Cow Milk Feeding Induces Antibodies to Insulin in Children – A Link Between Cow Milk and Insulin-Dependent Diabetes Mellitus?

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Exposure to cow milk (CM)-based formulas in early infancy has been associated with an increased risk of insulin-dependent diabetes mellitus (IDDM), but studies on the possible pathogenic mechanism(s) linking CM and IDDM are contradicting. We hypothesized that if CM formulas contained bovine insulin (BI), exposure to them could lead to immunization against insulin, which is the only known β-cell-specific autoantigen in IDDM. We measured immunoglobulin G (IgG) antibodies by enzyme immunoassay (EIA) to BI and human insulin (HI) in children who received, during the first 9 months of life, either a formula containing whole CM proteins or a formula containing hydrolyzed casein (HC) peptides. BI was detectable by radioimmunoassay (RIA) and immunoblotting in the CM-based formula. At 6 months of age the children who received CM formula had higher levels of IgG antibodies to BI than children who received either HC formula or children who were exclusively breast-fed (median levels 0.480 versus 0.185, $P = 0.04$; and 0.480 versus 0.160, $P = 0.04$; respectively). Also, at 9 months of age, children in the CM group differed from the HC group (0.403 versus 0.230; $P = 0.02$). Antibodies to BI and HI showed a positive correlation and cross-reacted in inhibition studies. The high incidence of insulin-binding antibodies in young children with IDDM may be explained by oral immunization to BI present in CM. Exposure to BI, which differs from HI only by three amino acids, may break the tolerance to insulin.

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INTRODUCTION

Insulin is the only known β-cell-specific autoantigen in insulin-dependent diabetes mellitus (IDDM). Insulin autoantibodies (IAA) are found in IDDM [1], and are associated, in particular, with IDDM diagnosed at young age [2–4]. Recently the importance of insulin in the pathogenesis of IDDM has been further supported by studies showing that insulin-reactive T-cell lines transfer the disease in an experimental animal model of IDDM [5].

Early exposure to cow milk (CM) proteins has been associated with an increased risk for IDDM in epidemiological studies [6]. Several pathogenic mechanisms, based mainly on molecular mimicry between CM proteins (bovine serum albumin, β-lactoglobulin, β-casein) and islet cell proteins (ICA69/p69, retinol-binding protein, GLUT-2 glucose transporter, respectively), have been proposed to explain this association [7–9], but there is no direct evidence for the pathogenic role of CM proteins in IDDM.

Because native CM contains bovine insulin (BI) [10], we studied whether CM formulas contain BI and whether exposure to CM formulas resulted in formation of antibodies to BI in children who received a CM-based formula, or a human casein (HC)-based formula after breast-feeding, when compared with children who were exclusively breast-fed.

SUBJECTS AND METHODS

Subjects. Twenty newborn infants of IDDM mothers were recruited into the first pilot study of a trial for the primary prevention of IDDM by the elimination of dietary CM proteins during early infancy [11, 12]. Exclusive breast-feeding was encouraged; thereafter, 10 infants received an adapted CM-based formula (Enfamil®, Mead Johnson, Evansville,

IN, USA; CM group) and 10 infants a HC formula containing casein peptides of a molecular weight less than 1200 (Nutramigen®, Mead Johnson; HC group) until the age of 9 months. The mothers of all infants were advised to eliminate infant food products containing CM or beef until the age of 9 months. After 9 months of age both groups started to get ordinary CM-based formulas. All infants except one received the test formula before the age of 6 months (mean age of exposure to the test formula was 1.2 months, range 0–7.5 months). No difference was observed in the duration of breast-feeding and the start of the substitution formula between the two groups. One infant in the CM group who was exposed to the CM formula for the first time at the age of 7.5 months was excluded from the analyses performed at the age of 6 months. Available plasma samples were studied at the age of 6 (CM group $n = 8$, HC group $n = 8$), 9 (CM group $n = 9$, HC group $n = 7$) and 12 months (CM group $n = 10$, HC group $n = 9$). One child in the HC group contracted manifest diabetes at the age of 14 months. One child in the CM group developed CM allergy at the age of 7 months and was excluded from the study after diagnosis. As control subjects, six 6-month-old children who were exclusively breast-fed were included. The study plan was approved by the ethical committee of the Children's Hospital, University of Helsinki.

Immunoblotting of BI in the test formulas. The presence of insulin in the CM formulas, Enfamil® and Nutramigen®, was studied by immunoblotting in non-reducing conditions. The ×10 concentrate of CM formula, compared with the dilution according to the manufacturer's protocol, was electrophoresed in 16% polyacrylamide. The proteins were transferred to a pure nitrocellulose membrane (Trans-Blot®Transfer Medium, Hercules, CA) and the membrane was blocked with 2% human serum albumin (HSA) in phosphate-buffered saline (PBS). The membrane strips were incubated with polyclonal guinea-pig anti-porcine insulin antiserum (Dako, Carpinteria, CA) diluted 1:1000 in 0.2% HSA, 0.05% Tween 20 in PBS. The second antibody was biotinylated goat anti-rabbit IgG (Dako). Alkaline phosphatase-streptavidin complex (Zymed, San Francisco, CA) was added and the reaction was developed with an alkaline phosphatase conjugate substrate kit (Bio-rad, Hercules, CA). BI (0.5 μg/lane) was run as a positive control protein in the immunoblotting experiment.

RIA for the detection of insulin in the CM products. The amount of immunoreactive insulin recognized by insulin antibodies in CM products was quantified by a commercial solid-phase ^{125}I radioimmunoassay (RIA) designed for the quantitative measurement of insulin in serum (Coat-A-Count®Insulin, Diagnostic Products Corp., Los Angeles, CA).

Enzyme immunoassay (EIA) for IgG antibodies to BI and HI. Polystyrene plates (Combiplate® Enhanced binding, Labsystems, Helsinki, Finland) were coated with BI or HI (Sigma, St. Louis, MO), both 1 μg/well. For residual coating, 1% HSA in PBS was used, and 0.05% Tween 20 in PBS was used as a washing buffer. The samples were diluted 1:20 in PBS containing 0.2% HSA, 0.05% Tween. Alkaline phosphatase-conjugated rabbit anti-human IgG antibodies (Jackson ImmunoResearch, West Grove, PA), were used as the secondary antibody. *P*-nitrophenyl phosphate (Sigma) was used as a substrate, and the absorbance was measured with an optical reader at 405 nm. The mean intra-assay variation of the method was 6.2% and interassay variation 11.5%. Two positive plasma samples from patients with newly diagnosed IDDM and two negative plasma samples from healthy children were run on all plates as quality controls.

Inhibition assays. For inhibition assays, 0.1, 1.0, 10 and 100 μg/ml BI, HI or B-chain of BI (Sigma) were incubated for 2 h at room temperature with the plasma samples before analysing the sample by the EIA (discussed above) for antibodies to BI.

Statistical analysis. The differences between different groups were analysed by the Mann–Whitney *U*-test. Correlations between different parameters were calculated by the Spearman correlation test.

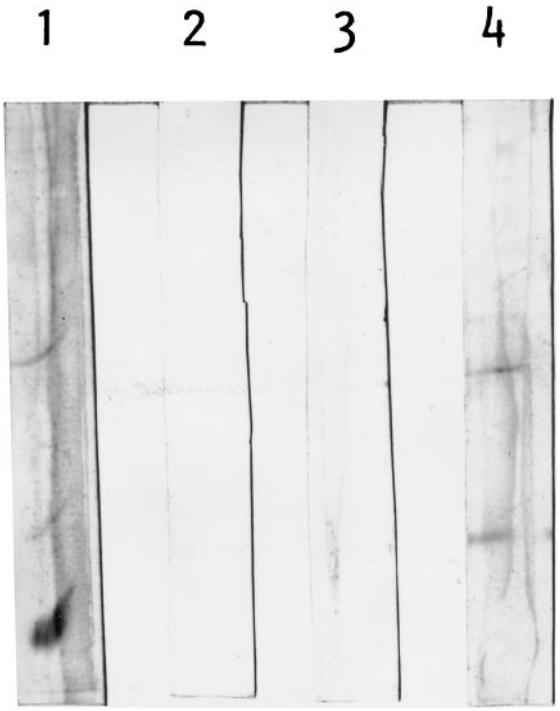

Fig. 1. Immunoblotting analysis of the presence of insulin in cow milk (CM) formulas used in the CM elimination study. Membrane strips were incubated with guinea-pig antiserum against human insulin. Lanes: 1, bovine insulin as control; 2, blank control; 3, Nutramigen®; 4, Enfamil®. In non-reducing circumstances insulin aggregates are seen.

RESULTS

BI was confirmed to be present by immunoblotting in Enfamil® but not in Nutramigen®, which contains only peptides smaller than 1200 MW (Fig. 1). The amount of immunoreactive insulin in Enfamil® was 5 mU/l by RIA. Immunoreactive peptides of insulin were found by RIA also in Nutramigen® (8 mU/l). The amount of insulin in native CM varied from 35 to 42 mU/l in four samples studied.

The IgG-antibody levels to BI at the age of 6 and 9 months in the children who received CM formula or HC formula and in the exclusively breast-fed children are shown in Fig. 2. At the age of 6 months, the children who received CM-based formula had higher levels of IgG antibodies to BI (median 0.480, range 0.213–0.656) than children who received the HC formula (median 0.185, range 0.112–0.539; $P = 0.04$) or who were exclusively breast-fed (median 0.160, range 0.134–0.293; $P = 0.04$). At the age of 9 months, children in the CM group had higher antibody levels to BI than children in the HC group (respective values: median 0.403, range 0.213–0.841, versus median 0.230, range 0.105–0.414; $P = 0.02$). After the end of the dietary manipulation, the difference between the CM and HC

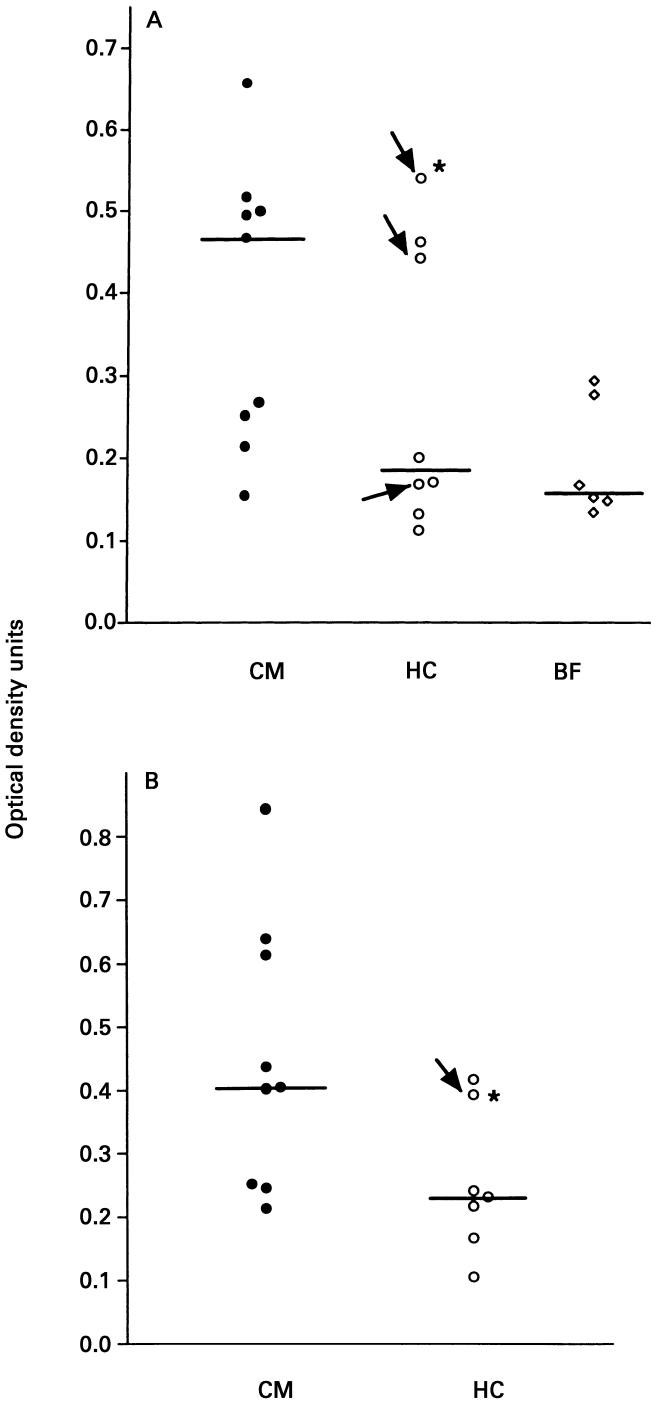

Fig. 2. The levels of IgG antibodies to bovine insulin at the age of 6 months (A) and at the age of 9 months (B) in children who had received either CM formula or HC formula after breast-feeding and in children who were breast-fed (BF) exclusively. The median value in each group is marked with a line. Children who had IAA are marked with an arrow. The child who developed IDDM at the age of 14 months is marked with an asterisk.

groups disappeared, being 0.364 (range 0.184–0.722) versus 0.308 (range 0.131–0.893) ($P = 0.81$) at 12 months of age.

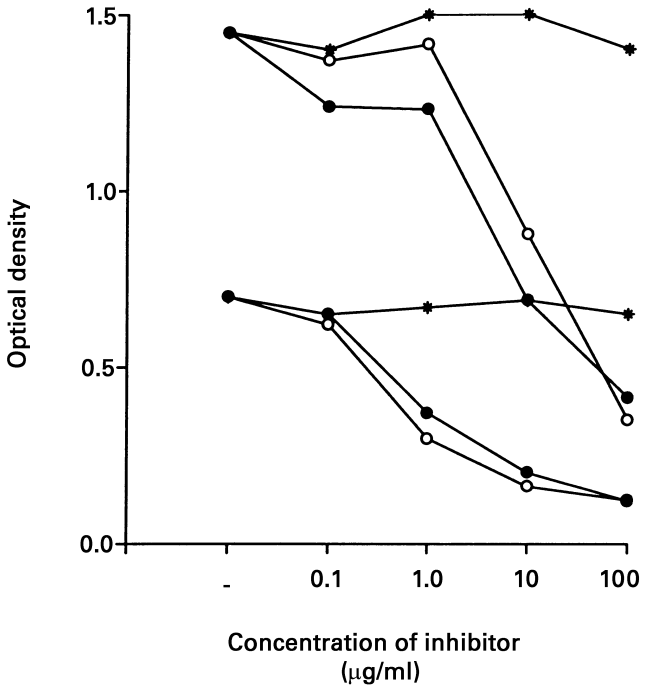

Fig. 3. The inhibition of IgG-antibody binding to solid-phase bovine insulin (BI) by: BI (open circle), human insulin (filled circle) or by the B-chain of BI (asterisk), incubated at different concentrations (x-axis) with the plasma samples from two newly diagnosed IDDM patients.

The levels of IgG antibodies to HI did not differ between the groups at any age (data not shown). However, EIA analysis showed significant correlation between the levels of antibodies to BI and HI ($r = 0.546$; $P < 0.0001$). The levels of antibodies to BI in children at the age of 6 months did not correlate with the age at the start of the formula feeding in the CM group.

At the age of 6 months, three children in the HC group had IAA. One developed IDDM at the age of 14 months and another had maternally transferred IgG antibodies (see Fig. 2). At the age of 9 months, only the child who later manifested IDDM had IAA. IgG-antibody levels to BI and HI in this child were 0.539 and 0.457, 0.390 and 0.374, 0.605 and 0.332 absorbance units at the ages of 6, 9 and 12 months, respectively. Data on IAA measurements have been published elsewhere [13].

In inhibition experiments, HI and BI inhibited the binding of IgG antibodies from patients with IDDM to solid-phase HI or BI in the same manner, but the B-chain of BI did not show inhibition (Fig. 3).

DISCUSSION

We demonstrated that CM formulas contain BI and that oral exposure to these formulas induces the production of IgG antibodies to BI in some children. The antibodies to BI cross-reacted with HI in inhibition studies and the levels of the two antibodies were correlated. BI and HI differ by three amino acids,

one difference being at position 30 in the B-chain, and the others at positions 8 and 10 in the A-chain. BI is known to be a potent immunogen in humans. Although insulin is highly conserved in mammals, treatment of patients with BI resulted in the production of high levels of antibodies to insulin and the antibody levels decreased when switching to HI [14].

This is the first study to demonstrate a natural way of immunization to insulin in humans. The detection of insulin-binding antibodies has been a controversial issue in IDDM research. The main purpose in the development of insulin antibody assays has been in improving their predictive value for IDDM. The frequent occurrence of insulin-binding antibodies in healthy children has limited the use of these antibodies alone as a risk marker for IDDM. For predictive studies, a competitive RIA for IAA is the best method of the various options available for IAA measurements [15]. In particular, the occurrence of IAA together with islet cell antibodies (ICA) is associated with impaired secretion of insulin by β-cells [3]. The frequency of IAA varies from 20 to 60% in patients with newly diagnosed IDDM and from 1 to 10% in healthy subjects, according to different studies [1–4]. In a prospective birth–cohort study, IAA appeared earlier and at a higher frequency than other autoantibodies and were detected in all five children of the cohort who progressed to clinical IDDM [16]. This suggests that immunization to insulin may indeed be one of the first events in the development of an autoimmune process against pancreatic -cells in humans.

Because our interest was focused on the possibility that BI in CM induces antibodies in infants, we studied the occurrence of IgG antibodies to BI and HI by EIA. In inhibition studies the binding of antibodies to solid-phase BI or HI were readily inhibited by BI or HI in the aqueous phase. This confirms that the specificity of these antibodies detected by our assay is against insulin. According to our study the induction of insulin-binding antibodies occurs in children when they are exposed to BI but these antibodies detected by EIA differ from the IAA detected by RIA, probably as a result of the differences in the affinity of the antibodies [15].

We have previously reported that some infants who had received a HC formula developed T cell and antibody responses to casein [12]. This indicates that orally given peptides can be immunogenic. It cannot be excluded that some children in the group who received the HC formula may have been immunized to the peptides of BI that were detectable in the formula by RIA. One child in this group developed IDDM at the age of 14 months. He already had elevated levels of IAA and antibodies to BI by EIA from the age of 6 months. It should be emphasized that this child may present an exceptional case because in the nationwide *Childhood Diabetes in Finland* (DiMe) study only 1.4% of 750 consecutive subjects diagnosed in the years 1986–89 were below 15 months of age at diagnosis (HK Åkerblom, unpublished data).

The demonstration of immunogenic BI in CM formulas raises the question of whether this priming may later be activated by autologous HI. Oral antigen stimulation has a dual nature. It may result in systemic immunity or tolerance but the mechanisms are poorly understood. In most children, tolerance to dietary proteins develops and harmful effects are exceptional. In a minority of children the sensitization to dietary antigens (not restricted to infancy), may, however, result in a disease (e.g. CM allergy and coeliac disease). By analogy, sensitization to dietary BI may lead to the development of immunity to insulin and, in the worst case, to the induction of autoaggressive β-cell-reactive T cells. Indeed, it has been recently shown that transgenic mice expressing ovalbumin in the β-cells developed cytotoxic ovalbumin-reactive lymphocytes and diabetes when fed with ovalbumin [17]. In one study, feeding insulin to non-obese diabetic mice induced $CD8^+$ T cells that enhanced the disease after adoptive transfer [18].

We have previously suggested that IDDM patients may have a general defect in the development of oral tolerance [8, 19]. Thus, immune responses to insulin in patients with IDDM could also be generated by dietary components. Of different CM proteins against which enhanced immune responses have been reported in IDDM [7–9], dietary BI may be the most potential trigger of IDDM. In addition, BI may form complexes with other CM proteins, such as caseins [10]. The presence of BI bound to CM antigens should be eliminated when immunity to other CM proteins is studied in IDDM, to exclude the possible false-positive results caused by reactivity against insulin.

We conclude that feeding CM induces insulin-binding antibodies in children. This provides an explanation of the elevated levels of antibodies to insulin, especially in young children with IDDM. Our observation also raises the possibility that oral administration of a heterologous insulin, which minimally differs from the autologous insulin, could in some cases result in the breakdown of immune tolerance to pancreatic β-cells. This hypothesis may also be of interest for studies on the etiology of autoimmune diseases other than IDDM.

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