Brief Report

Development of Type 1 Diabetes despite Severe Hereditary B-Cell Deficiency

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TYPE 1 diabetes results from an immune-mediated destruction of pancreatic beta cells. The disease can be transmitted by bone marrow transplantation in humans[1] and animals.[2,3] Furthermore, T cells that are reactive to several islet autoantigens have been identified in both mice and humans.[4,5] Although it is generally accepted that T cells have a role during the disease process, the possible role of B cells and autoantibodies in type 1 diabetes in humans has not been fully resolved. When they are activated, B cells can produce autoantibodies to pancreatic beta-cell antigens — such as glutamic acid decarboxylase 65 (GAD65), insulin, or the tyrosine phosphatase–like autoantigen IA-2 — and are able to take up and present autoantigen to T cells.[6,7]

Several effector mechanisms render autoantibodies potentially harmful. These include antibody-dependent, cell-mediated cytotoxicity; release of inflammatory mediators through stimulation of Fc receptors on natural killer cells, macrophages, or mast cells; opsonization of islet autoantigen, which promotes phagocytosis by macrophages; and complement activation with subsequent assembly of the membrane-attack complex.[8] In nonobese diabetic (NOD) mice, the presentation of antigen by B cells is required for the initiation of insulitis and sialitis,[9-11] and the presentation of antigen by the NOD major-histocompatibility-complex molecule I-Ag7 is critical in overcoming T-cell tolerance to islet beta cells.[12] Recently, it was shown in NOD mice that the initiation of GAD65-reactive T-cell responses requires only the presence of B cells as the antigen-presenting cells.[9]

X-linked agammaglobulinemia is a human immunodeficiency disease characterized by a blocking of B-cell differentiation that results in an arrest of the evolution of pre-B1a cells (low levels of cytoplasmic IgM and high levels of surrogate light chains) into later-stage B cells.[13] Male patients with X-linked agammaglobulinemia have very low serum levels of all classes of immunoglobulin and markedly decreased numbers of B cells in peripheral blood. The genetic defect has been localized to a cytoplasmic tyrosine kinase, designated as B-cell progenitor tyrosine kinase (BPK) or Bruton's tyrosine kinase (BTK),[14,15] which is expressed throughout B-cell differentiation and in myeloid cells but not in the T-cell lineage.

We report studies in a patient with X-linked agammaglobulinemia in whom insulin-dependent diabetes mellitus developed. The latter disease was identified as type 1 — that is, immune-mediated — diabetes. Hence, our data imply that neither autoantibodies nor B-cell function is critically involved in the pathogenesis of type 1 diabetes.

CASE REPORT

Immunodeficiency was diagnosed in a boy at three years of age on the basis of an absolute lack of B cells as well as immunoglobulins. Before the diagnosis, the boy had had several severe bacterial infections, and at the time of the diagnosis he had febrile conjunctivitis. An affected half-brother had died during a severe infection, and another affected half-brother is receiving infusions of immune globulin. A third half-brother is unaffected. None of the four fathers nor the mother is clinically immunodeficient.

Transient glucosuria was diagnosed in the patient at the age of 14; two months later, the diagnosis of diabetes was established when severe hyperglycemia (glucose concentration, >400 mg per deciliter) was observed during a middle-ear infection. Insulin treatment was initiated. Blood glucose values normalized with daily insulin doses of 0.9 U per kilogram of body weight per day, and the boy gained 4.5 kg. Ten days after the onset of diabetes and the subsequent establishment of metabolic control, residual insulin production by pancreatic beta cells was assessed by C-peptide measurements in peripheral blood (Biosource, Nivelles, Belgium) before and seven minutes after the intravenous administration of glucagon. At that time, the fasting blood glucose concentration was 141 mg per deciliter.

Since classic symptoms such as polydipsia, polyuria, and weight loss were not present at the time of diagnosis, insulin deficiency was verified by the finding of reduced basal C-peptide production (3 nmol per liter; normal range, 4.0 to 13.7) and reduced glucagon-stimulated C-peptide production (3.7 nmol per liter; normal range, 12.3 to 26.7). Nine months after the onset of diabetes the patient had an insulin requirement of 1.0 U per kilogram per day and a glycosylated hemoglobin value of 8.3 percent, indicating that hyperglycemia was not transient and supporting the diagnosis of type 1 diabetes.

METHODS

Peripheral blood was drawn from the patient for the analyses performed in this study after oral informed consent had been obtained from both the patient and his mother. Since the blood analyses were required to determine the nature of the immunodeficiency and the origin of diabetes in order to optimize therapy, no specific approval from our institutional ethics committee was required. The sampling took place 10 days after the onset of diabetes.

Autoantibody Analyses

Serum was analyzed for the major autoantibodies known to be associated with type 1 diabetes, including antibodies against islet cells, GAD65, IA-2, and insulin, as previously described.[16]

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T-Cell Proliferation

T-cell–proliferation assays were performed as previously described.[17] Peripheral-blood mononuclear cells (PBMCs) were stimulated by medium alone, by interleukin-2, by islet antigens such as GAD65, IA-2, 38-kD secretory granule protein, or insulin (10 μg per milliliter), or by the recall antigen tetanus toxoid (1.5 Lf units per microliter) as a control for memory–T-cell responses to recall antigen. Results were expressed in terms of the stimulation index (the counts per minute incorporated in the presence of antigen divided by the counts per minute incorporated in its absence).

Flow Cytometry

For flow-cytometric analyses, PBMCs were stained with monoclonal antibodies directed against the cell-surface molecules CD4, CD8, CD45RA, CD45RO, CD14, and CD20, as previously described.[18,19] Sensitive detection of B cells was achieved by means of an exclusion gate, in which CD3, CD14, CD15, CD16, and CD56 were used to exclude T cells, natural killer cells, monocytes, and granulocytes from the lymphocyte gate.[20] Analyses were performed with a fluorescence-activated cell sorter (FACSCalibur; Becton Dickinson, San Jose, Calif.).

Cytokine Analyses

Cytokine measurements for interleukin-4, interleukin-10, interleukin-13, interleukin-5, or interferon-γ were performed by means of enzyme-linked immunospot assays (Elispot, U-CyTech, Utrecht, the Netherlands) with the use of 3×10^6 PBMCs that were stimulated with medium alone, phorbol 12-myristate 13-acetate and ionomycin, or antigen.

DNA Sequencing

DNA was extracted from peripheral-blood granulocytes (QIAamp blood kit, Qiagen, Hilden, Germany), and polymerase-chain-reaction (PCR) analysis was performed as previously described.[21] Exons 1 through 13 and exon 19 of the *BTK* gene were amplified separately, whereas exons 14 through 18 were amplified en bloc. The sequences of the oligonucleotides used for the PCR amplification and sequencing of *BTK* have been described elsewhere.[22,23]

RESULTS

Characterization of Immunodeficiency

A PCR analysis of genomic DNA with subsequent sequencing was performed to determine the genetic defect that had caused X-linked agammaglobulinemia in this patient (Fig. 1). A deletion of nucleotides T and G at positions 54 and 55 in exon 8 of the *BTK* gene resulted in a frame shift at codon 214 in the TH domain and a premature stop codon at position 223 in the SH3 domain of the BTK protein (GenBank accession number AF375615).

Analysis of T-cell subgroups showed that the percentages of CD4 T cells and CD45RA (naive) cells were similar to those in 77 age-matched children with type 1 diabetes of recent onset. However, the patient had a higher level of CD4 cells or a lower level of CD45RA cells than 30 nondiabetic, age-matched subjects, including 16 children with unrelated chronic inflammatory problems. The patient had no abnormalities in the levels of CD8 T cells or memory lymphocytes (CD45RO) as compared with either the controls or the children with diabetes. The level of lymphocytes harboring surface markers consistent with recent primary activation (CD45RA/CD45RO) was higher than that in the other children with diabetes, who, in turn, had higher levels of CD45RA/CD45RO cells than the nondiabetic controls, regardless of the presence or absence of unrelated chronic inflammation. The patient had 66.4 percent CD4 cells, 16.5 percent CD8 cells, 56.5 percent CD45RA cells, 26.2 percent CD45RA/CD45RO cells, and 10.6 percent CD45RO cells (Table 1).[18] The level of CD20 B cells was below the limit of detection of

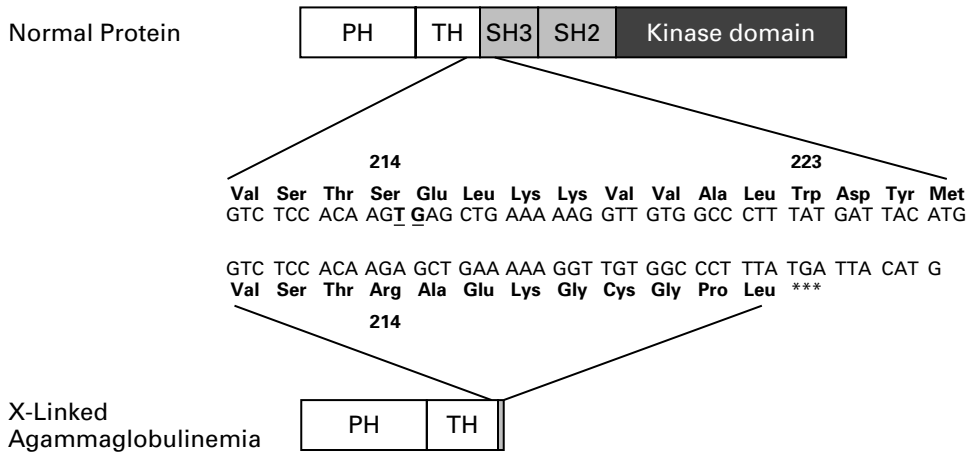

Figure 1. Structure of Bruton's Tyrosine Kinase (BTK) Protein and Sequencing of the *BTK* Gene.

Sequencing revealed a deletion of nucleotides T and G in exon 8 of the *BTK* gene resulting in a frame shift at codon 214 and a premature stop codon at position 223. The predicted BTK protein consists of the PH and TH domains and a short fragment of the SH3 domain.

TABLE 1. FLOW CYTOMETRY OF PERIPHERAL-BLOOD MONONUCLEAR CELLS.*

TYPE OF CELL	PATIENT WITH X-LINKED AGAMMAGLOBULINEMIA	OTHER CHILDREN WITH TYPE 1 DIABETES (N=77)	NONDIABETIC CONTROLS (N=30)
		percent	
CD4	66.4	51.9±10.8	45.4±10.0
CD8	16.5	19.3±7.0	19.4±4.9
CD45RA	56.5	61.6±11.8	69.4±8.3
CD45RA/CD45RO	26.2	16.9±7.2	11.8±6.1
CD45RO	10.6	16.1±7.6	15.8±6.0

*Plus–minus values are means ±SD.

0.4 percent. Serum immunoglobulins were not detectable.

Characterization of Diabetes

The possibility of an immune-mediated pathogenesis of diabetes was assessed by means of humoral and cellular immunologic assays. Autoantibodies associated with type 1 diabetes were undetectable, a result consistent with the diagnosis of X-linked agammaglobulinemia. In comparison with the responses to medium alone, there were increased T-cell proliferative responses after stimulation with GAD65 and IA-2 proteins, whereas responses to tetanus toxoid were in the same range as those in the nondiabetic controls (Fig. 2). There was no response to human insulin. Cytokine analyses were performed to determine the frequency of precursor cells that produce cytokines of the types produced by type 1 or type 2 helper T cells. Nonspecifically induced cytokine responses were no different from those in the nondiabetic controls, whereas all cytokines were detectable after stimulation with either tetanus toxoid or autoantigen (data not shown). The patient's HLA type, DR3,DQ2,DR4,DQ8, is the one that is associated with the highest genetic risk of type 1 diabetes.

DISCUSSION

Several lines of evidence support a diagnosis of type 1 diabetes in this patient with X-linked agammaglobulinemia. In addition to the presence of insulin dependence, deficient beta-cell function was suggested by the markedly reduced basal insulin level and the minimal response to stimulation by intravenous glucagon. An immune-mediated pathogenesis was demonstrated by proliferative responses to islet-specific autoantigens. As expected, autoantibodies normally associated with type 1 diabetes were not detectable, as a consequence of the patient's genetic inability to produce immunoglobulin. Finally, the patient had the HLA class II alleles known to confer the highest risk for type 1 diabetes, DR3,DQ2,DR4,DQ8.[24]

X-linked agammaglobulinemia may result from a variety of genetic mutations and immunologic defects.[25] Patients with classic X-linked agammaglobulinemia have less than 1 percent B cells and undetectable serum immunoglobulins, and those with so-called leaky X-linked agammaglobulinemia have more than 1 percent B cells and detectable immunoglobulins.[26] These phenotypes result from mutations that have been identified at different sites throughout the *BTK* gene. The patient described here has a deletion of two base pairs in exon 8, resulting in a stop codon at position 223 in the SH3 domain of the *BTK* gene — a novel mutation. Two phenotypically similar cases with a stop codon at amino acid residue 255, 32 amino acids downstream of the mutation reported here, have been reported previously.[27,28] These mutations resulted in the production of a severely truncated protein lacking the SH2 and kinase domains, leading to the phenotype of classic X-linked agammaglobulinemia. At the time of diagnosis, the patient's serum did not contain detectable immunoglobulins or B cells; flow cytometry revealed B cells below the limit of detection of 0.4 percent, confirming the diagnosis of classic X-linked agammaglobulinemia.

Despite the lack of B cells in the patient's peripheral blood, he had normal T-cell proliferation in response to nonspecific stimuli and to the recall antigen tetanus toxoid. Hence, earlier exposure to tetanus antigen during routine vaccination had induced normal T-cell memory, which is indicative of normal T-cell and antigen-presenting functions. This observation is in accordance with earlier findings that the *BTK* gene is expressed throughout B-cell differentiation and in myeloid cells but not in the T-cell lineage.[14,15]

The role of B cells and immunoglobulins during the pathogenesis of type 1 diabetes remains unresolved. There is solid evidence that autoantibodies serve as markers of the development of the disease, and in the majority of patients, one or more autoantibodies against islet cells are present before or at the time of the clinical onset of the disease.[29] B-cell lines transformed by Epstein–Barr virus process IA-2 and present naturally processed peptides to autoreactive T cells, a finding that confirms that these peptides serve as T-cell epitopes.[30] Increased antigen uptake and heterogeneity in the specificity of the autoantibodies might therefore provide a mechanism for an antibody-facilitated T-cell response that influences the progression of type 1 diabetes.

Our data imply that autoantibodies are not required for either the initiation or the progression of type 1 diabetes. This conclusion is further supported by the findings that islet-cell autoantibodies were not affected by cyclosporine therapy and that their presence in patients with type 1 diabetes of recent onset

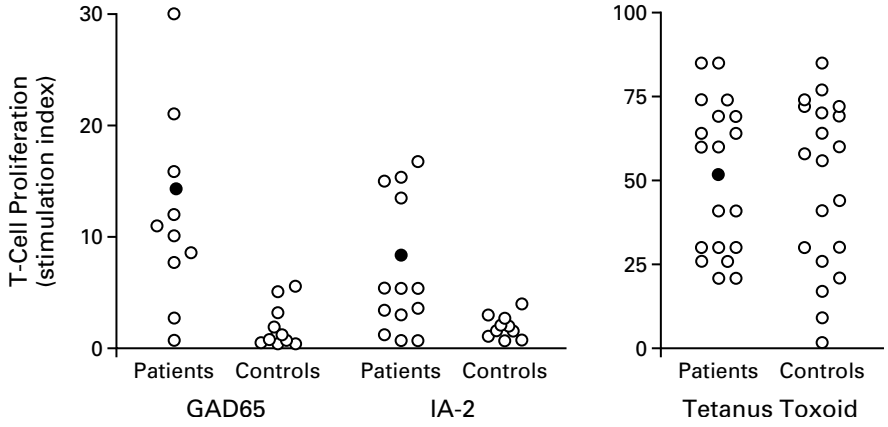

Figure 2. T-Cell Proliferation in the Peripheral-Blood Mononuclear Cells (PBMCs) of the Patient with Diabetes and X-Linked Agammaglobulinemia (Solid Circles), Other Children with Type 1 Diabetes of Recent Onset (Patients), and Nondiabetic Controls after Stimulation with Glutamic Acid Decarboxylase 65 (GAD65), IA-2 Proteins, and Tetanus Toxoid.

The stimulation index is defined as the number of counts per minute incorporated in the presence of antigen divided by the number of counts per minute incorporated in its absence.

who were treated with cyclosporine or placebo for 12 months was not related to the subsequent remission of insulin-requiring diabetes or to the loss of glucagon-stimulated C-peptide response.[31] Moreover, in cyclosporine-treated patients, neither the prevalence nor the titer of islet-cell antibodies at the time of diagnosis correlates with beta-cell dysfunction.[32] Cyclosporine-induced remission of type 1 diabetes was not predicted by or coincident with the disappearance of islet-cell antibodies. Despite the high specificity and sensitivity of multiple autoantibodies for predicting the development of type 1 diabetes, the majority of subjects with diabetes-associated autoantibodies remain healthy.

We conclude that type 1 diabetes can develop in the absence of both autoantibodies and B cells. This aspect of its pathogenesis places type 1 diabetes in marked contrast to spontaneous autoimmune diabetes in NOD mice, which has been claimed to be B-cell–dependent.[9-11] Although we do not wish to argue that B cells or autoantibodies cannot contribute to the pathogenesis of type 1 diabetes, our findings help explain why immunotherapy directed specifically toward B cells or autoantibodies may not be effective in preventing the destruction of beta cells.

Supported by grants from the Deutsche Forschungsgemeinschaft (MA 1260/4); the Ministerium für Wissenschaft und Forschung des Landes Nordrhein-Westfalen, Düsseldorf; the Bundesministerium für Gesundheit, Bonn; the Diabetes Funds Netherlands; the Royal Academy of Arts and Sciences, the Netherlands; and the Juvenile Diabetes Foundation International.

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