Antibodies to Bovine Serum Albumin in Brazilian Children and Young Adults With IDDM

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OBJECTIVE — To evaluate the prevalence of IgG antibodies to bovine serum albumin (BSA) in a cohort of Brazilian children and young adults with IDDM.

RESEARCH DESIGN AND METHODS — Sera from 81 subjects with <1 year of IDDM (group 1), 111 subjects with >1 year of IDDM (group 2), and 207 normoglycemic subjects were tested using an immunofluorimetric assay. A receiver-operating–characteristic curve was used to establish the threshold of anti-BSA antibody titers defining the positivity of the assay.

RESULTS — The distribution of the fluorimetric index (FI) of anti-BSA antibodies did not have a gaussian profile. Rank sum of FI was significantly higher in patients than in control subjects ($P < 0.0001$). Average logFI values of both IDDM groups were significantly higher than that of the control group ($P < 0.005$ for both groups). There was a trend toward higher FI levels in group 1 than in group 2 ($P = 0.06$). A FI cutoff of 0.7 optimized the ratio of true-positive to false-positive of the assay, with the best equilibrium between sensitivity and specificity. The prevalence of anti-BSA antibodies was 52% in group 1, 47% in group 2, and 28% in the control group ($P = 0.0001$). An independent association between anti-BSA antibodies and IDDM, with an odds ratio of 3.03 ($P < 0.0001$), was observed in a logistic regression analysis. However anti-BSA antibodies explained only 5% of the variability of IDDM versus NIDDM.

CONCLUSIONS — Our results confirm that the prevalence of anti-BSA antibodies is higher in IDDM subjects than in control subjects, even after 1 year of diabetes. However, a large overlap of antibody titers is observed in patients and control subjects, suggesting that anti-BSA antibodies are neither sensitive nor specific markers of IDDM.

IDDM is a multifactorial autoimmune disease with both polygenic inherited susceptibility (1–5) and environmental determinants (6) contributing to the expression of diabetes. The nature of these environmental determinants is currently unknown, but viruses, toxins, and dietary factors have been suspected (7). Epidemiological data suggest that early exposure (<3 months of age) to cow's milk might be an important determinant of subsequent IDDM (8). These data are supported by results from intervention studies in rodent models of IDDM that show that the incidence of diabetes can be prevented (9) or reduced (10) by removing intact cow's milk protein from the diet.

The fraction of cow's milk that evokes the diabetogenic response is not clearly defined. IgG antibodies to bovine serum albumin (BSA) and to a 17–amino acid sequence of BSA (ABBOS), which share a common epitope with the pancreatic β-cell surface protein p69, were observed in 100% of IDDM subjects and in only 3.8% of control subjects in a cohort of Finish children (11). However, discordant results have been reported in other Finish (12), French (13), and American (14) cohorts of patients with recent-onset IDDM.

These contrasted results might be partly ascribed to the differences in the studied populations, the techniques of antibody assays, and the choice of cutoff values of antibody titers to define positivity. We here report the assessment of IgG antibodies to BSA in a cohort of Brazilian children and young adults with IDDM. The use of a receiver-operating characteristic (ROC) curve (15) made it easier to establish the threshold of anti-BSA antibody titers defining the positivity of the assay.

RESEARCH DESIGN AND METHODS

Population

The study was performed in 192 children and young adults (<25 years old) with IDDM attending a diabetes vacation camp or being followed as outpatients in the diabetes department of the Escola Paulista de Medicina (Paulista School of Medicine), Sao Paulo, Brazil. Subjects were divided into two groups according to duration of diabetes (Table 1). Group 1 consisted of 81 individuals who had been diagnosed with IDDM <12 months before the inclusion in the protocol. Group 2 consisted of 111 subjects with >12 months of diabetes. IDDM was defined according to the National Diabetes Data Group criteria (16). The control group was composed of 207 children and young adults with fasting plasma glucose <6.1 mmol/l, either attending a school in the neighborhood of Escola Paulista de Medicina or enlisted as blood donors in this institution. The protocol was approved by the Escola Paulista de Medicina Ethical Committee. Subjects or their parents gave fully informed written consent to take part in the study.

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Received for publication 9 May 1995 and accepted in revised form 28 September 1995.

ANOVA, analysis of variance; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FI, fluorimetric index; ROC, receiver-operating characteristic.

Table 1—*Clinical characteristics of IDDM and control subjects*

	IDDM subjects				
	Group 1	Group 2	All	Control group	*P* value
n	81	111	192	207	—
Sex (M/F)	48/33	48/63	96/96	108/99	0.66*
Age (years)	11.8 ± 0.5 (0.3–25)	12.5 ± 0.4 (4–25)	12.2 ± 0.3 (0.3–25)	12.7 ± 0.3 (2–25)	0.19*
Duration of IDDM (months)	4 ± 3 (0–12)	61 ± 3 (13–168)	37 ± 3 (0–168)	—	—
Dose of insulin (U/day)	22 ± 2 (0–70)	36 ± 1 (5–80)	31 ± 16 (0–80)	—	<0.0001†

Age, duration of IDDM, and dose of insulin are expressed as means ± SE (range). *Comparisons between all IDDM and control groups. †Comparison between groups 1 and 2.

Determination of serum anti-BSA antibodies

Blood samples were obtained after an overnight fast. IgG anti-BSA antibodies were assayed in serum by a method of time-resolved fluorescence (17) using europium as a tracer (18). Microplates (Nunc-Imuno Plates, 96 wells, Intermed, Roskilde, Denmark) were incubated overnight at 4°C with 200 μl of BSA dissolved in a carbonate-bicarbonate buffer (8 and 10 g/l, respectively; BSA final concentration, 200 mmol/l, pH 9.5). The wells were then washed with a solution of Tris-HCl buffer (50 mmol/l, pH 7.75) containing NaCl (9 g/l), Tween 20 (0.05%), sodium azide (0.5 g/l), and trizma base (6 g/l). There was 200 μl of 2% egg yolk extract in Tris-HCl buffer added into the wells for a 1-h incubation at 37°C. Egg yolk extract was used to block nonspecific binding; its preparation is described elsewhere (19). After washing, 200 μl of serum from patients or control subjects was added to the wells in a 1/100 dilution in 2% egg yolk extract and left 2 h for incubation at 37°C. Samples were assayed in duplicate. After washing, 200 μl of antihuman IgG monoclonal antibody labeled with europium in 1/1,000 dilution in 2% egg yolk extract was placed into wells and incubated for 1 h at room temperature, protected from light. The wells were washed and fluorescence developed by adding to each well 200 μl of an enhancement solution (Wallac, Turku, Finland). Fluorescence activity was read with a fluorometer (Delphia system; Wallac) operating in the time-resolved fluorescence mode for europium. Anti-BSA antibody titers were expressed as a fluorimetric index (FI) calculated as the average fluorescence activity of the duplicate of each subject divided by the average fluorescence activity of a standard serum sample. Inter- and intra-assay coefficients of variability were 4.8 and 11.5%, respectively.

Statistical analysis

Data are expressed as means ± SE, unless stated otherwise. Mann-Whitney *U* and Kruskal-Wallis tests were used when comparing two or three groups, respectively. Analysis of variance (ANOVA) with Tukey-Kramer harmonic Studentized distribution (HSD) test (20) was carried out after logarithmic transformation of FI values. Qualitative traits were analyzed by contingency table χ^2 tests. A ROC curve (15) was constructed to test the ability of anti-BSA antibody titers to discriminate IDDM and control subjects and to establish the best cutoff value of FI to define the positivity of the assay. The ROC curve is a graphical plot of all of the sensitivity/specificity pairs resulting from continuously varying the cutoff value of positivity over the range of results observed in a test. Logistic and linear regression analyses were performed to evaluate associations of clinical and biological parameters; quantitative data were log-transformed for these analyses. Statistics were performed with the JMP software (SAS Institute, Cary, NC).

RESULTS — The distribution of the FI of anti-BSA antibodies did not have a gaussian profile and was skewed toward lower values: the average FI was higher than the median in the three groups (Table 2). FI was significantly higher in IDDM patients than in control subjects (Kruskal-Wallis, $P < 0.0001$). ANOVA after log-transformation of data yielded similar results (Table 2). Average logFI values of both IDDM groups were significantly higher than that of the control group ($P < 0.005$ for both groups; Tukey-Kramer HSD test after ANOVA). There was more of a trend toward higher FI in patients with recent-onset IDDM than in subjects with >1 year of diabetes ($P = 0.06$). No sex-related differences were observed in any of the groups.

To determine the best cutoff value of FI to differentiate antibody-positive from antibody-negative subjects, a ROC curve was constructed (Fig. 1) by computing sensitivity and specificity of anti-BSA detection for a spectrum of 16 values of FI taken as cutoff (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0, 2.5, 2.9, 3.0, and 4.1). When the cutoff value is varied over the spectrum of possible FI values, sensitivity and specificity move in

Table 2—*Anti-BSA antibodies in IDDM and control subjects*

	Anti-BSA antibodies		
	FI	FI (median)	logFI
Group 1 (IDDM <1 year)	1.73 ± 0.40	0.74	−0.0863 ± 0.0514
Group 2 (IDDM >1 year)	1.29 ± 0.17	0.65	−0.1229 ± 0.0439
All IDDM	1.47 ± 0.20	0.68	−0.1074 ± 0.0333
Control group	0.89 ± 0.11	0.43	−0.3383 ± 0.0321

Data for FI and logFI are means ± SE. Kruskal-Wallis and ANOVA were used to compare groups 1, 2, and control. Kruskal-Wallis, $P < 0.0001$; ANOVA, $P < 0.0001$. Tukey-Kramer HSD test after ANOVA: group 1 vs. control group ($P < 0.005$). Group 2 vs. control group ($P < 0.005$). Group 1 vs. group 2 ($P < 0.06$).

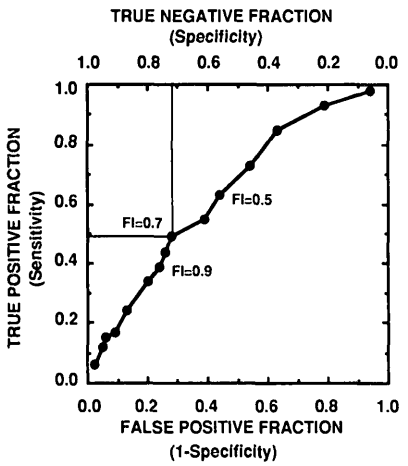

Figure 1—*ROC curve showing sensitivity and specificity of anti-BSA antibody detection for 15 values of FI taken as the cutoff of positivity. ●, from bottom left corner to upper right corner, represent the following FI values: 4.1, 3.0, 2.5, 2.0, 1.5, 1.0, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, and 0.1.*

opposite directions. There is thus an important tradeoff between sensitivity and specificity when choosing the cutoff value, suggesting that anti-BSA antibodies have a low accuracy as a biological marker of IDDM. We have chosen a cutoff of 0.7, as it optimizes the ratio of true-positive to false-positive (1.75 vs. 1.43 for FI = 0.5, 1.41 for FI = 0.6, and 1.68 for FI = 0.8) among the cutoffs that present the best equilibrium between sensitivity and specificity. At this cutoff (0.7), sensitivity and specificity of anti-BSA antibody detection were 0.49 and 0.72, respectively, and the frequency of anti-BSA antibody positivity was 52 and 47% in IDDM subjects from groups 1 and 2, respectively, versus 28% in control subjects (χ^2 contingency table, $P = 0.0001$).

The frequency of anti-BSA antibody positivity was significantly higher in IDDM than in control subjects over the whole spectrum of cutoff values. Frequencies of antibody positivity in groups 1 and 2 were not significantly different. A logistic regression analysis, with the diabetes status as the categorical dependent variable (IDDM/nondiabetic) and age, sex, and anti-BSA antibody titers as independent variables, was performed. An association between anti-BSA antibody titers and IDDM was observed, with an odds ratio of 3.03 (95% CI 1.86–4.92; $P < 0.0001$), while the other parameters were excluded as contributors to the risk of IDDM. It is noteworthy that this model, although highly significant ($P < 0.0002$), explained only 5% of the variability of IDDM versus nondiabetic.

A multiple regression analysis, with anti-BSA antibody titers as the dependent variable, showed a significant inverse association with age (odds ratio 0.59; 95% CI 0.44–0.79; $P < 0.0004$; r^2 for the model 0.08) but not with duration of diabetes in the IDDM group. A trend toward an association with age was observed in the control group (odds ratio 0.64; 95% CI 0.40–1.01; $P = 0.054$).

CONCLUSIONS — We report significantly higher titers of IgG antibodies to BSA in Brazilian children and young adults with IDDM than in unrelated normoglycemic control subjects. However, the range of anti-BSA antibody titers overlapped in patients and control subjects, and no clear cutoff value of FI to define positivity was recognized. Nevertheless, all over the spectrum of possible cutoff values, the frequency of anti-BSA antibody positivity was consistently and significantly higher in all IDDM patients than in control subjects. Moreover, high titers of anti-BSA antibodies were associated with an odds ratio of 3.03 to present with IDDM, which attests to the robustness of the results. Age, but not duration of diabetes, was independently and inversely associated with anti-BSA antibody titers in these patients—an association that was also reported in Finish IDDM children (12). Incidentally, these results are consistent with the observation that the association of IDDM and early cow's milk exposure decreases with increasing age of diagnosis of diabetes (8). We would like to point out that our study was not performed in a population-based cohort, and thus the control subjects might not be fully representative for the group of cases. However, as our observations are in agreement with previous reports (12,13), it is unlikely that bias in the selection of cases and control subjects has had a major effect in the results.

Data on the literature on the prevalence of anti-BSA antibodies in IDDM are controversial. Karjalainen et al. (11) reported high levels of IgG and IgA anti-BSA antibodies in Finnish children with recent-onset IDDM. There was practically no overlapping of antibody ranges in patients and control subjects, and the frequency of positivity in patients was 100 and 60% for IgG and IgA antibodies, respectively. Positivity was defined as values >2 SE above the mean of control subjects. These clear-cut results were not confirmed in another Finnish study (12) that showed that although IgG anti-BSA antibody levels were higher in children with recent-onset IDDM, the distribution of IgG and IgA antibodies overlapped in patients and control subjects. The positivity for IgA anti-BSA antibodies, defined as any value above the level of sensitivity of the assay, was 48 and 27% for patients and control subjects, respectively. Recently, Krokowski et al. (13) observed high levels of anti-BSA antibodies in French young adults with IDDM, but in only 17% of the patients, the titers were above the 95th percentile of control values (13). Still more controversial were the results of Atkinson et al. (14) who found no difference either in the anti-BSA antibody levels or in the frequency of antibody positivity in American children and young adults with recent-onset IDDM as compared with control subjects. Positivity, defined as values >2 SE above the mean of nondiabetic subjects, was observed in 10% of patients only but was significantly higher in their first-degree relatives.

These discrepancies might be related, at least partly, to differences in age and duration of diabetes but also in the ethnic origin of the studied populations. The inherited susceptibility to IDDM is polygenic, with a large genetic effect contributed by a gene in the HLA region in chromosome 6p (1, 2) and minor genetic effects contributed at least by six other loci in several chromosomes (3–5). Populations with different genetic admixtures might present different humoral responses in IDDM. At this regard, Krokowski et al. (13) have observed that anti-BSA antibody titers were higher in HLA-DR3 than in non-DR3 French IDDM young adults, while no such difference was observed in Finnish IDDM children (11). It is also noteworthy that the different studies used dissimilar techniques of antibody assay, such as particle concentration fluoroimmunoassay (11,14), radioimmunoprecipitation assay (13), and enzyme-linked immunosorbent assay (ELISA) (12). It has been suggested that these assays detect distinct subsets of anti-BSA antibodies, that their results might not correlate, and that fluoroimmunoassay might be more sensitive and specific than ELISA for detecting IgG anti-BSA an-

tibodies (21). Furthermore, the choice of different decision thresholds to define antibody positivity makes it difficult to compare these various results. The ROC curve is an interesting alternative to define an optimum cutoff value of an assay, especially when dealing with distributions that largely overlap in cases and controls. It provides a comprehensive picture of the ability of a biological marker to detect or predict a disease (22,23) and takes into account the sensitivity and specificity of the assay in a particular population when determining the best decision threshold.

In conclusion, our results confirm that the prevalence of anti-BSA antibodies is higher in IDDM subjects than in normoglycemic control subjects. It also confirms that there is a large overlap of antibody titers in IDDM patients and control subjects. This overlap might be due to a small contribution of cow's milk protein to the heterogeneous, multifactorial, and polygenic pathophysiological mechanisms leading to IDDM; to the lower sensitivity and specificity of currently available assay techniques to detect the subset of antibodies related to the disease; or to both. Whatever the reason, anti-BSA antibody titers explained only 5% of the variability of IDDM versus nondiabetic in our sample and thus seem to be of limited use as markers to the disease. Comparison and standardization of the different assay techniques might be useful for further studies. In this regard, reporting results as a ROC curve provides a clear picture of the assay accuracy and makes it easier in the comparison of results from different studies.

Acknowledgments— We thank Tereza Kasamatsu and Ilda Kunii for excellent technical assistance and Dr. Marco Antônio Vívolo and the staff (doctors, nurses, and residents) at the Nosso Recanto diabetes vacation camp for help in collecting data.

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