# A BOVINE ALBUMIN PEPTIDE AS A POSSIBLE TRIGGER OF INSULIN-DEPENDENT DIABETES MELLITUS

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**Abstract** *Background.* Cow's milk has been implicated as a possible trigger of the autoimmune response that destroys pancreatic beta cells in genetically susceptible hosts, thus causing diabetes mellitus. Studies in animals have suggested that bovine serum albumin (BSA) is the milk protein responsible, and an albumin peptide containing 17 amino acids (ABBOS) may be the reactive epitope. Antibodies to this peptide react with p69, a beta-cell surface protein that may represent the target antigen for milk-induced beta-cell–specific immunity.

*Methods.* We used immunoassays and Western blot analysis to analyze anti-BSA antibodies in the serum of 142 children with insulin-dependent diabetes mellitus, 79 healthy children, and 300 adult blood donors. Anti-ABBOS antibodies were measured in 44 diabetic patients at the time of diagnosis, three to four months later, and one to two years later.

*Results.* All the diabetic patients had elevated serum concentrations of IgG anti-BSA antibodies (but not of antibodies to other milk proteins), the bulk of which were specific for ABBOS. The mean (±SE) concentration was 8.5±0.2 kilofluorescence units (kfU) per microliter, as compared with 1.3±0.1 kfU per microliter in the healthy children. IgA antibodies were elevated as well, but not IgM antibodies. The antibody concentrations declined after diagnosis, reaching normal levels in most patients within one to two years. The initial decline involved anti-ABBOS–specific antibodies almost exclusively. Much lower serum concentrations of anti-BSA antibodies were found in all 379 control subjects, but only 2.5 percent of them had small amounts of ABBOS-specific IgG.

*Conclusions.* Patients with insulin-dependent diabetes mellitus have immunity to cow's-milk albumin, with antibodies to an albumin peptide that are capable of reacting with a beta-cell–specific surface protein. Such antibodies could participate in the development of islet dysfunction. (N Engl J Med 1992;327:302-7.)

THE destruction of the pancreatic beta cells that occurs in patients with insulin-dependent diabetes mellitus is autoimmune in nature, but the cause of the autoimmune process is not known. Several recessive genes map the risk of diabetes,[1] but the concordance in identical twins is less than 50 percent,[2] suggesting that one or more environmental factors trigger the disease.[3]

Diabetes does not occur in diabetes-prone rodents reared on a diet free of cow's milk for the first two to three months of life,[4-6] indicating that cow's-milk proteins can trigger the disease. Epidemiologic and serologic data in humans also suggest a relation between cow's milk and diabetes.[7-11] The diabetogenic triggering event occurs early in life; exclusive breast-feeding with delayed exposure to infant formula based on cow's milk significantly reduced the risk of diabetes in Finnish children.[12] The whey protein bovine serum albumin (BSA) has been suggested to be the trigger molecule in the development of diabetes, because early induction of tolerance to BSA prevents diabetes and immunization accelerates it in BioBreeding (BB) rats,[13-15] and serum concentrations of anti-BSA antibodies are significantly higher in diabetic rodents than in control animals.[6,16] The anti-BSA antibodies found in diabetic BB rats[14] as well as in patients[13] bind to p69, a pancreatic beta-cell surface protein 69 kd in size that is inducible by interferon gamma. We therefore proposed that a BSA-induced, p69-crossreactive immune response could be the link between milk protein and beta-cell–specific immunity in patients with diabetes.[13,15] We subsequently identified a sequence of BSA containing 17 amino acids (ABBOS, a region of the BSA molecule extending from position 152 to position 168) that differed from the sequence of human, mouse, and rat albumins. Immunization with synthetic ABBOS induced the formation of antibodies capable of reacting with p69, thus focusing our search for the postulated link between bovine albumin and beta-cell–specific immunity on a small peptide.[13]

We developed the following hypothesis[15]: the ABBOS peptide is immunogenic only in hosts with diabetes-associated HLA Class II (DR/DQ)haplotypes able to bind and present this antigen. Since ABBOS and p69 share a common epitope, p69 should boost and sustain ABBOS-specific immune memory even after gut maturation, when large fragments of cow albumin cease to be absorbed. The ABBOS-specific immune effector function would mediate beta-cell destruction. Unrelated infectious events that generate the systemic release of interferon gamma induce the expression of p69 on the surface of beta cells, transiently exposing some of these cells to immune attack.[17-19] The long course preceding clinical diabetes could be explained by the temporary nature of such episodes of p69 expression on beta cells.[20-22]

To determine whether this model could be relevant to insulin-dependent diabetes in humans, we tested the serum of patients with newly diagnosed insulin-

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dependent diabetes for the presence of anti-BSA and anti-ABBOS antibodies.

## Methods

### Patient Populations

We studied 142 Finnish children (83 boys and 59 girls; mean [±SD] age, 8.4±4.3 years) with newly diagnosed insulin-dependent diabetes mellitus. Fifty patients had diabetic ketoacidosis, 48 had diabetic ketosis only, and the remainder had hyperglycemia alone. All were dependent on at least one daily injection of human insulin and had increasing dependence on insulin after their diagnoses. We also studied 79 healthy children matched for age, sex, and region of residence who were admitted for minor surgery (42 boys and 37 girls; mean age, 8.4±3.1 years) and 300 adult blood donors (in Toronto). Blood samples were obtained from the patients before their first insulin injection, three to four months later, and in a randomly selected subgroup of 44 patients, one to two years later. The serum samples from the two groups of Finnish children were coded and sent to Toronto.

The patients' clinical assessment included the taking of a history and measurements of autoantibodies to insulin and islet cells, identified by either indirect immunofluorescence or complement fixation.[23-25] Because the samples obtained earliest were of insufficient volume for a full titration, the results for islet-cell antibodies were expressed as positive or negative only. The HLA-A, B, C, and DR/Dw haplotypes of all patients were determined as described elsewhere.[23]

### BSA Antibodies

Anti-BSA antibodies were measured by particle-concentration fluoroimmunoassays.[26] Twenty microliters of serum (dilution, 1:100 to 1:1000) was added to 96-well unidirectional-flow vacuum-filtration plates (IDEXX, Portland, Me.), each containing 20-μl microspheres (0.125 percent wt/vol) covalently conjugated with BSA. Ovalbumin (0.1 percent wt/vol, Sigma) in phosphate-buffered saline containing 1 percent Tween-20 was used to prevent nonspecific binding. After one to three minutes of incubation and washing, unbound proteins were removed by vacuum filtration, and 100 μg of affinity-purified, fluorescein-conjugated goat antihuman IgG, IgA, IgM, or IgD was added to each well (Bio/Can, Mississauga, Ont.). Bound fluorescence was read and transmitted to Macintosh computers for automated data processing.

A calibrated pool of serum from the diabetic patients was used as the standard in each plate. Each microliter of this standard contained 12.3 kilofluorescence units (kfU) of IgG anti-BSA antibodies, 4.2 kfU of IgA anti-BSA antibodies, and 4.9 kfU of IgM anti-BSA antibodies. The anti-BSA assays had sensitivities of 1.0 ng of IgG or IgA per milliliter of sample and 10.0 ng of IgM per milliliter; the intraassay and interassay coefficients of variation ranged from 8 percent to 10 percent. The addition of BSA (but not that of ovalbumin) blocked antibody binding in a dose-dependent fashion. Concentrations of anti-BSA antibody that were more than 2 SD above the mean in the 79 normal children were defined as elevated.

Monoclonal anti-BSA and anti-ABBOS antibodies were generated from patient B lymphocytes transformed by Epstein–Barr virus.[27] These monoclonal antibodies, as well as polyclonal rat anti-ABBOS antiserum,[14] reacted negatively in tests for insulin and islet-cell autoantibodies. Conversely, the addition of up to 1000 μg and 100 μg of BSA and ABBOS peptide, respectively, did not alter the results of assays for islet-cell antibodies and insulin autoantibodies in the serum of all 15 diabetic patients tested.

### Studies of Specificity

Additional studies were done with serum samples from 44 children with diabetes and 44 normal children. IgG antibodies to casein (Sigma) and β-lactoglobulin (Sigma) obtained from cow's milk were measured with coated microspheres, as described for the measurement of BSA antibodies. The ABBOS peptide and the homologous region of rat serum albumin (the ABRAS peptide) were synthesized with a C-terminal cysteine residue not present in the natural sequence, for biotinylation and binding to streptavidin-coated microspheres.[26] Twenty microliters (0.5 percent wt/vol) of microspheres conjugated with either ABBOS or ABRAS was mixed with 3 μl of serum from patients or normal subjects in a final volume of 0.3 ml or 3 ml, respectively. After incubation at 4°C for 15 minutes, the mixtures were centrifuged, and 20 μl of the supernatant was used to measure residual anti-BSA antibodies.

### Protein-Expression Studies

Cell-membrane proteins from rat insulinoma cells incubated with interferon gamma and tissue extracts from rats given continuous infusions of interferon gamma for one week were subjected to sodium dodecyl sulfate–polyacrylamide-gel electrophoresis, blotted onto nitrocellulose membranes, and incubated with polyclonal rat anti-ABBOS antiserum.[14] The blots were developed with alkaline phosphatase–conjugated antirat IgG and scanned with an Apple OneScan instrument.

### Statistical Analysis

Antibody concentrations are expressed in kilofluorescence units per microliter relative to the standard serum pool. The results were analyzed by the chi-square test, parametric one-way analysis of variance, and Student's unpaired t-test for normally distributed values. The distribution of anti-BSA concentrations was normal for each isotype. In the case of a skewed distribution, the Mann–Whitney U test and Spearman's rank-correlation test were used. Concentrations of anti-BSA antibody after the time of diagnosis were evaluated by paired t-tests.

## Results

### Anti-BSA Antibodies

The serum concentrations of IgG anti-BSA antibody in the patients with diabetes were considerably higher than those in the normal children (Fig. 1), the mean concentration being almost seven times higher ($P<0.001$) (Table 1). Among the diabetic patients, the concentrations were lower in the boys than in the girls ($P<0.02$). According to our definition, all the diabetic children had elevated IgG anti-BSA concentrations, whereas only two normal children had elevated values ($P<0.001$) (Table 2). The elevated serum concentrations of IgG anti-BSA in the diabetic patients did not reflect generalized immune responses against nutritional antigens, since the patients and the normal children had similar serum concentrations of IgG antibodies to the cow's-milk proteins casein and β-lactoglobulin (Table 1).

The mean serum concentration of IgA anti-BSA antibodies was higher in the diabetic patients than in the normal children ($P<0.001$) (Table 1), but the values overlapped (Fig. 1). Two thirds of the diabetic patients (and two normal children) had elevated concentrations ($P<0.001$) (Table 2). The diabetic patients who had elevated levels of IgA anti-BSA antibodies were older than those with normal concentrations (mean age, 10.1±3.4 vs. 6.0±4.6 years, respectively; $P<0.001$).

The mean serum concentrations of IgM anti-BSA antibodies in the diabetic patients were slightly lower than those in the normal children ($P<0.05$) (Table 1). Less than 1 percent of the patients had elevated concentrations of these antibodies, as compared with 8 percent of the normal children ($P<0.01$) (Table 2

and Fig. 1). No patient with diabetes or normal child had IgD anti-BSA antibodies, and in a random subgroup of patients no IgE anti-BSA antibodies were detected.

Low concentrations of anti-BSA antibodies (mainly IgM) were detected in both the patients with diabetes and the normal children, but high concentrations of IgG and IgA anti-BSA antibodies were found only in the diabetic children. The latter findings indicated the existence of a close (100 percent) association between BSA-specific immune responses and the clinical expression of insulin-dependent diabetes. In a manner consistent with an active, antigen-driven immune response against BSA in diabetes, IgM and IgG anti-BSA concentrations were significantly correlated ($r_s = 0.77$; $P<0.001$).

The concentration of IgG anti-BSA antibodies remained elevated in the diabetic patients three to four months after diagnosis, whereas that of the IgA anti-BSA antibodies became lower over this period ($P<0.001$) (Table 3). In the 44 patients studied one to two years after diagnosis, the concentrations of all three types of anti-BSA antibodies were lower ($P<0.001$), and in most patients they were normal (IgG, normal in 27; IgA, normal in 43) (Table 2).

Table 1. Concentrations of Anti–Cow's-Milk Antibodies at the Time of Diagnosis in Children with Insulin-Dependent Diabetes Mellitus and Normal Children.*

| Antibody | Children with Diabetes | | | Normal Children | | | P Value |
|---|---|---|---|---|---|---|---|
| | Mean | Range | 95% Confidence Interval | Mean | Range | 95% Confidence Interval | |
| | *kilofluorescence units per microliter* | | | | | | |
| **Anti-BSA** | | | | | | | |
| IgG | 8.5±0.2 | 3.6–18.2 | 7.6–8.9 | 1.3±0.1 | 0.7–3.5 | 1.2–1.4 | <0.001 |
| IgA | 3.2±0.1 | 1.4–7.6 | 2.9–3.4 | 1.8±0.1 | 0.8–3.5 | 1.6–1.8 | <0.001 |
| IgM | 3.4±0.1 | 1.1–6.8 | 3.2–3.6 | 3.8±0.2 | 1.6–9.5 | 3.5–4.1 | <0.05 |
| **Anti-β-lactoglobulin** | | | | | | | |
| IgG | 3.3±0.2 | 1.4–6.9 | 3.0–3.6 | 3.0±0.1 | 1.3–5.1 | 2.8–3.3 | NS |
| **Anti-casein** | | | | | | | |
| IgG | 4.4±0.2 | 2.0–8.1 | 4.0–4.8 | 3.9±0.2 | 2.1–7.0 | 3.6–4.3 | NS |

*The assays for anti-BSA antibodies were performed in 142 children with diabetes and 79 normal children. The assays for anti-β-lactoglobulin and anti-casein were performed in 44 children with diabetes and 44 normal children. Plus–minus values are means ±SE. NS denotes not significant.

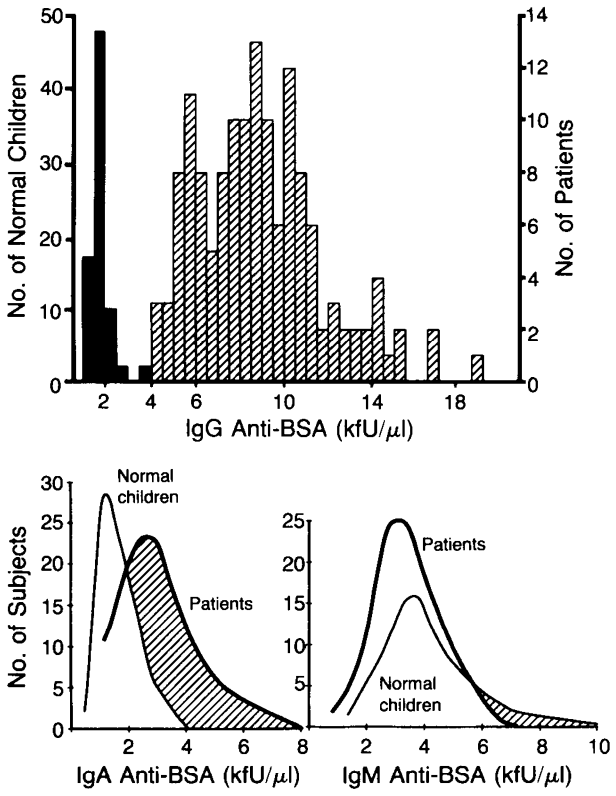


Figure 1. Serum Concentrations of IgG Anti-BSA Antibody in 142 Children at the Time of Diagnosis of Insulin-Dependent Diabetes (Hatched Bars) and in 79 Normal Children (Solid Bars).

The distribution of serum concentrations of IgM and IgA anti-BSA antibodies is shown in the lower panels after normalization by smoothing.

### Anti-ABBOS Antibodies

Anti-BSA antibodies were measured before and after the exposure of the serum samples to solid-phase ABBOS peptide (Fig. 2) to determine the proportion of the anti-BSA antibodies that specifically bound the ABBOS region of BSA. These studies were done with serum from 44 diabetic patients with high, moderate, and relatively low anti-BSA concentrations and an average concentration near the overall mean (17 representative samples are shown in Fig. 2). The concentration of IgG anti-BSA antibodies decreased by two thirds (range, 30 percent to 90 percent) after the incubation of serum with ABBOS peptide. Similarly, 23 to 71 percent of the IgA and IgM anti-BSA antibodies were ABBOS-specific, indicating a bias for this short sequence, which represents less than 2 percent of the BSA molecule. The amount of anti-BSA antibody removed by the incubation of serum with the ABRAS peptide was within the normal variation of the assay (approximately 10 percent).

The post-diagnosis decrease in concentrations of anti-BSA antibody began with the disappearance of ABBOS-specific antibodies (Table 3). One to two years after diagnosis, only 7 of the 44 patients studied had anti-BSA antibodies of any isotype that were specific for the ABBOS peptide, and 17 of the 44 had slightly elevated anti-BSA concentrations. Serum samples from the 17 normal children with the highest anti-BSA concentrations were studied in a similar fashion after their serum was incubated with the solid-phase ABBOS peptide. Concentrations of anti-BSA antibody were not significantly reduced in the absorbed serum, and only two serum samples contained detectable IgG or IgA anti-ABBOS

Table 2. Proportion of Subjects with Elevated Serum Concentrations of Anti-BSA Antibodies.

| Type of Anti-BSA Antibody | Children with Diabetes (N = 142) | | | Normal Children (N = 79) | Adult Blood Donors* (N = 300) |
|---|---|---|---|---|---|
| | At diagnosis | At 3–4 mo | At 1–2 yr† | | |
| | *percent* | | | | |
| IgG | 100‡ | 99.3‡ | 38.6‡ | 3.8 | 3.3 |
| IgA | 60.6‡ | 37.3‡ | 2.3 | 2.5 | ND |
| IgM | 0.7§ | 1.4§ | 0§ | 7.6 | ND |

*ND denotes not determined.
†Data in this column were obtained from studies of 44 children.
‡P<0.001 for the comparison with normal children.
§P<0.01 for the comparison with normal children.

antibodies. In the 300 adult blood donors from Toronto, the range and mean of IgG anti-BSA–antibody concentrations were similar to those in the 79 normal Finnish children (Table 3), and IgG anti-ABBOS antibodies were found in 3 percent of the samples.

Anti-ABBOS antibodies precipitated the beta-cell surface protein p69, whereas no comparable antigenic activity was found in other tissues (Fig. 3). This exclusivity of p69 expression could explain the decline in anti-ABBOS concentrations that occurred together with the disappearance of beta cells after the diagnosis of diabetes, provided that p69 (and not dietary protein) was the antigenic stimulus sustaining these disease-associated antibodies in the patients with diabetes. Nutritional information obtained from a subgroup of patients did not indicate that milk consumption had decreased in the diabetic children.

### Anti-BSA Antibodies and Disease Markers

No relations were found between the concentrations of either anti-BSA or anti-ABBOS antibodies and the severity of disease presentation (as indicated by blood glucose, hemoglobin $A_1$, or serum C-peptide concentrations), the duration of symptoms before diagnosis, or the severity of diabetic ketosis or acidosis. The specificities, concentrations, and isotype distributions of the antibodies were similar in families with one and families with more than one member with Type I diabetes.

At the time of diagnosis, 78 percent of the patients were positive for islet-cell antibodies, 58 percent had complement-fixing islet-cell antibodies, and 47 percent had insulin autoantibodies. Anti-BSA concentrations, isotype diversity, and specificity were not associated with the presence or absence of islet-cell or insulin autoantibodies.

Children heterozygous for HLA-DR3/4 or Dw3/4 initially had more severe diabetes (with higher concentrations of blood glucose and hemoglobin $A_1$ and lower serum concentrations of C peptide) than those negative for these haplotype combinations, but the frequencies or concentrations of insulin and islet-cell autoantibodies were similar in both haplotype groups. The concentrations of antibodies to BSA and ABBOS were comparable among the diabetic children with or without HLA-DR3/4 or Dw3/4, as well as among those with HLA-DR3/x, Dw3/x, DR4/x, and Dw4/x.

## Discussion

All the diabetic patients studied initially had high serum concentrations of anti-BSA antibodies. These antibodies reacted primarily with the small ABBOS peptide and were mostly of the IgG isotype. The lack of association between diabetes and the presence of IgG antibodies to other milk proteins and the absolute association between diabetes and anti-BSA or anti-ABBOS antibodies link the BSA and ABBOS molecules closely to insulin-dependent diabetes in these patients.

Our patients were typical in all respects. They all had increasing insulin dependency during the first six months. At the time of the patients' diagnosis, 80 to 90 percent of islet beta cells are estimated to have been destroyed, and this process continues at a considerable pace.[28,29] The decline of antibody concentrations in parallel with beta-cell destruction in the presence of continued consumption of cow's milk is difficult to understand if dietary protein was still driving the immune response. The results suggest that after its initiation by BSA and ABBOS in early infancy, the immune response is sustained by an endogenous antigen that is present in all patients at diagnosis but that disappears within one to two years. We propose that it is the cross-reactive beta-cell p69 that maintains the antibody response until the destruction of islet cells is complete and p69 is no longer available.[13,15]

The presence in all patients of elevated serum concentrations of IgG and IgA but not IgM anti-BSA antibodies is a profile most consistent with an antigen-driven immune response, in which relevant clones are continuously transferred from immature

Table 3. Decline in Serum Concentrations of Anti-BSA Antibodies and in Proportions of ABBOS-Specific Anti-BSA Antibodies after the Diagnosis of Insulin-Dependent Diabetes Mellitus.*

| Type of Anti-BSA Antibody | Children with Diabetes (N = 44) | | | Normal Children (N = 79) | Adult Blood Donors (N = 300) |
|---|---|---|---|---|---|
| | At diagnosis | At 3–4 mo | At 1–2 yr | | |
| | *kilofluorescence units per microliter (percent of antibodies specific for ABBOS)* | | | | |
| IgG | 6.3±0.2 (61) | 5.9±0.2 (27) | 2.2±0.1† (6) | 1.3±0.1 (4) | 1.3±0.02 (3) |
| IgA | 2.9±0.1 (31) | 2.5±0.1† (11) | 0.9±0.1† (7) | 1.8±0.1 (0) | ND |
| IgM | 2.9±0.1 (46) | 2.9±0.1 (20) | 1.3±0.1† (9) | 3.8±0.2 (0) | ND |

*Plus–minus values are means ±SE. ND denotes not determined.
†P<0.001 for the comparison with the values obtained at diagnosis.

IgM-expressing B-cell compartments to pools of IgG-secreting or IgA-secreting cells.[27,30] This view is supported by the close relation between the serum concentrations of IgM and IgG anti-BSA antibodies and the similarity of the bias toward ABBOS among the different isotypes in the same patient. The transient exposure to p69 antigen proposed in our pathogenetic model would be consistent with this serologic profile.

Serum IgG anti-BSA antibodies were found in both diabetic and normal children, but in the normal children the concentrations were low and distributed in a narrow range. Not unexpectedly,[31] the serum samples from 300 adult Canadian blood donors also contained low concentrations of anti-BSA antibodies, similar to those in the normal Finnish children. These results establish the normal concentrations for these antibodies and emphasize the abnormality in the patients with diabetes.

The presence in every diabetic patient of an increased serum concentration of IgG anti-BSA antibodies makes their frequency higher than that of

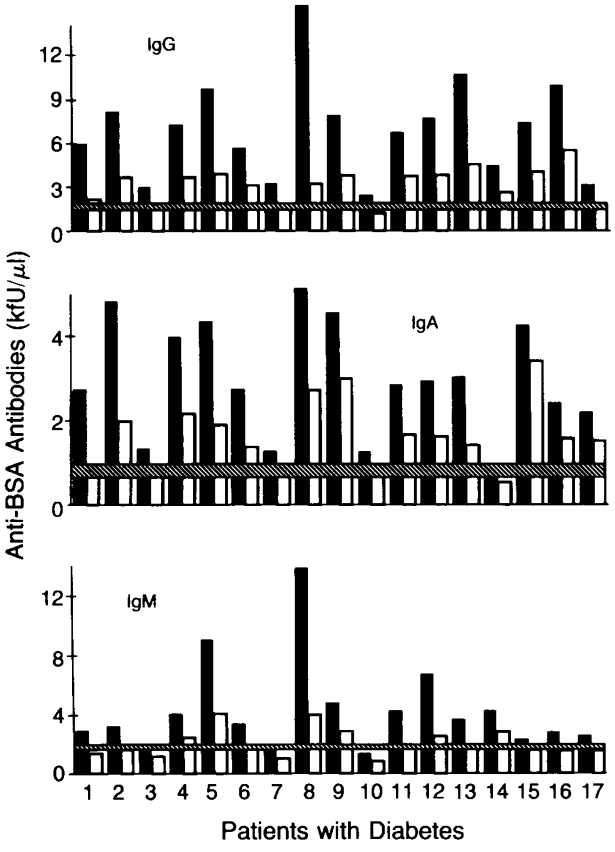


Figure 2. Total and ABBOS-Specific Anti-BSA Antibodies in 17 Patients with Diabetes.

The solid bars represent anti-BSA levels in the patients, and the open bars the levels remaining after the removal of ABBOS-specific antibodies. The hatched horizontal bars indicate the mean anti-BSA concentrations in 17 normal children (upper line) and the concentrations after the removal of anti-ABBOS antibodies from the same serum samples.

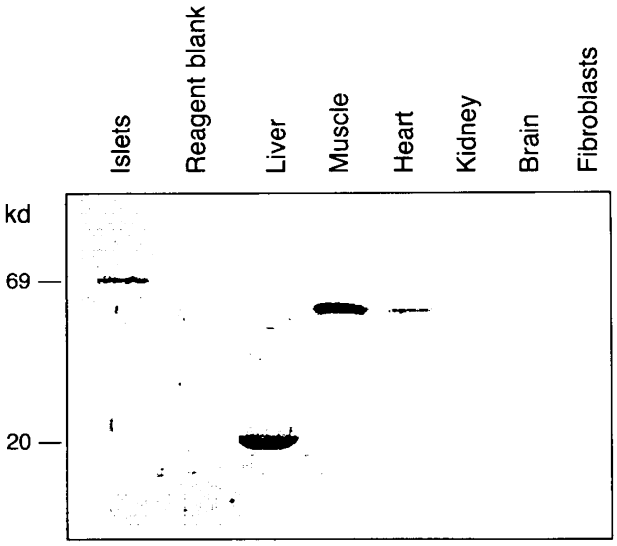


Figure 3. Western Blot Analysis of p69 Expression in Various Rat Tissues.

Enriched cell-membrane proteins from the tissues shown were separated by sodium dodecyl sulfate–polyacrylamide-gel electrophoresis, blotted, and probed with polyclonal rat anti-ABBOS antiserum. The rats from which the tissues were obtained were given infusions of interferon gamma for one week with implanted pumps. The blot shows that the antiserum reacted with a 69-kd protein in the islet cells, and with smaller proteins in the liver, muscle, and heart.

the classic diabetes-associated autoantibodies to insulin and cytosolic islet-cell antigens. There was no association between the concentrations of anti-BSA antibodies and any diabetes-associated combination of HLA haplotypes, a finding that parallels an earlier observation with regard to antibodies to cow's milk.[8] The lack of correlation between concentrations of anti-BSA antibodies and HLA haplotypes is reminiscent of the dissociation between islet-cell antibodies and HLA haplotypes.[32,33] HLA molecules are probably required during the initiation of the immune response specific to BSA and ABBOS, but they do not appear to determine its intensity.

We postulated that in an immune system able to process, present, and recognize ABBOS, specific immune memory is established at the time of dietary exposure to cow's milk. In this context the timing of gut closure, the predominance early in life of digestive enzymes such as trypsin[34] (which leaves much of the ABBOS sequence intact) and perhaps gastrointestinal infections, as well as the maturation of oral tolerance mechanisms, must collaborate to allow the initiation of the anti-ABBOS response. Thereafter, the induction of p69 expression by interferon gamma generated during variable, etiologically unrelated infectious episodes would become the slow pacemaker of the development of diabetes.

This model describes a slow, rather inefficient process, consistent with the fact that clinical disease develops in only about 5 to 6 of 1000 hosts with the relevant genetic predisposition.[35,36] Taken as a whole, our find-

ings suggest that an active, antigen-driven immune response against the BSA-derived ABBOS peptide is a feature of the autoimmune response in patients with insulin-dependent diabetes. This likens the disease in humans to that in diabetes-prone rodents, in which the prevention of exposure to cow's milk early in life prevents the development of the disease.[15]

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## A BOVINE ALBUMIN PEPTIDE AS A POSSIBLE TRIGGER OF DIABETES MELLITUS

*To the Editor:* Karjalainen et al. (July 30 issue)* hypothesize that since antibodies to bovine albumin that cross-react with a beta-cell antigen are found in higher titers in children with insulin-dependent diabetes mellitus than in normal children and adults, exposure to cow's milk early in life predisposes a person to the development of diabetes. An alternative hypothesis is that there are multiple antibodies to beta-cell antigens at the onset of diabetes and that the homology of the beta-cell antigen and bovine serum albumin (BSA) is clinically irrelevant.

The authors could make their case stronger by demonstrating that the children with the antibodies to BSA were exposed to cow's milk at an early age. Since the average age of their diabetic patients was eight years, the authors should have provided historical data on this point. Can they do so now?

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*Karjalainen J, Martin JM, Knip M, et al. A bovine albumin peptide as a possible trigger of insulin-dependent diabetes mellitus. N Engl J Med 1992; 327:302-7.

The authors reply:

*To the Editor:* Dr. Chideckel is correct in reminding us that patients with Type I diabetes have autoantibodies to a number of components of islet cells. The purpose of our study, however, was to provide a link between Type I diabetes in humans and a model of diabetes previously established in diabetes-prone animals. The important aspect of our study is the link it provides between Type I diabetes in humans and immune responses to the dietary bovine albumin molecule.

We and others previously established the diabetes-triggering effect of BSA in diabetes-prone rodents and demonstrated that withholding dietary bovine albumin, as well as inducing tolerance to the molecule early in life, prevented Type I diabetes in these animals.[1-3] Epidemiologic evidence in humans, such as the diabetes-protective effect of exclusive breast feeding and the close relation between the consumption of cow's milk and the risk of diabetes, is compatible with, but not proof of, a link between dietary bovine albumin and diabetes.[4,5] We demonstrated not only that there was a close association between diabetes and elevated titers of anti-BSA antibodies, but also that most of these antibodies were directed against the small ABBOS peptide, the predicted focus of our model; that such antibodies were detected rarely in normal subjects; and that the antibody titers followed the course of the disease, declining after diagnosis, in parallel with the death of residual islet cells.

Although these findings fall short of proving that exposure to cow's milk leads to Type I diabetes, they lead to the question, Will the avoidance of exposure to cow's milk during the first months of life reduce the incidence of diabetes? We do not have data on milk consumption in early life in the patients we studied. The pilot phase of a trial asking this question is now under way.

We think it unlikely that our findings of dietary milk–related immune responses are "clinically irrelevant." None of the islet-cell autoantigens have been proved irrelevant, and in our case the diabetes-triggering role of bovine albumin is well established in animal models of Type I diabetes.

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## LACK OF IMMUNITY TO BOVINE SERUM ALBUMIN IN INSULIN-DEPENDENT DIABETES MELLITUS

*To the Editor:* Atkinson and colleagues (Dec. 16 issue)[1] report a lack of B-cell and T-cell immunity to bovine serum albumin (BSA) in children with insulin-dependent diabetes mellitus (IDDM). Their results are in contrast with several epidemiologic, ecologic, time-series, and experimental studies.[2,3]

Exposure during the first months of life to infant formulas based on cow's milk carries a significantly elevated risk of IDDM (mean odds ratio in case–control studies, 1.6; 95 percent confidence interval, 1.2 to 2.2).[3] We reported elevated levels of IgG anti-BSA antibody in Finnish children with diabetes of recent onset,[4] an observation confirmed in a recent large study of French children (unpublished data).

The low titers of BSA antibodies in the patients with IDDM studied by Atkinson et al.[1] are surprising. The values were twice as high in the study of French children and four times higher in the diabetic children in Finland.[4] BSA antibodies resemble anti-insulin autoantibodies in that they are difficult to detect in standard immunoassays; technical aspects of the assay may contribute to the discrepancy. In addition, Atkinson et al. used a BSA-specific mouse monoclonal antibody to standardize their assay, which is unlikely to recognize epitopes related to diabetes in humans. Also, the secondary antihuman antibodies used contained BSA as a stabilizer, which competes for anti-BSA and reduces the sensitivity of the assay.

We found that T cells from 88 percent of 43 diabetic Canadian children were sensitized to BSA, ABBOS, and the p69 islet-cell protein (mean [±SD] stimulation index, 4.8±0.7).[5] The absence of any T-cell responses to BSA in the study by Atkinson et al. raises the question of problems with culture conditions. The excellent, high-insulin culture medium used has a short shelf life, and we have found it necessary to prepare fresh stocks frequently.

The findings of Atkinson et al. emphasize the need for collaborative efforts to standardize assays. Their negative results signal the need for caution but do not disprove positive results from an increasing number of studies of BSA immunity in patients with IDDM.

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The authors reply:

*To the Editor:* We find the epidemiologic association between the consumption of cow's milk and IDDM intriguing.[1] We sought to confirm an immunologic basis for the association,[2] but found none.

We believe the statements by Dosch et al. about our ability to detect BSA antibodies are unfounded and provide insufficient explanation of the differences between their results[2] and ours. First, although the fluorescence activity of our positive serum samples was not as high as that reported by Dosch and colleagues, the titers of anti-BSA antibodies in these samples were not necessarily low, as their letter suggests. Antibody titer and mean intensity of fluorescence are different measurements, and the determination of titer requires an assay of serially diluted serum samples that was not reported in our study.[1]

Second, we used the BSA-specific monoclonal antibody to ensure that BSA was covalently linked to the polystyrene beads and not to standardize immunologic detection by human IgG. Our assay did in fact detect anti-BSA antibodies, since the antibody reactions could be inhibited by exogenous BSA. The antibodies, however, were not specific for IDDM, being detected in both high- and low-risk nondiabetic relatives of probands with IDDM, as well as patients with other autoimmune disorders.

Third, we disagree with the statement that standard immunoassays do not detect insulin autoantibodies. Finally, the secondary antihuman antibodies we used contained ovalbumin as a stabilizer, not BSA.

With respect to the detection of T-cell responses to BSA, we used only freshly prepared medium. Furthermore, the immunologic association between anti–islet-cell p69 autoantibodies[3] and anti-BSA immunity is increasingly unclear, because detection of the former is not inhibited by the addition of cow's milk.[3] In addition, since epidemiologic data suggest that breast-feeding is associated with a decreased incidence of IDDM, it may be that this practice protects against the development of IDDM rather than that IDDM is induced by the ingestion of cow's milk.

The association of environmental factors with the pathogenesis of IDDM remains the subject of controversy. Further epidemiologic, immunologic, and genetic information is required to identify the cause of this disease.

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