Diabetologia (1992) 35: 985–990

Diabetologia

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Disease-associated anti-bovine serum albumin antibodies in Type 1 (insulin-dependent) diabetes mellitus are detected by particle concentration fluoroimmunoassay, and not by enzyme linked immunoassay

J. Karjalainen[1,4], T. Saukkonen[3], E. Savilahti[2] and H.-M. Dosch[1]

[1] Department of Immunology and Cancer, The Hospital for Sick Children, Toronto, Ontario, Canada, [2] First and [3] Second Department of Paediatrics, University of Helsinki, Helsinki, [4] Department of Medical Microbiology and Paediatrics, University of Oulu, Oulu, Finland

Summary. We recently developed a particle concentration fluoroimmunoassay for the measurement of serum antibodies to bovine serum albumin in patients with Type 1 (insulin-dependent) diabetes mellitus. We observed elevated IgG-anti-bovine serum albumin antibodies in 100% of newly-diagnosed diabetic children and in 2.5% of matched control children. Here we compare the fluoroimmunoassay and the more commonly available enzyme linked immunoassay technique, exchanging coded serum samples from 40 newly-diagnosed diabetic children and 179 control children between two laboratories. Particle concentration fluoroimmunoassay detected elevated IgG-anti-bovine serum albumin antibodies in all diabetic children, enzyme immunoassay in 25% ($p < 0.0001$). Fluoroimmunoassay detected elevated levels in 2.2% and enzyme immunoassay in 10% of control children ($p < 0.002$). Elevated IgA-anti-bovine serum albumin antibodies in patients were slightly more often detected by fluoroimmunoassay than by enzyme immunoassay, while in control children enzyme immunoassays detected elevated levels three times more often ($p < 0.01$). Values measured in either assay showed overall no correlation in either patient (IgG: $r_s = 0.28$; IgA: $r_s = 0.11$) or control sera (IgG: $r_s = 0.02$; IgA: $r_s = -0.05$). Fluoroimmunoassay for IgG was 100% disease-sensitive (enzyme immunoassay: 25%, $p < 0.0001$) and more disease-specific (IgG; $p < 0.02$). Our findings demonstrate that these assay techniques detected distinct subsets of anti-bovine serum albumin antibodies with little (IgG) or some (IgA) overlap. In fluoroimmunoassay procedures, antigen:antibody binding occurs within 1–2 min while hours are allowed in an enzyme immunoassay. Antibodies with high on-off binding rates typical for immune responses following hyperimmunization are therefore measured preferentially by particle concentration fluoroimmunoassay and it is these antibodies which appear to be associated with diabetes. These observations emphasize the need for epidemiological surveys to validate immunoassay procedures used for clinical purposes.

Key words: Bovine serum albumin antibodies, Type 1 (insulin-dependent) diabetes mellitus, enzyme linked immunoassay, particle concentration fluoroimmunoassay.

Based on observations in diabetes-prone BB-rats, we recently identified a small peptide ("ABBOS-peptide") in bovine serum albumin (BSA, pos. 153–168, GenBank access number: A03232) as a candidate trigger of Type 1 (insulin-dependent) diabetes mellitus [1]. BSA and in particular the ABBOS peptide share an immunologically cross-reactive epitope with p69, a γ-interferon inducible islet Beta-cell surface protein detected by anti-BSA and anti-ABBOS antibodies which occur in all Type 1 diabetic patients or in animals immunized with BSA or ABBOS [2, 3]. Neonatal tolerization with BSA and removal of dietary whey protein prevents diabetes in BB rats and NOD mice [1, 3–5]. We proposed that dietary BSA/ABBOS initiates an immune response in genetically susceptible hosts (DR3, DR4, DR3-DR4, DQβnon-Asp^{57}) that culminates in the destruction of insulin producing Beta cells [6].

To evaluate the relevance of this hypothesis in man, we developed a particle concentration fluoroimmunoassay technique (PCFIA) [7–11] for the measurement of serum BSA antibodies the bulk of which were found to be ABBOS-specific by solid phase epitope mapping [6]. We found that 100% of 142 newly-diagnosed diabetic children had significantly elevated IgG-anti-BSA antibodies (61% for the IgA isotype) compared to below 3% of matched control children and adult control subjects [6].

Active BSA-specific serology is typical for diabetic NOD-mice and BB-rats [3, 12, 13], and was preferentially observed in a proportion of children and adults with Type 1 diabetes [14, 15]. In addition, antibodies to cow's milk proteins have been associated with Type 1 diabetes [16, 17]. These studies employed enzyme immunoassay (EIA) techniques and differences between diabetic sub-

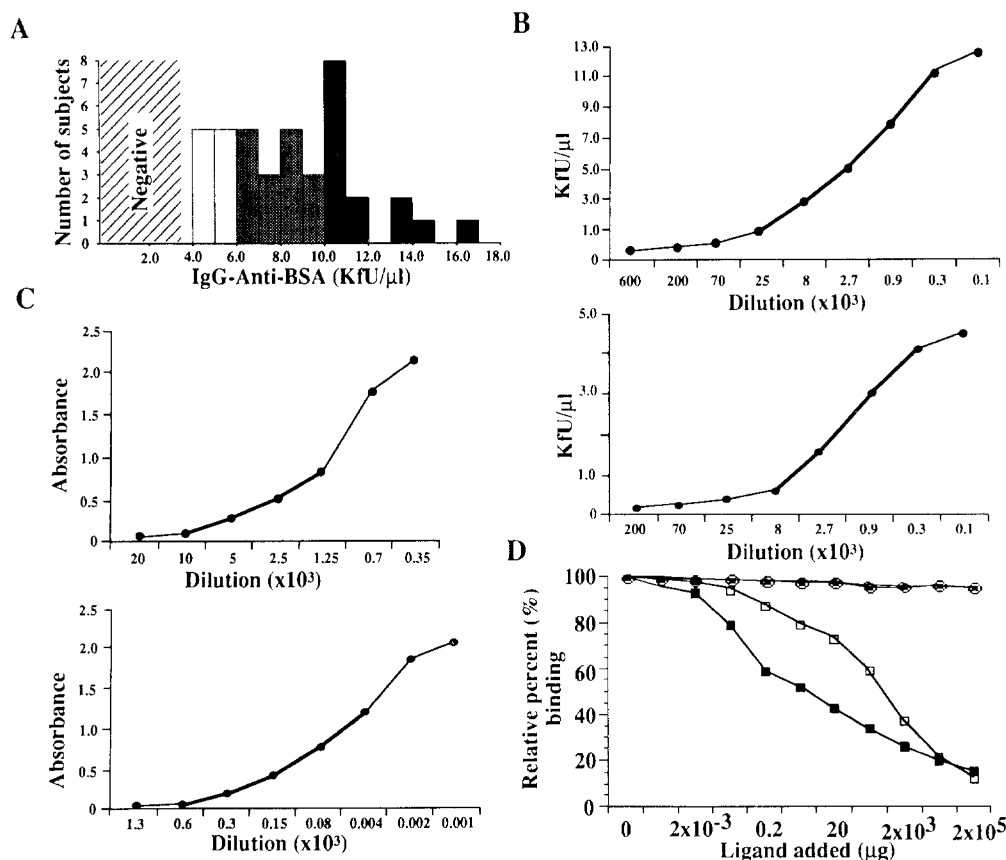

Fig. 1. A Distribution of moderately- (white bars) and highly elevated (black bars) IgG-anti-bovine serum albumin (BSA) antibodies in 40 diabetic children as determined by particle concentration fluoroimmunoassay (PCFIA). **B**: PCFIA standard curve for a serum pool (from diabetic children) containing 12.3 KfU/μl IgG-(top panel) and 4.2 KfU/μl IgA-anti-BSA antibodies. **C**: Anti-BSA standard curves for enzyme immunoassay (EIA). KfU, Kilo fluorescence units. **D**: Binding competition with increasing amounts of free ovalbumin/Tween-20 for IgG- (-⊕-) and IgA- (-◆-) anti-BSA antibodies, as well as with increasing amounts of free BSA for IgG- (-□-) and IgA- (-■-) anti-BSA antibodies

jects and control subjects were more subtle than those observed in our study [6].

We modified an anti-β-lactoglobulin EIA procedure [18] for the measurement of BSA antibodies and compared it and the PCFIA procedure in their ability to detect diabetes-associated BSA antibodies. We found that each technique detected a different subset of anti-BSA antibodies. PCFIA measured highly disease-associated BSA antibodies, whereas EIA measured antibodies more common in the general population.

Subjects and methods

Study population

Forty Finnish diabetic children (22 males, mean ± SD age 6.2 ± 4.5 years, range 0.9–15.5 years) from our previous study [6] were randomly selected for assay comparison and contained a typical range of elevated levels of BSA antibodies (Fig. 1 A). Samples were drawn at the time of diagnosis of diabetes, and selected sera were sent coded back to the laboratory in Finland (E.S.) to be analysed by EIA. Control subjects comprised 179 age- and sex-matched non-diabetic Finnish children (98 males, mean ± SD age 6.2 ± 3.6 years, range 0.9–15.9 years) whose samples were shared between the laboratories. All samples were studied without knowledge of the sample origin.

Particle concentration fluoroimmunoassay (PCFIA)

PCFIA is a solid-phase immunoassay technique employing 96-well unidirectional flow vacuum filtration plates and a very high-surface-area solid phase (carboxylated polystyrene microspheres) covalently conjugated with the antigen [7]. Phase separation procedures are carried out by robotic Screen-Machine Instruments (IDEXX, Portland, Me., USA), which is progammable for reagent additions, timed incubations, phase separations, washings and measurements of particle-bound fluoresceinated secondary antibody [10, 19].

Two-hundred microlitres of BSA (Grade V; Sigma Chemical Co., St Louis, Mo., USA, 10 % in phosphate-buffered saline (PBS; 40 g NaCl, 1 g KCl, 1 g KH_2PO_4, 5.75 g Na_2HPO_4, 0.5 g $CaCl_2$, 0.5 g $MgCl_2$/5 l distilled water, pH 7.2)) was coupled covalently (100 μl of 10 mg/ml 1-ethyl-3-(3-dimethylaminopropyl)-carbodimide) onto 400 μl (5 % stock; IDEXX) carboxylated polystyrene beads (diameter 0.75 μm). Subsequently, 10 % Tween-20 in 1.0 % ovalbumin-PBS was used as blocking agent. After repeated washings, beads were stored in 1 % Tween-20-PBS. Over a period of 9 months the activity of the beads remained unchanged.

Twenty microlitres of test serum dilutions (1:100-1:1,000) were added to microwells containing 20 μl of 1:20 diluted BSA-coated microspheres (initial 2.5 % weight/volume). Up to ten plates were inserted into the Screen-Machine for programmed phase separations, washings and addition (100 ng/well) of affinity purified, custom BSA-free fluorescein-conjugated goat anti-human IgG, IgA, and IgM (F_c-fragment specific; BioCan, Mississauga, Ontario, Canada). Drying and precipitation of serum protein was avoided by short (1 min) incubation, phase separation and washing procedures at low (5 mm Hg) vacuum pressure. Prior to reading, wells were vacuum dried for 1 min and fluorescence emission was read under high vacuum from the concentrated particle cake at the bottom of the well. Kinetics of the system have been published [7]. We have processed up to 3,000 replicate samples per day per instrument per operator. The PCFIA assay had a sensitivity of less than or equal to 1 ng antibody per ml and analysis of 400 consecutive assays delineated intra- and inter-assay variations of 8.9 % and 9.8 %, respectively.

An in-house (positive) standard serum pool from diabetic children was used and a standard curve prepared in every plate (Fig. 1 B). Competition experiments showed satisfactory specificity for BSA. Free BSA blocked antibody binding in a dose-dependent

Table 1. Frequency of elevated anti-bovine serum albumin antibodies in diabetic ($n = 40$) and control children ($n = 179$) as defined by particle concentration fluoroimmunoassay (PCFIA) and by enzyme immunoassay (EIA)

Isotype	Positive diabetic children n (%)		p	Positive control children n (%)		p
	PCFIA	EIA		PCFIA	EIA	
IgG	40[a] (100%)	10[b] (25%)	< 0.0001	4 (2.2%)	18 (10%)	< 0.002
IgA	20[a] (50%)	17[a] (42%)	NS	6 (3.3%)	18 (10%)	< 0.01

[a] $p < 0.0001$, [b] $p < 0.05$ for difference between antibody positive diabetic patients and control children using PCFIA and EIA

fashion, whereas neither ovalbumin nor Tween-20 nor a combination of the two were able to displace the antibody (Fig. 1 D). Results are expressed as kilo fluorescence units (KfU) per microlitre based on instrument gain (5×), serum dilution and assay volume as derived from the standard containing 12,300 KfU/ml IgG- and 4,200 KfU/ml IgA-anti-BSA antibodies. Due to the linearity of fluorescence emission energy the values were normally distributed. Elevated antibody levels were defined to exceed the mean level in control subjects plus 2 SD.

Enzyme linked immunosorbent assay (EIA)

Our EIA for the measurement of anti-BSA is a conventional three-layer solid-phase procedure modified from Tainio et al. [18]. The method employs polystyrene Microstrip wells (Labsystems, Helsinki, Finland) that are processed in an automatic EIA analyser (Auto-EIA II; Labsystems, Helsinki, Finland), which can process up to three plates (66 samples) per day. The plates were coated with 100 µl (2 mg/ml) BSA (A-4378; Sigma) in 0.1 mol/l PBS-5 mmol/l NaN_3 (pH 7.4) overnight at room temperature. After washing with PBS-NaN_3, the wells were saturated with 1% gelatin-PBS-NaN_3 for 1 h at 37 °C and stored at + 4 °C until used.

Serum samples were diluted 1:40 in 0.5% gelatin-PBS-NaN_3 prepared in 0.05% Tween-20. Three replicates of 100 µl of serum dilutions were plated, two in coated wells and one in a non-coated well. After a 60-min incubation at 37 °C wells were washed four times. Diluted alkaline phosphatase conjugated anti-human IgG or -IgA (cat. no. 67806 and 67808; Orion Diagnostica, Espoo, Finland) was added and incubated for 60 min followed by four washes. After 45-min incubation of substrate (2 mg/ml p-nitrophenyl phosphate in N-N-diethylaniline buffer) the reaction was stopped with 1 mol/l NaOH. Absorbances (optical density 405 nm) in non-coated wells were subtracted from test values.

Intra-assay- and inter-assay variations were 9.3% and 15.8%, respectively. For serum assays, serial dilutiona of a BSA-antibody positive standard were run on each plate (Fig. 1 C) and the results expressed as percent binding of the standard serum. Values for both IgG- and IgA were skewed despite log-transformation, and thus the limit for positivity was set at the 90th percentile of the values in control subjects. This limit was selected after examining a series of cut-off values as giving the highest sensitivity with acceptable specificity. Sera having an absorbance of 3.9% for IgG and 14.2% for IgA of the standard were considered as positive.

Statistical analysis

Statistical analysis was performed using cross-tabulation, chi-square statistics and Student's unpaired t-test in the case of normally distributed variables. Since the distribution of BSA antibody levels obtained by EIA was skewed despite transformation, the difference between diabetic and control children was evaluated by a Mann-Whitney U-test. Mann-Whitney U-test and Spearman's rank-correlation test were used to compare antibody levels between EIA and PCFIA. Sensitivity and specificity of the assays was determined, and the results evaluated by cross-tabulation with chi-square statistics. Results are presented as means ± SEM.

Results

The 40 sera from diabetic children contained a range of elevated IgG-anti-BSA^{PCFIA} antibodies; however, only 25% were found positive in EIA (Table 1, $p < 0.0001$). Conversely, IgG-anti-BSA^{EIA} antibodies in control subjects were elevated more frequently than those detected by PCFIA (Table 1, $p < 0.002$). Elevated IgA-anti-BSA antibodies in diabetic children were found in 50% and 42% (p = NS), however only 3.3% and 10% of control children were positive in PCFIA and EIA, respectively (Table 1, $p < 0.01$). These results demonstrated that PCFIA but not EIA preferentially detects disease-associated BSA antibodies in children with Type 1 diabetes. In contrast, EIA shows preference for detection of antibodies more prevalent in the general population. Neither procedure detected all BSA antibodies.

BSA antibody levels in diabetic children are shown in Figure 2. There was a significant difference in the levels of both IgG-, and IgA-anti-BSA antibodies between diabetic and control children when determined by PCFIA ($p < 0.001$ and $p < 0.001$). In contrast, the levels of IgG-anti-BSA^{EIA} antibodies were roughly similar in diabetic and control children. IgA-anti-BSA^{EIA} antibodies were higher in diabetic children, but the difference ($p < 0.01$) was less prominent than in PCFIA ($p < 0.001$). These findings suggest a quantitative difference in the subsets of antibodies detected by PCFIA, and this difference distinguishes diabetic and control children.

Individual PCFIA- and EIA values are compared in Figure 3 for diabetic and control children. Shaded areas indicate the levels considered as negative (see Methods). The correlation between PCFIA and EIA was very poor ($-0.05 \leq r_s \leq 0.28$, $0.09 \leq p \leq 0.1$). In diabetic subjects only a subset of sera (IgG: ~20% and IgA: ~32%) gave relatively low or high anti-BSA values in both assays i.e. showed correlation. Moreover, among control subjects only one IgA sample (0.3%) was positive in both assays.

BSA-antibodies in control sera bound significantly more frequently in EIA than in PCFIA (IgG: $p < 0.002$ and IgA: $p < 0.01$, Table 1) and only one out of the 18 control subjects positive for IgG- or IgA-anti-BSA^{EIA} was positive by PCFIA. On the other hand, of the four IgG- and six IgA-positive control sera detected by PCFIA, only one was positive by EIA (Fig. 3). Therefore, most disease-associated BSA-antibodies were only detected by PCFIA. The sensitivity of PCFIA in detecting disease-associated IgG anti-BSA antibodies was excellent when compared to EIA (100% vs 25%,

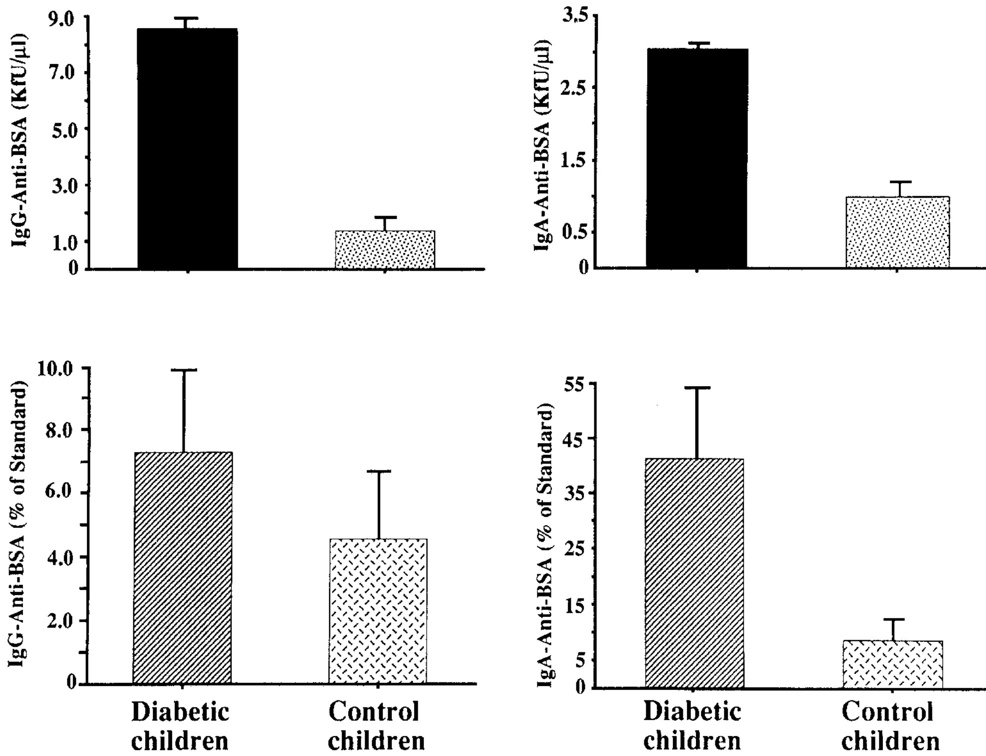

Fig. 2. Mean levels (±SEM) of anti-bovine serum albumin (BSA) antibodies in Type 1 (insulin-dependent) diabetic- and matched control children as detected by particle concentration fluoroimmunoassay (PCFIA, upper panels) and enzyme immunoassay (EIA, lower panels). Difference between diabetic and control children: PCFIA: IgG, $p < 0.0001$; IgA, $p < 0.001$. EIA: IgG, NS; IgA, $p < 0.01$

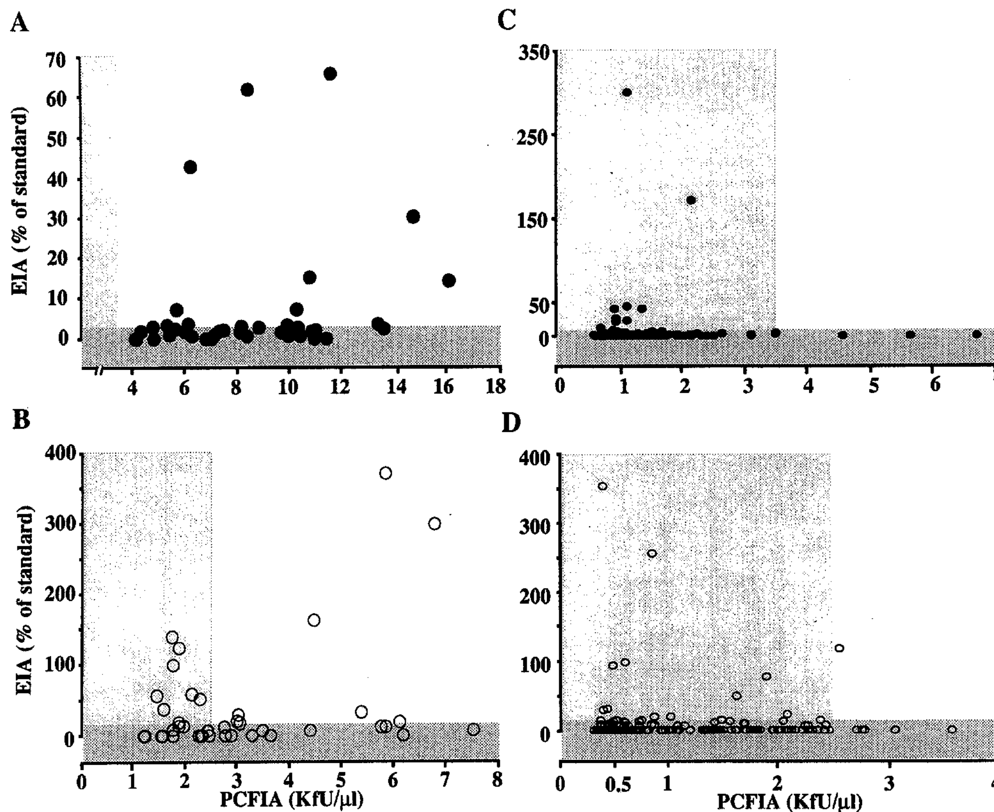

Fig. 3 A–D. Correlation between the levels of anti-bovine serum albumin (BSA) antibodies as determined by enzyme immunoassay (EIA) and particle concentration fluoroimmunoassay (PCFIA) in diabetic- and control children. Shaded areas represent BSA-antibody levels considered as "non-elevated" (▒ negative for BSA antibodies by PCFIA, ▒ negative for BSA antibodies by EIA).
A: IgG in diabetic children, $n = 40, r_s = 0.28, p = 0.09$;
B: IgA in diabetic children, $n = 40, r_s = 0.11, p = 0.48$;
C: IgG in control children, $n = 179, r_s = 0.02, p = 1.0$;
D: IgA in control children, $n = 179, r_s = -0.05, p = 1.0$.
Correlation coefficients were determined by Spearman's rank correlation

Table 2; $p < 0.0001$) and the disease-specificity was higher for PCFIA (Table 2; $p < 0.02$). For the IgA isotype assay results were comparable with respect to disease-sensitivity, but in EIA this was at the cost of disease specificity ($p < 0.05$). These findings suggest that PCFIA and EIA preferentially detect different subsets of BSA antibodies with no (IgG) or some overlap (IgA). Antibodies detected in EIA are poorly associated with Type 1 diabetes and are found commonly in the general population.

Table 2. Sensitivity and specificity of particle concentration fluoroimmunoassay (PCFIA) and enzyme immunoassay (EIA) in detecting anti-bovine serum albumin antibodies

	Sensitivity (%)			Specificity (%)		
	PCFIA	EIA	p	PCFIA	EIA	p
IgG	100	25	< 0.0001	98	90	< 0.02
IgA	50	42	NS	97	90	< 0.05

Discussion

Epidemiological evidence in man [4, 20–22] and data from animal feeding studies [13, 23–25] have suggested a diabetogenic effect of dietary cow's milk proteins. Supportive serological findings have been identified in animals [12, 13] and humans [15–17] associating immunity to cow's milk proteins with Type 1 diabetes. The most direct evidence for a pathogenic link between cow's milk proteins and diabetes comes from a family study in Finland, where exclusive breast-feeding for the first 3–4 months of life was found to protect from later development of diabetes [22].

Most of these studies did not identify a specific cow's milk protein or explain the near global increase in diabetes incidence despite emphasis on breast-feeding. However, these latter observations [22] are consistent with the view that in humans (as in diabetes-prone rats [13]) a diabetes associated immune response to BSA/ABBOS is triggered in the early post-natal period [1, 26]. The link of BSA/ABBOS to human diabetes was made by finding elevated antibody levels against BSA in all newly-diagnosed patients, the bulk of this response specific for the ABBOS epitope [6].

In order to compare the PCFIA assay procedure to the more commonly available EIA, we analysed a large number of samples using both techniques. The comparison revealed unequivocal differences with diabetes-associated anti-BSA molecules detected almost exclusively by PCFIA. Both, levels and frequency of positive responses among diabetic children were significantly higher in PCFIA than EIA. The wide scatter of antibody levels in EIA for both patients or control subjects caused major overlap between the groups and made differences statistically insignificant. A large proportion of samples were below the detection limit of EIA, causing considerable skewness for measurement of both isotypes examined.

PCFIA detected BSA antibodies in children with diabetes, whereas only a few non-diabetic children were positive. In contrast, only a small proportion of the antibodies were disease-associated in EIA, and levels were elevated more often in non-diabetic subjects. With only one out of the 18 controls positive for IgG- or IgA-anti-BSA^{EIA} elevated by PCFIA as well, the dichotomy was clear between antibody subsets detected in either procedure. Interestingly, the single elevated IgG and IgA values detected in both procedures derived from the same serum sample, suggesting that the host determines the choice of antibody species utilized in the common immune response to dietary BSA.

The BSA molecule consists of 608 amino acids and there are several areas where the sequence differs from human serum albumin. One of those is the described ABBOS peptide (pre-BSA position 153–169 [2]). As we have recently shown [6], most of the diabetes-associated antibodies detected by PCFIA in diabetic children are directed against this epitope, whereas in non-diabetic control subjects the major epitopes are different, with less than 3% of donors able to recognize ABBOS [6]. Since the ABBOS epitope is immunologically cross-reactive with a (Beta-cell) autoantigen, p69 [1, 3], the poor immunogenicity of this epitope in the general population is not surprising and clearly identifies the diabetic population. We have tentatively linked this principal difference to efficient antigen presentation of ABBOS by diabetes-associated MHC class II molecules coupled with a delay in oral (or mucosal) tolerance development in diabetic subjects [1, 3]: our focus on the latter was triggered by the report that the single highest marker of diabetes risk (DQβ non-ASP^{57}) also marks susceptibility for IgA deficiency, a regulatory abnormality of mucosal immunity [27, 28].

An antigen such as BSA has several epitopes which can induce a wide spectrum of high and low affinity antibodies. Our results are very reminiscent of the observation that insulin-autoantibodies (IAA) detected by EIA are poorly disease-associated [29] and have a low predictive value compared to fluid-phase radiobinding assays (RIA) [30–32]. EIA has been characterized by low sensitivity and an unacceptably high rate of false positives [29], similar to the results obtained in this study. EIA detects mainly low affinity IAA that have high binding capacity, whereas RIA detects high affinity, low binding capacity and strongly disease-associated subset(s) of IAA [32]. The same could apply to a fluid-phase assay such as PCFIA, in which accessibility to the epitopes may be different from EIA. The same epitopes may not be available PCFIA and EIA due to different binding procedures. Disease-associated epitopes may not be accessible if bound to EIA plate [33]. On the other hand, an excess of adhesive antigens on EIA-plate surface may bias binding of non-disease-associated, low affinity antibodies with high binding capacity as reported for IAA in healthy blood donors [34].

The striking lack of correlation between the two assay systems is less suggestive of gradual differences in average antibody affinity, but indicates absolute distinctions in the quality of antibodies detected. Maturation of an antigen driven (hyper-) immune response produces an antibody repertoire that is not only of high affinity but also favours immunoglobulins with fast binding kinetics [35], i. e. antibodies characterized by high on-off antigen binding rates and release required, for example, for rapid opsonization of pathogens and re-utilization of antibody. We speculate that the combination of large-surface area of antigen-conjugated microspheres, consequent ease of antigen accessibility and the fast dynamics of PCFIA (1 min binding periods) all contribute to the preferential detection of antibodies with a high on-off binding rate. If correct, then the presence of such antibodies supports our claim that these diabetes-associated antibodies are the product of a stringently antigen-driven immune response [6]. In any case, the observations presented here emphasize the im-

portance of clinical validation for serological assay procedures which rarely cover all possible immunoglobulin repertoires able to associate with a given antigen.

Acknowledgements. This work was supported by the Medical Research Council of Canada, the Research and Science Foundation of Farmos, Turku, and the Foundation for Paediatric Research, Helsinki, Finland. J. K. is a postdoctoral fellow of the Sigrid Juselius Foundation, Helsinki, Finland.

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Received: 24 March 1992
and in revised form: 12 June 1992

Dr. H.-M. Dosch, Division of Immunology and Cancer
The Hospital for Sick Children, 555 University Avenue
Toronto, Ontario M5G 1X8
Canada