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Immunology

Heterophile antibodies segregate in families and are associated with protection from type 1 diabetes

JIN-XIONG SHE[†‡], TAMIR M. ELLIS[†‡], S. BRIAN WILSON[§], CLIVE H. WASSERFALL[†], MICHELE MARRON[†], SHARON REIMSNEIDER[†], SALLY C. KENT[¶], DAVID A. HAFLER[¶], DONNA S. NEUBERG[‖], ANDY MUIR[†], JACK L. STROMINGER[§††], AND MARK A. ATKINSON[†‡‡]

[†]Departments of Pathology, Immunology, and Laboratory Medicine, University of Florida, Gainesville, FL 32610; Departments of [§]Cancer Immunology and AIDS, and [‖]Biostatistical Science, Dana Farber Cancer Institute, Boston, MA 02115; [¶]Center for Neurologic Disease, Department of Neurology, Brigham and Women's Hospital, Boston, MA 02115; and [††]Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA, 02138

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ABSTRACT **Markedly elevated levels of serum IL-4 were reported previously in 50% of a small group of type 1 diabetes nonprogessors. To determine the patterns of expression for this phenotype, a larger cohort of 58 families containing type 1 diabetic patients was examined. Analysis of the two-site ELISA assay used to measure serum IL-4 revealed evidence for heterophile antibodies, i.e., nonanalyte substances in serum capable of binding antibodies mutivalently and providing erroneous analyte (e.g., IL-4) quantification. Interestingly, relatives without type 1 diabetes were significantly more likely to have this phenotype than were patients with the disease ($P = 0.003$). In addition, the trait appears to have clustered within certain families and was associated with the protective MHC allele DQB1*0602 ($P = 0.008$). These results suggest that heterophile antibodies represent an *in vivo* trait associated with self-tolerance and nonprogression to diabetes.**

Autoimmune (type 1) diabetes is a disease caused by T cell-mediated destruction of insulin-producing pancreatic beta cells (1, 2). The development of type 1 diabetes is influenced by a number of susceptibility genes (3–6), whereas other genes may confer dominant protection (7, 8). Longitudinal studies indicate that many high-risk subjects do not develop overt disease (9). Although epigenetic events may explain incomplete penetrance of genetic risk in type 1 diabetes, it is less clear why autoreactive T cells and antibodies are detectable in the circulation of at-risk first-degree relatives as well as in healthy HLA- and age-matched nondiabetic control subjects that do not go on to develop type 1 diabetes. These observations suggest that the presence of autoreactive T cells and antibodies are not sufficient to confer disease but that additional immune abnormalities must occur to result in the beta cell destruction characteristic of type 1 diabetes.

The discovery of T helper 1 (Th1) and Th2 subsets of $CD4^+$ T cells has helped explain the cellular basis for the diversity of T and B cell responses (10). Th1 cells are biased toward secretion of IFN-γ, tumor necrosis factor β, and IL-2 and promote inflammatory cellular immune responses. Th2 cells are biased toward secretion of IL-4, IL-5, IL-6, IL-10, and IL-13, induce humoral immunity, and inhibit Th1 responses. Although lymphocyte cytokine production in type 1 diabetes exhibits a bias toward the Th1 cytokine IFN-γ, the cellular mechanisms integrating the drive to Th1 or Th2 effector cell differentiation are poorly understood. In our study of Vα24JαQ T cells, evidence was presented to support the hypothesis that a defect in IL-4 secretion from these clones is associated with susceptibility to type 1 diabetes (11). Furthermore, markedly elevated levels of serum IL-4 were reported in 50% (7/14) of a small group of high-risk nonprogressors (11). To further study this observation in relation to disease progression, we sought to determine serum IL-4 levels in a cohort of 58 families containing type 1 diabetic patients.

MATERIALS AND METHODS

Antibodies. Anti-human cytokine capture and/or detection antibodies were purchased from PharMingen (anti-IL-4: catalogue nos. 18651D and 18502D; anti-tumor necrosis factor β: no. 18912D; anti-IL-13: no. 23222D) and Endogen (anti-IFN-γ: no. M700A).

Two-Site ELISA Assay. As described (11), capture anticytokine (e.g., IL-4) antibody was absorbed (overnight, 4°C, 1.0 μg/ml in 1.0 M $NaHCO_3$, pH 8.2) to ELISA plates. Wells were blocked as described in the text (2 hr, 37°C, 1.0% BSA or 10% FBS in PBS, pH 7.4) followed by addition of serum samples. After incubation (overnight, 4°C), steps involving addition of biotinylated detection antibody, avidin-horseradish peroxidase (Sigma), and tetramethylbenzidine (Kirkegaard & Perry Laboratories) reactions were performed. Cytokine concentrations were determined against OD readings obtained from standard curves using recombinant cytokines purchased from the above manufacturers.

Genotyping. HLA-DQB1 alleles were determined as described (12). The CTLA4 and insulin gene polymorphisms were analyzed as described (13, 14).

Patients. Serum samples and DNAs were obtained from a previously characterized cohort of multiplex and simplex families harboring diabetic probands and individuals with other autoimmune disorders (5, 15).

RESULTS AND DISCUSSION

Previous studies have shown that interference with two-site immunoassays can be caused by an endogenous human antibody that imparts Ig self-aggregation by binding to both the capture and detection antibodies (16). Because the elevated IL-4 phenotype previously was associated with type 1 diabetes nonprogression (11), it was important to confirm the identity of the immunoreactive substance in the serum as IL-4. To identify any assay interference and/or false-positives afforded by immunoreactive substances other than IL-4, immunoassays were performed in the presence of BSA (as in our previous experiences, ref. 11) as well as FBS (George Eisenbarth, personal communication) (17, 18). The addition of FBS is recognized to markedly reduce the false detection of analyte provided by Ig-reactive substances in human serum (19, 20).

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Abbreviation: Th, T helper.

[‡]J.X.S. and T.M.E. contributed equally to this work.

[‡‡]To whom reprint requests should be addressed at: Department of Pathology, University of Florida, 100275 JHMHC, Gainesville, FL 32610. e-mail: atkinson@ufl.edu.

When a set of samples from eight subjects preselected to represent stratified levels of serum IL-4 were tested and calculated by OD versus standard curves, the substitution of FBS for BSA in these two-site ELISA immunoassays effectively eliminated "serum IL-4" (Fig. 1*a*).

An additional test for the presence of Ig crossreactive substances (e.g., heterophile antibodies) interfering with serum IL-4 measurement involved determinations using the correct IL-4 capture and detection reagent pairs (e.g., anti-IL-4 and anti-IL-4) in comparison to the signal detected with antibody mismatch pairs (e.g., anti-IL-4 and anti-tumor necrosis factor β, capture and detection, respectively). When these mismatch analyses were expanded to a set of samples from 16 subjects stratified according to presumed serum IL-4 levels, proportionally equivalent OD levels were identified in cases of correct and mismatched antibody pairings. Specifically, the anti-IL4–anti-IL-4 assay was compared with the anti-IL-4 and anti-tumor necrosis factor β (capture and detection, respectively) $r^2 = 0.94.$, $P < 0.0001$; and to anti-IL-4 capture with anti-IL-13 detection, $r^2 = 0.97$, $P < 0.0001$. Similar IL-4 values also were obtained in cases of reversed cytokine antibody mismatch (e.g., anti-IFNγ capture with anti-IL-4 detection; $r^2 = 0.98$, $P < 0.0001$) to the appropriate matching antibodies. As was observed in cases of correct IL-4 antibody pairings, signal from the mismatched antibody pairings also was eliminated with the substitution of FBS for BSA (Fig. 1 *b* and *c*).

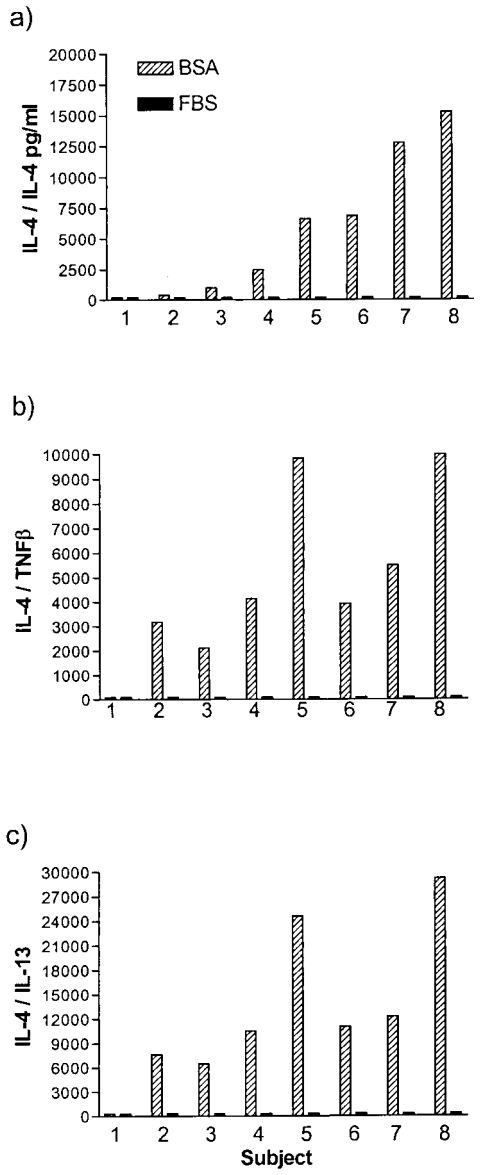

FIG. 1. Analysis of serum cytokine and demonstration of heterophile antibody. The quantity of serum cytokine determined through two-site ELISA testing is influenced by the addition of serum from appropriate species (e.g., FBS) and may be inaccurately estimated as evidenced by analyses using mismatched anticytokine antibody pairings. Serum samples were obtained from a previously characterized series of diabetic probands and nondiabetic relatives (5, 15). By using a set of samples from eight subjects stratified according to presumed serum IL-4 levels, OD levels were identified in cases of (*a*) correct (i.e., IL-4 capture, IL-4 detection) or mismatched (*b*) [IL-4 capture, tumor necrosis factor β (TNF-β) detection] and (*c*) (IL-4 capture, IL-13 detection) antibody pairings. Assays were performed in the presence of BSA as well as FBS, as indicated.

Studies predominantly within the clinical chemistry literature have reported such interference in immunological testing because of endogenous "heterophile antibodies." Heterophile antibodies may cause interference because of Ig aggregation, binding of the capture antibody as a result of rheumatoid factor (Fab-Fc) reactions, Fc-Fc dependent mechanisms, or idiotypic antibody (Fab-Fab) interactions. The heterophile antibodies observed in this system did not appear Fc specific as determinations using anti-IL-4 F(ab) fragment detection antibodies resulted in only a partial loss in IL-4 determination (data not shown).

To determine whether IL-4 could be measured without such interference in human serum, a series of samples from individuals with and without type 1 diabetes were subjected to two-site ELISA testing in the presence of FBS. In these analyses, elevated IL-4 (>39 pg/ml) was observed in equivalent frequencies in healthy controls (3/21; 14%; range 107-2982 pg/ml) and new-onset type 1 diabetes patients (3/20; 15%; 100-2503 pg/ml). When these six samples were subject to mismatch analysis (i.e., anti-IL-4 capture and anti-IL-13 detection antibodies), no signal representing cytokine was observed. The target of such assays was further confirmed as IL-4 as shown through quantitative spike recovery experiments with recombinant IL-4 in cases of both correct antibody pairings as well as in cases of antibody mismatch (data not shown).

The segregation within families of this heterophile antibody trait was monitored in multiplex families with type 1 diabetes to observe a component of genetic control for this phenotype. Heterophile antibodies (as defined through this two-site ELISA assay using correct IL-4 antibody pairs in BSA) were determined by ELISA for 340 members (128 patients and 212 relatives) of 58 multiplex diabetic families, 89 patients with other autoimmune diseases (Hashimoto's thyroiditis, Graves disease, or vitiligo), and 75 normal controls (Table 1). Con-

Table 1. Number of subjects analyzed and the frequency of heterophile antibodies

Groups	*N*	Heterophile antibody +	% of heterophile antibody +
Normal controls	75	3	4.0
Hashimoto's thyroiditis (HT)	30	0	0
Graves disease (GD)	10	0	0
Vitiligo	34	0	0
Relatives of HT, GD, and vitiligo	45	0	0
All diabetic families*			
Type 1 patients	139	6	4.3†
Relatives	222	31	14

*These samples include 128 patients and 212 relatives from this study as well as four diabetic patients and seven of their relatives previously reported (11).

†$P < 0.003$; statistical significance was determined by a two-tailed χ^2 test.

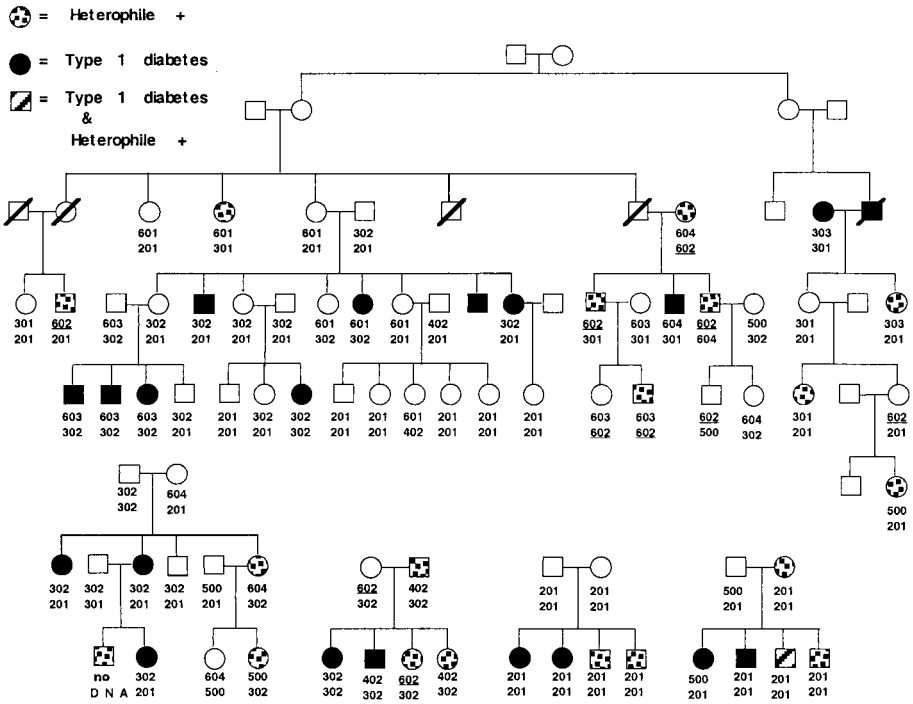

FIG. 2. The phenotype of heterophile antibodies clusters in families with type 1 diabetes. Serum samples and DNAs were obtained from a previously characterized cohort of multiplex and simplex families harboring diabetic probands and individuals with other autoimmune disorders (5, 15). Heterophile antibody levels were determined as described (11). The pedigrees for the five families that had two or more subjects with heterophile antibodies are pictured. Only one individual in these families had both type 1 diabetes and heterophile antibodies. The HLA-DQB1 alleles for each subject are pictured. Individual alleles for the DQB1*05 family were not further subtyped and are represented as 500. In addition, the diabetes-protective DQB1*0602 alleles are underlined (602).

sistent with our previous findings, heterophile antibodies were observed in three of 75 (4.0%) normal controls (11). No heterophile antibodies were detected in the sera obtained from patients with other autoimmune diseases (Table 1). The frequency of subjects with heterophile antibodies was significantly greater in the population of nondiabetic relatives (31/222, 14.0%) than in patients with type 1 diabetes (6/139, 4.2%) ($P = 0.003$) (Table 1). Sixteen of 58 (27.6%) families had at least one subject positive for heterophile antibodies. Twenty cases clustered into five families with more than one subject with heterophile antibodies (Fig. 2). These results suggest that a phenotype of serum heterophile antibodies segregated in families and was associated with nonprogression to type 1 diabetes. However, the detection of heterophile antibodies in the serum was not an absolute predictor of nonprogression because the antibody was detected in the serum of some diabetic patients (Table 1).

The segregation within families of heterophile antibodies indicated a genetic contribution to the phenotype. Therefore, associations between the heterophile antibody phenotype and several diabetes susceptibility genes (HLA-DR B1 and DQB1, INS, CTLA4) or candidate protective alleles such as DQB1*0602 were investigated (12–14, 16). Interestingly, the protective DQB1*0602 allele was found in 10 of 29 (34.5%) heterophile antibody-positive relatives compared with 28 of 207 (13.5%) heterophile antibody-negative relatives from the diabetic families ($P = 0.008$). This association appears to be caused in large part by one pedigree (Fig. 2 and Table 2), in which five of nine (55.6%) of the heterophile antibody-positive subjects had the DQB1*0602 allele compared with three of 36 (8.3%) of heterophile antibody-negative individuals (relative risk = 14, $P = 0.004$). Further analysis of a much larger data set, including careful longitudinal analysis, will be necessary to rigorously evaluate the suggested heritable component. None-

Table 2. Gene frequencies in heterophile antibody-positive and-negative members of families with type 1 diabetes

Genes	Heterophile antibody +	Heterophile antibody −	Control population	*P*
DQB1*0602				
Relatives	10/29 (34.5%)	28/207 (13.5%)	25.0%	<0.008†
CR pedigree	5/9 (55.6%)	3/36 (8.3%)	–	<0.004‡
CTLA4 (G/G)				
Relatives	6/29 (20.7%)	22/159 (13.8%)	12.8%	NS
Relatives + diabetics	9/35 (25.7%)	35/258 (13.6%)	12.8%	<0.03†
Diabetes genotypes for relatives				
DQB1*0302	12/29 (41.4%)	84/207 (40.6%)	15.0%	NS
DQB1*0201/0201	5/29 (17.3%)	32/207 (15.5%)	5.7%	NS
INS(+/+)	21/29 (72.4%)	126/173 (72.8%)	61.0%	NS

The CTLA4 and insulin gene polymorphisms were analyzed as described (12, 13). HLA-DQB1 alleles were determined as described (13). NS, not significant.
†Statistical significance was determined by a two-tailed χ^2 test.
‡Statistical significance was determined by a Fisher's exact test.

theless, these results indicate that in some families, DQB1*0602 or a closely linked gene was important for the production of heterophile antibodies. It also should be noted that testing of the large pedigree (Fig. 2) for serum IL-4 in the presence of FBS completely eliminated detection of these heterophile antibodies (i.e., they had limited or no demonstrable serum IL-4). Differences in the frequencies of susceptibility alleles between family members with heterophile antibodies compared with those without offered a potential explanation for the apparent protective effect of the phenotype. In fact, the CTLA4 susceptibility genotype G/G was found modestly more frequently in all heterophile antibody-positive subjects (25.7%, $P = 0.03$) compared with heterophile antibody-negative family members (13.6%), and the general population (12.8%) (Table 2). Moreover, a higher frequency of diabetes susceptibility alleles or genotypes (DQB1*0302, DQB1*0201/0201, INS+/+) was seen in heterophile antibody-positive subjects compared with normal controls, but no significant difference was observed between heterophile antibody-positive subjects and their heterophile antibody-negative relatives (Table 2). The sharing of genetic factors by the two phenotypes (heterophile antibody production and type 1 diabetes) was expected because heterophile antibody-positive subjects were found more frequently in families with diabetes.

Antibodies that interfere with immunoassays can be monospecific, produced in response to immunizations (e.g., anti-animal antibodies in animal care workers, patients treated with animal globulins for immunotherapeutic or diagnostic purposes), whereas others are characterized by nonspecificity being developed in response to no clear immunogen. The broad reactivities of polyspecific antibodies may play an important role in normal immunity as such antibodies appear predominant in newborns and are useful in initial defense mechanisms against multiple bacterial and viral antigens. To what degree these factors may cause alterations in immunoregulation leading to autoimmune disease is unclear, but the possibility that heterophile antibodies are involved in immunoregulation is intriguing. The results presented here suggest a relationship between heterophile antibodies and resistance to progression of an autoimmune disorder. Hence, these studies suggest that genetic control of serum heterophile antibodies may be an important regulatory event in the development of type 1 diabetes.

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