT8A) were measured by radioimmunoassay in sera collected from children and young adults with newly diagnosed type 1 diabetes between 1985 and 2002. The influence of date of diagnosis on prevalence and level of autoantibodies was investigated by logistic regression with adjustment for age and HLA class II genetic risk. Prevalence of IA-2A and ZnT8A increased significantly over the period studied, and this was mirrored by raised levels of IA-2A, ZnT8A, and IA-2B autoantibodies (IA-2B A). IAA and GADA prevalence and levels did not change. Increases in IA-2A, ZnT8A, and IA-2B at diagnosis during a period of rising incidence suggest that the process leading to type 1 diabetes is now characterized by a more intense humoral autoimmunity response. Understanding how changes in environment or lifestyle alter the humoral autoimmune response to islet antigens should help explain why the incidence of type 1 diabetes is increasing and may suggest new strategies for preventing disease.

The incidence of type 1 diabetes is rising by 3–4% per year across Europe, North America, and Australia, with the greatest increase in children diagnosed at <5 years of age (1,2). These increases have been accompanied by a reduced frequency of the highest-risk HLA class II diabetes susceptibility genotype in patient populations and greater penetrance of lower-risk genotypes (3). The reason for the rise in incidence remains obscure but has been attributed to changes in environment and/or lifestyle (4). As type 1 diabetes results from autoimmune destruction of the pancreatic β-cells, increases in incidence could be associated with changes in the pattern of autoimmunity.

Autoantibodies to insulin (IAA), GAD (GADA), islet antigen-2 (IA-2A), and zinc transporter 8 (ZnT8A) are the most reliable markers of humoral autoimmune activity in diabetes and may offer key insights into changes in disease pathogenesis. As islet autoimmunity evolves, there is typically spreading of reactivity to different target autoantigens and epitopes (5). IAA are generally the first autoantibodies to be detected in infancy, followed by GADA (6), while IA-2A and ZnT8A tend to appear later and may mark a critical turning point in the process leading to β-cell destruction (5,7,8). Spreading of autoreactivity within antigens shows a distinct pattern; autoantibodies to epitopes in the juxtamembrane region of IA-2 (JMA) often arise early and develop independently of responses to protein tyrosine phosphatase epitopes of IA-2 (9), while antibodies to IA-2β (IA-2B A) may signal advanced autoimmunity and impending clinical onset of diabetes (10).

Despite the importance of autoantibodies in characterizing disease, changes in these markers at diagnosis have not been examined over the period of increasing type 1 diabetes incidence. We therefore investigated the prevalence and levels of autoantibodies at diagnosis, controlling for age and HLA-determined genetic risk, in a cohort of patients recruited to a U.K. population-based study of childhood diabetes during a time when type 1 diabetes incidence in the region rose by 2.2% per year (1,2).

RESEARCH DESIGN AND METHODS

Newly diagnosed patients were recruited to the Bart’s-Oxford (BOX) study of childhood diabetes (11), a prospective, population-based study that has identified >95% of children and young people below age 21 years with type 1 diabetes diagnosed in the Oxford region, U.K., since 1985. Between 1985 and February 2002, 1,801 patients were referred to the study. Sera collected within 3 months of diagnosis (median 1 day [range –61 to 90]) were available from 613 of these patients (median age 11.0 years [0.7–20.9]) (12) (Table 1). GADA, IA-2A, and ZnT8A were tested in all sera. To avoid detecting antibodies to exogenous insulin, IAA testing was limited to 423 sera collected <2 weeks after diagnosis. IA-2A–positive sera were tested for IA-2B A and JMA. The BOX study was approved by local research ethics committees.

Autoantibody assays. Autoantibodies to insulin, full-length GAD65, and intracytoplasmic (606–979) or juxtamembrane (609–631) regions of IA-2 were assayed by radioimmunoassay as previously described (12–14). IA-2B A and ZnT8A were assayed using similar protocols with plasmids encoding the protein tyrosine phosphatase domain of IA-2B A (723–1015) or C-terminus of ZnT8 (285–369, 325R, or 325W) provided by Dr. Noyo Lampasona. ZnT8A were determined by combining results from separate assays for autoantibodies recognizing either arginine (ZnT8RA) or tryptophan (ZnT8WA) at position 325 (15). IAA, GADA, IA-2B A, and ZnT8A results were expressed in arbitrary units and IA-2A in DK units/mL (14) derived from standard curves, with extrapolation of values above the top standard. JMA results were calculated as an index (12). Thresholds were set at the 97.5th percentile of 2,860 schoolchild sera for IAA, GADA, and IA-2A and of 523 schoolchild sera for ZnT8A. Thresholds for IA-2B A and JMA were set at the mean ± 3 SDs of 270 schoolchild sera.

Laboratory-defined sensitivity and specificity for assays in Diabetes Antibody Standardization Program workshops over the period of sample testing were as follows: IAA, 36 and 100% (2001); GADA, 84 and 92% (2001); IA-2A, 68 and 99% (2010); and ZnT8A, 60 and 100% (2010), respectively.

Interassay coefficients of variation of moderate/high and low positive samples, respectively, were 21 and 21% for IAA, 16 and 17% for GADA, 14 and 21% for IA-2A, 20 and 16% for ZnT8A, 23 and 27% for ZnT8WA, 18 and 24% for JMA, and 14 and 19% for IA-2B A.

Genotyping. HLA class II DRB1, DQA1, and DQB1 analysis was performed on blood and mouth swab DNA with sequence-specific primers as previously described (16). Haplotypes were established based on common patterns of linkage disequilibrium; those of interest were DBR1*04-DQA1*0501-DQB1*0201 (DB3-DQ2) and DRB1*04-DQA1*0301-DQB1*0302 (DB4-DQ4). Statistical analysis. Patients were divided into quartiles based on diagnosis date (Table 1). Genetic risk was analyzed as DR3-DQ2/DR3-DQ4, DR4-DQ4/ DR4-DQ8, DR3-DQ2/ DR3-DQ4, DR4-DQ4/DR4-DQ8, XR (where X was not DR3-DQ2 or DR4-DQ8). Changes in autoantibody prevalence, sex,
levels of patients had IA-2A, compared with 83% in the last. This increase in prevalence was accompanied by higher IA-2A levels (P = 0.025) (Fig. 1A).

Of the 478 IA-2A–positive patients, 228 (48%) had JMA and 408 (85%) had IA-2B. There was no change in the prevalence or levels of JMA with diagnosis quartile. In contrast, the median level of IA-2B increased twofold among IA-2A–positive patients over the period studied (P < 0.001, Fig. 1B). ZnT8A prevalence also increased from 64 to 75% over the study period (P = 0.022). This was paralleled by rises in levels of antibodies to both ZnT8 isoforms (325R and 325W) tested (P < 0.001 for both) (Figs. 1C and D).

Logistic regression showed that after correction for age at diagnosis, sex, and DR-DQ genotype, diagnosis quartile was an independent determinant of prevalence of IA-2A (hazard ratio 1.34 [95% CI 1.03–1.62], P = 0.002) and ZnT8A (1.20 [1.03–1.41], P = 0.021) (Table 3). In contrast, the prevalence and levels of IAA and GADA were stable across the diagnosis quartiles even after adjustment for age, sex, and genotype (Fig. 1E and F).

The number of antibodies detected increased across the quartiles (P = 0.026) (Table 2). Logistic regression showed that date of diagnosis quartile was an independent predictor for the presence of all four antibodies (P = 0.016).

### DISCUSSION

We have shown that, during a period when the incidence of type 1 diabetes was rising in the Oxford region of the U.K. (1), the prevalence and levels of IA-2A and ZnT8A in patients at diagnosis increased. These increases were paralleled by a shift in autoantibody profile, with a higher

#### Table 1

| Factor                        | Overall | Quartile 1 | Quartile 2 | Quartile 3 | Quartile 4 | P     |
|-------------------------------|---------|------------|------------|------------|------------|-------|
| BOX subjects                  | 613 (34.0) | 153 (30.2) | 154 (37.1) | 153 (37.4) | 153 (28.6) |       |
| Minimum date of diagnosis     | July 1985 | July 1985  | April 1990 | February 1994 | July 1997 | NA    |
| Maximum date of diagnosis     | February 2002 | March 1990 | February 1994 | June 1997 | February 2002 | NA    |
| Age (years)                   | 408 (85%) | 407 (96%)  | 408 (85%)  | 407 (96%)  | 408 (85%)  |       |
| Disease duration (days)*      | 3 (–61 to 90) | 3 (–61 to 90) | 3 (–61 to 90) | 3 (–61 to 90) | 3 (–61 to 90) | 0.078 |

Data are n (%) unless otherwise indicated. P value indicates the trend over the diagnosis quartiles. *Negative values indicate samples obtained prior to diagnosis from siblings who developed diabetes in the BOX prospective family study.

#### Table 2

| AUTOANTIBODY PROFILE OF PATIENTS SUBDIVIDED BY DATE OF DIAGNOSIS |
|---------------------------------------------------------------|

| AUTOANTIBODIES | Overall | Quartile 1 | Quartile 2 | Quartile 3 | Quartile 4 | P     |
|----------------|---------|------------|------------|------------|------------|-------|
| IAA*           | 295 (69.7) | 63 (67.0)  | 80 (70.8)  | 76 (72.4)  | 76 (68.5)  | 0.860 |
| GADA           | 487 (79.4) | 121 (79.1) | 127 (82.5) | 123 (80.4) | 116 (75.8) | 0.405 |
| IA-2A          | 475 (78.0) | 106 (69.3) | 118 (76.6) | 127 (83.0) | 127 (83.0) | 0.002 |
| ZnT8A          | 429 (70.0) | 98 (64.1)  | 105 (68.2) | 112 (73.2) | 114 (74.5) | 0.022 |
| 0 autoantibodies* | 16 (3.8)  | 4 (4.3)    | 4 (3.5)    | 4 (3.8)    | 4 (3.6)    |       |
| 1 autoantibody* | 31 (7.3)  | 9 (6.6)    | 9 (8.8)    | 7 (6.7)    | 6 (5.4)    |       |
| 2 autoantibodies* | 72 (17.0) | 23 (24.5)  | 18 (15.9)  | 12 (11.4)  | 19 (17.1)  | 0.026 |
| 3 autoantibodies* | 150 (35.5) | 32 (34)    | 44 (38.9)  | 38 (36.2)  | 36 (32.4)  |       |
| 4 autoantibodies* | 154 (36.4) | 26 (27.7)  | 38 (33.6)  | 44 (41.9)  | 46 (41.4)  |       |

Data are n (%) unless otherwise indicated. P value indicates the trend over the diagnosis quartiles. *In 423 patients with samples suitable for IAA testing.
There was an increase in the genotype, which could potentially drive changes in islet autoimmunity in patients diagnosed more recently. The autoantibodies were tested in IA-2A-positive patients, levels for four quartiles based on date of diagnosis. Median antibody levels are shown by the line in the box, the interquartile range is represented by the box, and the whiskers represent 1.5 times the interquartile range. The levels of IAA and GADA were stable, but levels of IA-2A, ZnT8A, ZnT8WA, and IA-2βA increased significantly with diagnosis quartile.

A strength of this study is that the patients were recruited as part of the long-running BOX study of childhood diabetes, which is population based and has high levels of case ascertainment, allowing reliable monitoring of childhood type 1 diabetes incidence in the Oxford region since 1985. The size and detailed characterization of this cohort enabled us to investigate changes in islet autoantibodies while adjusting for age at diagnosis and HLA Class II genotype in our analysis; therefore, changes in these factors do not explain our findings, although we have not examined potential changes in the frequency of non-HLA genes.

The BMI of U.K. children has increased during this period, which suggests that in recent cases IA-2 and ZnT8 are not commonly accompanied by inter- and intramolecular autoimmune response but that disease progression is more severe metabolic decompensation, and rates of ketoacidosis at diagnosis in the BOX region did not alter between 1986 and 2005.

Our findings imply more intense and mature humoral autoimmunity in patients diagnosed more recently. The antibodies that increased over the study period, IA-2A and ZnT8A, usually appear after GADA and IAA. Furthermore, in the IA-2A–positive patients, levels of IA-2βA, which are associated with a more advanced phase of disease pathogenesis, rose in tandem with the increase in IA-2A, whereas JMA, which are often detected as part of the initial IA-2A response, appeared to be stable. IA-2A and ZnT8A were rarely found in the absence of IAA or GADA, which suggests that in recent cases IA-2 and ZnT8 are not being targeted more frequently as part of the primary autoimmune response but that disease progression is more commonly accompanied by inter- and intramolecular antigen spreading. This change to a more florid immunophenotype is consistent with the observation that progression...
from islet autoantibody positivity to diabetes was faster in young children born in 2004–2010 than in those born in 1989–2000, despite a similar frequency of islet autoantibodies (25). Understanding how changes in environment or lifestyle alter the humoral autoimmune response to islet antigens should help explain why the incidence of type 1 diabetes is increasing and may suggest new strategies for preventing disease.

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A.E.L. and A.J.K.W. researched data, contributed to the discussion, and wrote the manuscript. K.M.G. researched data, contributed to the discussion and edited the manuscript. P.J.B. researched data, contributed to the discussion, wrote the manuscript, and coordinated the BOX study. A.J.K.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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