Cellular Immune Response to Cow's Milk β-Lactoglobulin in Patients With Newly Diagnosed IDDM

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Elevated levels of antibodies to cow's milk proteins, i.e., β-lactoglobulin (BLG) and bovine serum albumin (BSA), have been associated with IDDM. We observed enhanced cellular immune response by a proliferation test of peripheral blood mononuclear cells to BLG in 22 of 40 (55%) patients with newly diagnosed IDDM compared with 7 of 32 healthy children (22%) ($P = 0.004$, χ^2 test). The median stimulation index to BLG was 3.3 in patients and 1.5 in healthy children ($P = 0.003$, Mann-Whitney U test). No difference was found in cellular reactivity to other cow's milk proteins, such as BSA or α-casein, or to a dietary immunogenic protein, ovalbumin. Cellular responsiveness to BLG was not associated with HLA-DQB1* risk alleles of IDDM, which suggests that immune response to the protein does not only reflect the accumulation of these HLA alleles in the patients with IDDM. We suggest that enhanced cellular immune response to dietary BLG may reflect a disturbance in the regulation of immune response to oral antigens in IDDM. This kind of defect may play a fundamental role in the development of β-cell autoimmunity in IDDM. *Diabetes* 45:178–182, 1996

Enhanced humoral immune response to protein fractions in cow's milk (CM) has been reported in patients with newly diagnosed IDDM (1–6). Elevated levels of IgG and IgA antibodies, as detected by enzyme-linked immunosorbent assay to β-lactoglobulin (BLG) and bovine serum albumin (BSA), have been found, especially in young patients with IDDM (2,4,5). In a series of Swedish patients with newly diagnosed IDDM, IgA-class antibodies to BLG were independent risk factors for IDDM (2). The role of CM as a possible trigger for the autoimmune process leading to IDDM is further supported by epidemiological data showing that short breast-feeding time and early introduction of CM formula carry a risk for IDDM in several populations (7–9).

T-cells are considered to be responsible for the destruction of pancreatic β-cells in IDDM (10). Proliferation of peripheral blood mononuclear cells (PBMCs) to BSA was recently reported in 90% of patients with IDDM but not in control subjects (11). In contrast, Atkinson et al. (12) could not find any difference in humoral or cellular response to BSA between newly diagnosed patients with IDDM and healthy adults. We studied the proliferation of PBMCs from newly diagnosed Finnish IDDM patients and healthy children to different CM proteins, such as BSA, BLG, and α-casein (CAS), and to ovalbumin (OVA), which is also an oral antigen in humans.

RESEARCH DESIGN AND METHODS

Peripheral venous blood samples were obtained with parents' consent from 40 unselected patients with newly diagnosed IDDM (<4 weeks after diagnosis) from the Children's Hospital at the University of Helsinki, Aurora Hospital, and Jorvi Hospital. The mean age of the patients was 7.1 years (range 1–17). Of the unrelated control subjects without acute infections or autoimmune diseases, 32 were randomly chosen from nondiabetic patients undergoing minor elective surgery at the Children's Hospital, University of Helsinki. Mean age of the control subjects was 9.1 years (range 1–17). The study plan was approved by the hospitals' ethical committees. Testing of patients and control subjects was always done on fresh blood samples.

Proliferation test of PBMCs. PBMCs were separated from heparinized blood by Ficoll-Hypaque (Pharmacia) density centrifugation. Cells were washed three times in phosphate-buffered saline and diluted to 1×10^6 cells/ml in RPMI-1640 (Gibco) containing 5% pooled human AB^+ serum (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) and 4 mmol/l L-glutamine; 1×10^5 cells in 200-μl volume per well were cultured in U-bottomed microwell plates (Nunc, Roskilde, Denmark). A CM-derived antigen, such as BSA, BLG, CAS, and OVA (all obtained from Sigma; catalog numbers A2058, L0130, C6780, and A5378, respectively), or tetanus toxoid (TT) without thiomersal (National Public Health Institute, Helsinki, Finland) was added (20 μl) to quadruplicate wells to provide final concentrations of 2, 20, and 200 μg/ml for all antigens except TT, which was used in the final concentration of 8 μg/ml. After 6 days of incubation in 5% carbon dioxide at 37°C, 1 μCi of tritiated thymidine (Amersham) was added to each well. Cultures were harvested 16 h later semiautomatically, and thymidine incorporation was measured by liquid scintillation counting. Proliferation was expressed as stimulation index (SI) equal to median counts per minute incorporated in the presence of antigen divided by counts per minute incorporated in the absence of antigen (medium value). The intra-assay variation was 25%.

Inhibition of the proliferation of PBMCs to BLG (at the concentration of 200 μg/ml) was studied by adding monoclonal anti-DR antibodies (Becton Dickinson) to the cultures in the beginning of the proliferation assay. The antibodies were dialyzed to remove preservatives, diluted in culture medium, and used at final dilutions of 1/400 and 1/100.

IgG- and IgA-class antibodies. IgG- and IgA-class antibodies to BLG were detected by enzyme-linked immunosorbent assays as previously described (2,4). The level of antibodies was considered positive when it was above the sensitivity of the assay (>0.01% of standard serum with high levels of antibodies against BLG).

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BLG, β-lactoglobulin; BSA, bovine serum albumin; CAS, α-casein; CM, cow's milk; OVA, ovalbumin; PBMC, peripheral blood mononuclear cell; SI, stimulation index; TT, tetanus toxoid.

TABLE 1
Proliferation of PBMCs from patients with IDDM and healthy children (control subjects) to different dietary antigens expressed as SI

Antigen	Group	*n*	Median	SD
BLG	IDDM patients	40	3.3	2.6
	Control subjects	32	1.5	2.6
BSA	IDDM patients	30	1.0	0.1
	Control subjects	19	1.0	0.2
CAS	IDDM patients	27	1.5	2.6
	Control subjects	19	1.1	1.7
OVA	IDDM patients	20	2.1	2.0
	Control subjects	19	1.8	2.4

HLA-DQB1* typing. HLA-DQB1* typing was made using a technique developed for screening of IDDM susceptibility based on HLA-DQB1* alleles associated with a significant risk (0302, 0201) or protection (0602/3, 0301) against the disease. The HLA-DQB1* gene segment of interest was amplified using biotinylated 3′ primer, and the amplification product was bound by streptavidin-coated microtiter plate wells. The hybridization of different lanthanide-labeled short allele specific oligonucleotide probes is measured using time-resolved fluorometry (13).
Statistical analysis. The difference in SI between patients and control subjects was analyzed by the nonparametric Mann-Whitney *U* test and χ^2 test by using an SI of 3 as the cutoff limit for positive response.

RESULTS

Proliferation of PBMCs (expressed as SI) to different antigens used in the study are shown in Table 1. The median SI to BLG at a concentration of 200 μg/ml for patients and control subjects differed significantly (3.3 vs. 1.5; $P = 0.003$, Mann-Whitney *U* test). In contrast, proliferation of PBMCs to other CM proteins, such as BSA and CAS, did not differ between patients with IDDM and control subjects, *P* values being 0.49 and 0.60, respectively. The proliferative response to BSA was low for both patients and control subjects (median SI = 1.0 for both groups at the concentration of 200 μg/ml). The proliferation of PBMCs to CAS (200 μg/ml) was increased in two patients and two control subjects, the median SI being 1.5 for patients and 1.1 for control subjects. The median SI to OVA (200 μg/ml) for patients and control subjects did not differ significantly (2.1 vs. 1.8, respectively). We found a proliferative response of PBMCs to BLG (SI >3) in 22 of 40 (55%) children with newly diagnosed IDDM but only in 7 of 32 (22%) nondiabetic children (χ^2 test, $P = 0.004$). The distribution of the SI to BLG in patients with IDDM and healthy subjects is shown in Fig. 1. The highest SI was detected at a concentration of 200 μg/ml, although in most cases proliferation was seen already at a concentration of 20 μg/ml. A dose-response curve of proliferation of PBMC to BLG in five patients with IDDM is shown in Fig. 2. The median SI to TT was 21 in control subjects and 16 in IDDM patients, and the groups did not differ significantly (Mann-Whitney *U* test, $P = 0.44$).

The inhibition of the proliferation of PBMCs to BLG was studied in two patients with previously detected positive SIs to BLG. In both patients, anti-DR antibodies inhibited the proliferation to BLG (Fig. 3).

IgG- and IgA-class antibodies to BLG were measured in 37 patients and 31 control subjects. No significant difference was found in the levels of antibodies to BLG between the groups. The relation between IgG-class BLG antibodies and T-cell reactivity to BLG, however, seemed to be different between patients and control subjects (Table 2). Of the 11 control subjects with IgG-class BLG antibodies, 8 did not have detectable cellular response against BLG. In patients with IDDM, only 4 of 12 patients with IgG-class BLG antibodies had no detectable cellular response to BLG. Instead, 13 of 21 patients with BLG-reactive T-cells had no detectable levels of antibodies to BLG.

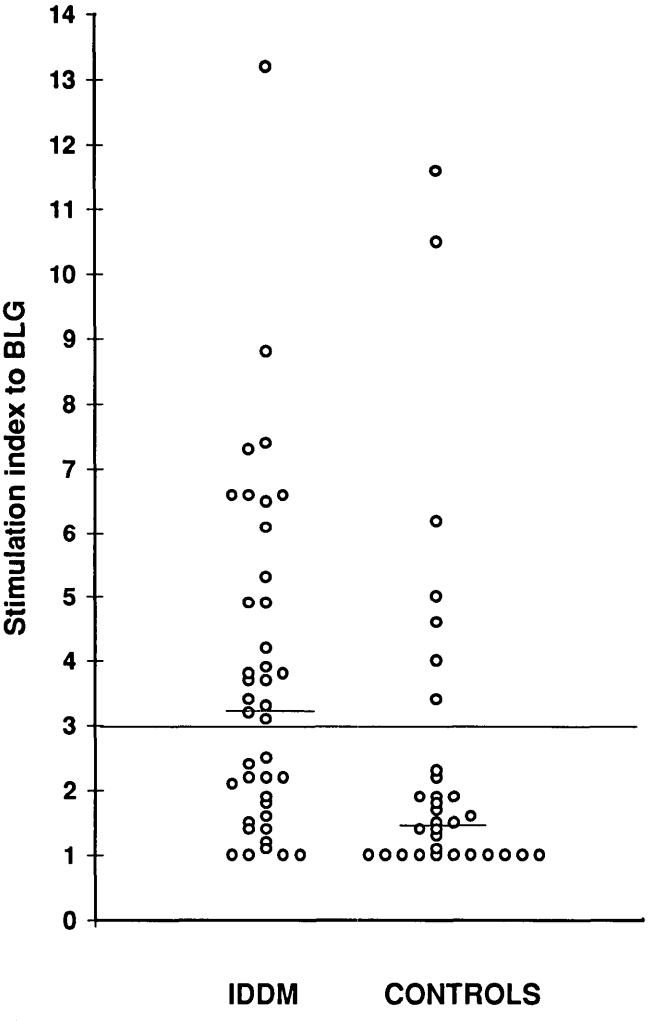

FIG. 1. Proliferation of PBMCs to BLG in patients with IDDM ($n = 40$) and control subjects ($n = 32$) is presented as SI. SI of 3 is marked as the cutoff point for positivity; median of each group is shown.

HLA-DQB1* risk alleles were determined from 29 randomly selected patients with IDDM and 14 healthy control subjects. The median SI to BLG did not differ significantly according to HLA-DQB1* risk alleles (Fig. 4*A*). Both patients with the highest risk genotypes (0302/0201) had positive SIs to BLG, whereas 8 of 14 (57%) patients with 0302 risk allele, 3 of 6 (50%) patients with 0201 risk allele, and 4 of 6 (67%) patients without the risk alleles had SIs of >3 to BLG. Three of 14 HLA-typed nondiabetic children had a positive stimulation to BLG, two of them having IDDM risk alleles (0302/0201 and 0201/x) (Fig. 4*B*).

DISCUSSION

In agreement with the earlier reports showing that patients with newly diagnosed IDDM have augmented immune response to BLG (1,2,4), we found an enhanced cell-mediated immune response to BLG in our series of newly diagnosed IDDM patients. This further emphasizes the view that immunity to oral protein(s) is altered in patients with newly diagnosed IDDM.

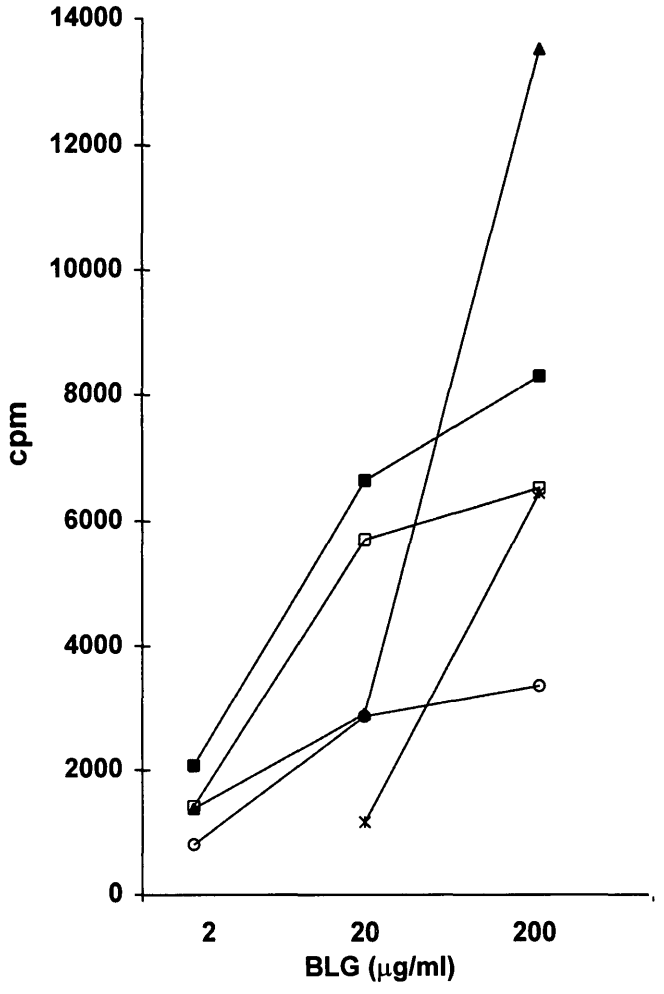

FIG. 2. Dose-response curve of proliferation of PBMCs to BLG in five patients with IDDM. The incorporation of [H_3]-thymidine is expressed as counts per minute at different concentrations of BLG.

We could not find an enhanced cellular reactivity to other proteins in CM, such as BSA or CAS. CAS is a component in the casein fraction of CM, and an immune response to it has not been associated with IDDM (3). In contrast, elevated levels of antibodies to BSA and enhanced cellular immunity to it have been reported in IDDM (3,5,6,11), although a contradictory report exists (12). Antibodies to BSA have been shown to recognize an islet-cell autoantigen with a molecular weight of 69 kD, p69 (3). This protein has been

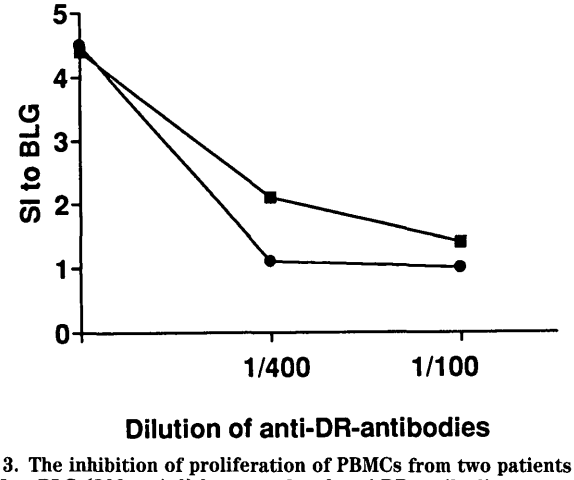

FIG. 3. The inhibition of proliferation of PBMCs from two patients with IDDM to BLG (200 mg/ml) by monoclonal anti-DR antibodies.

TABLE 2
The relationship between proliferation response of PBMCs to BLG and IgG antibodies to BLG in patients with IDDM and in nondiabetic children

	SI > 3		SI < 3	
	IDDM patients	Control subjects	IDDM patients	Control subjects
Antibodies to BLG	8	3	4	8
No antibodies to BLG	13	4	12	16
Total	21	7	16	24

cloned independently by two groups and shown to share short sequence homology areas with BSA (14,15). It has been suggested that children who are exposed to BSA in early infancy develop an immune response to BSA that later is reactivated by virus-induced expression of islet-cell protein p69 (3,11,16). In our study series, proliferative responses of PBMCs to BSA were low in all subjects studied, as reported by Atkinson et al. (12). In the same culture conditions, we have also studied children with CM allergy and have found proliferative responses to BSA (SI >3) in some of them (data not shown). Thus, our proliferation test does detect responses to BSA, although epitopes related to CM allergy may differ from those in IDDM. However, methodological differences may explain discrepant findings between the study by

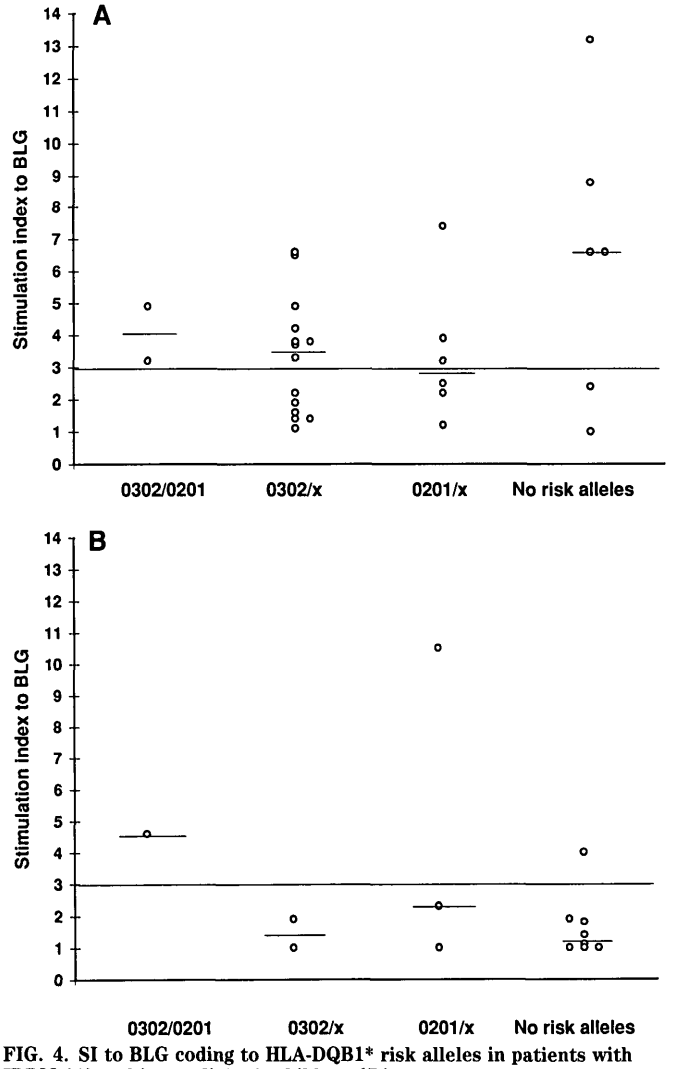

FIG. 4. SI to BLG coding to HLA-DQB1* risk alleles in patients with IDDM (*A*) and in nondiabetic children (*B*).

Cheung et al. (11) and our study. They studied proliferation of PBMCs to BLG in the serum-free culture medium but could not find any response to BLG in patients or control subjects (11).

We could not find differences in stimulation response to BLG between patients having different HLA-DQB1* risk alleles. Four of six patients without risk alleles had SIs of >3 to BLG. However, two of three stimulation test–positive control subjects had DQB1* risk alleles. The immunization to BLG in patients with IDDM seems to occur irrespective of the HLA haplotype. This may be due to the presence of several immunogenic epitopes in BLG having binding capacities to different HLA alleles. On the other hand, it has been reported that immunodominant DR-restricted peptides in a protein antigen (e.g., TT and acetylcholine receptor) have the capacity to bind to several HLA alleles and are recognized by T-cells in the context of different DR alleles (17,18). The DR restriction of cellular response to BLG was demonstrated in two patients with IDDM (Fig. 3). Although HLA typing was done only in a subgroup of patients, it seems that cellular immunity against BLG is not a secondary phenomenon connected to the presence of HLA-DQ risk alleles but is rather associated as such with IDDM.

In previous reports based on large epidemiological case-control studies, the difference in the levels of BLG antibodies between patients with IDDM and healthy children was seen in young children (2,4). The mean age of patients was 7.1 years in our study, which may explain why no difference was found in BLG-antibody levels between patients and control subjects. Interestingly, the relation between BLG antibodies and BLG-reactive T-cells seemed to differ between patients and control subjects. Antibodies to BLG were found in the control subjects mostly without detectable BLG-reactive T-cells (Table 2), suggesting a Th2-type responsiveness. In patients with IDDM, this kind of response was rare, and cellular reactivity without antibody response was often seen. This suggests that BLG-reactive T-helper cells may be functionally different in children with IDDM when compared with healthy children.

Observed immune responsiveness to BLG may reflect a defect in the development of immune tolerance to oral antigens in IDDM. We have reported that both humoral and cellular reactivity against BLG appeared in healthy infants who were exposed to BLG orally in CM formula (19), whereas no response was seen in infants who received only casein-hydrolysate formula. During the follow-up, T-cell response to BLG declined, suggesting the development of oral tolerance in healthy infants.

The immunogenic properties of dietary BLG may differ from those of other dietary proteins and explain why enhanced cellular reactivity was seen with BLG only in patients with IDDM. BLG is a component of whey proteins in CM. It stays soluble at low pH, is not precipitated in the stomach, and reaches the intestine in an intact form (20). In patients with CM allergy, humoral immunity to BLG is also seen in the majority of cases (21).

The role of the mucosal immune system in induction of tolerance to different antigens has received a lot of attention during the past years (22). Patients with IDDM have an increased incidence of celiac disease (23,24), in which immune tolerance to dietary wheat protein (glutelin) is broken. In addition, T-cell lines isolated from islets of a newly diagnosed patient with IDDM showed strong adherence to the endothelium of the diabetic pancreas and the mucosal lymphoid tissue, suggesting that lymphocytes derived from the mucosal lymphoid tissue may be involved in the pathogenesis of IDDM (25). There is increasing evidence that intraepithelial lymphocytes of the small intestine can mature and undergo selection in the gut without thymic influences (26).

On the other hand, BLG shows a structural similarity with retinol-binding protein (27), which has been localized in human islet cells (28). Most of the cells containing retinol-binding protein also contained insulin, but the distribution of protein was not restricted to insulin-producing cells (28). Cell-mediated immunity to retinol-binding protein may be provoked during the destruction of β-cells, and the same cell population may cross-react with BLG. Because of this cross-reactivity, the role of BLG in the autoimmune process leading to IDDM may be either initiating or sustaining and should be carefully studied.

In conclusion, the findings of altered immune responsiveness to dietary antigens in IDDM suggest that the regulation of mucosal immunity may be disturbed in IDDM. It is possible that this regulatory defect has a fundamental role in the pathogenetic process leading to IDDM.

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