Quantitation of Bovine Serum Albumin in Cow's-Milk-Based Infant Formulas and Removal of Bovine Serum Albumin from Cow's Milk and Whey Protein Isolates

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QUANTITATION OF BOVINE SERUM ALBUMIN IN COW'S MILK-BASED INFANT FORMULAS AND REMOVAL OF BOVINE SERUM ALBUMIN FROM COW'S MILK AND WHEY PROTEIN ISOLATES

by

Patricia Z. Marincic

A dissertation submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Nutrition and Food Sciences

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

1997
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ABSTRACT

Quantitation of Bovine Serum Albumin in Cow’s-Milk-Based Infant Formulas and Removal of Bovine Serum Albumin from Cow’s Milk and Whey Protein Isolates

by

Patricia Z. Marincic, Doctor of Philosophy
Utah State University, 1997

Major Professor: Dr. Deloy Hendricks
Department: Nutrition and Food Science

Early introduction of cow’s-milk-based infant formulas, in particular the ABBOS epitope of bovine serum albumin (BSA), has been implicated as an autoimmune trigger in the pathogenesis of insulin dependent diabetes mellitus (IDDM). A direct enzyme-linked immunosorbant assay (ELISA), using polyclonal anti-BSA antibodies, was developed to determine the BSA content of cow’s milk and 15 infant formulas. Powdered high-whey (60%) formulas averaged 41 mg BSA/100 ml; 2% milk contained 52 mg BSA/100 ml; and the high-casein formulas averaged 13 mg/100 ml. BSA content of powdered polymeric formulas and cow’s milk varied directly with the whey protein concentration (correlation coefficient = 0.8445, p = 0.008). BSA was not detected in any hydrolyzed powdered formula or commercially sterile liquid preparation regardless of protein composition. The absence of BSA was confirmed by polyacrylamide gel electrophoresis. It is unlikely that the ABBOS epitope is present in the formulas testing negative for BSA due to enzymatic hydrolysis and heat denaturation of these formula preparations.

A laboratory technology was developed that could be upgraded to produce BSA free protein bases used in the manufacture of infant formula. Affinity chromatography,
using paramagnetic beads with an immobilized antibody against BSA, was applied to extract BSA from cow's milk and whey isolates. Monoclonal and polyclonal antibody-activated beads were used to capture BSA from samples. The capture efficiency in milk was 11% and 19% for polyclonal beads, and 59% for monoclonal beads. Capture efficiency of monoclonal beads of 91% was significantly greater in both acid and sweet whey compared to the polyclonal beads exhibiting a capture efficiency 31% and 24% in acid and sweet whey, respectively. Capture efficiency of monoclonal and polyclonal beads did not differ significantly in milk, acid whey, or sweet whey. Removal of BSA from a known sample of 25ng of BSA treated with polyclonal beads was 70% effective with a capture efficiency of 35%. A net reduction of 99.9% of the BSA could be expected by coupling immunocapture with molecular sieving. Immunocapture was most effective in removing BSA when only small amounts were present in the sample.
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Patricia Z. Marincic
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Insulin dependent diabetes mellitus (IDDM) is a chronic autoimmune disease that results in destruction of the insulin-producing beta-cells of the islets of Langerhans of the pancreas in genetically susceptible individuals. While 25% of the population carries genetic markers of IDDM, only 5% of those with genetic predisposition will develop IDDM (American Academy of Pediatrics, 1994). Since genetic predisposition alone does not confer progression to clinical disease, recent research has focused on identifying specific environmental determinants that may also be involved in the immunopathogenesis of IDDM.

The environmental contribution of infant feeding practices was brought to the forefront of diabetes research by Borsch-Johnson and colleagues (1984), who reported epidemiological evidence that a decrease in the incidence of breast-feeding in Scandinavia since World War II has been paralleled by an increase in the incidence of IDDM. Infants who are not breast fed or who receive supplemental breast milk substitutes are exposed to cow's-milk-based infant formula and other potential dietary antigens at an early age. Risk of IDDM has been shown to be two-fold higher in infants who receive dairy products prior to 2 months of age (Virtanen et al., 1993) and proportional to the total amount of cow's milk protein in the diet (Cerrato, 1993). These observations have led to the current hypothesis that cow's milk may precipitate IDDM (Drash, 1990; Gerstein, 1994; Karjalainen, Martin, et al., 1992; Kostraba et al., 1993; Virtanen et al., 1992).

The whey protein, bovine serum albumin (BSA), in particular, the ABBOS epitope (pre-BSA position 153-169), is thought to cross react with a protein on the surface of the beta-cell and trigger an autoimmune response that progressively leads to IDDM (Cheung, Karjalainen, Vandermeulen, Singal, & Dosch, 1994). Exposure of infants to cow's milk
and infant formula, prior to "gut closure," which occurs between 3 and 12 months of age (McCarren, 1992), may be a contributing factor in the pathogenesis of IDDM. The amount of BSA required to elicit the autoimmune response has not been established.

Cow's-milk-based infant formulas vary in their casein-to-whey ratio, degree of thermal processing, and extent of enzymatic hydrolysis. Each of these factors is likely to affect total BSA content and residual antigenicity. Epidemiological studies, to date, which have attempted to establish the impact of infant feeding practices and early exposure to cow's-milk-proteins (e.g., BSA) in the pathogenesis of IDDM, have failed to consider the potential heterogeneity of infant formula. Reports in the literature of the BSA content of cow's milk vary by four-fold (Robin, Turgeon, & Paquin, 1993; Swaisgood, 1982; Whitney, 1988). Although BSA has been detected in a number of infant formulas, the quantitative BSA content of commercial infant formulas has not been well defined. There is no method that has been employed for consistent and sensitive quantitation of BSA in cow's milk and infant formula. The relative contribution of BSA to the infant's diet from cow's milk and cow's-milk-based infant formulas is unknown.

The described research was undertaken to provide a comprehensive quantitative assessment of BSA content of cow's milk and cow's-milk-based infant formulas. Both direct and indirect enzyme linked immunosorbant assays (ELISA) were evaluated as a method for quantitating BSA. A sensitive, direct ELISA was developed to specifically detect and quantitate the amount of BSA present in cow's milk and infant formula preparations. The information obtained provides a consistent, relative quantitative measure of the contribution of cow's milk and cow's-milk-based infant formula to the BSA antigen load in the diet of infants.

The objective of the second component of the described work was to produce a BSA-free polymeric protein base. Current technology to decrease antigen load in infant formulas involves extensive enzymatic hydrolysis. This affects all proteins in the
preparation, is costly, and decreases nutritional quality and palatability. Of the major milk proteins, BSA has been shown to be the most resistant to enzymatic hydrolysis by trypsin and other proteases (Jost, Monte, & Pahud, 1987). An immunomagnetic capture system was developed and utilized to selectively extract BSA from cow's milk and whey protein isolates that are used in the manufacture of commercial infant formula.

Assessment of the BSA content of cow's milk and commercial infant formula preparations will provide critical information to assist professionals and public health agencies in making sound nutritional recommendations to help reduce the incidence of IDDM in those infants not exclusively breast-fed for the first year of life. The American Academy of Pediatrics (1994) "strongly encourages" families with a history of IDDM to adopt the practice of breast-feeding and avoid cow's milk and products containing intact cow's-milk-protein during the first year of life. Since routine screening to detect the "diabetic genes" is not practical at this time as a component of public health policy, it may be appropriate for all infants to avoid BSA. Development of technology to commercially produce an affordable BSA-free breast-milk substitute for use in feeding all infants not exclusively breast-fed may prophylactically help to reduce the incidence of IDDM. The results of the research reported within this dissertation provide vital information that infant formula is not heterogeneous in terms of its BSA content. This information can be used to further evaluate infant feeding practices and the contribution of BSA to the immunopathogenesis of IDDM.
Insulin dependent diabetes mellitus (IDDM), Type I, or Type 1 has historically been referred to as juvenile diabetes since it manifests itself in early childhood and peaks in adolescence. Research over the past 20 years has clearly demonstrated that IDDM is an autoimmune disorder with a genetic bias (Alberti, 1993). There is mounting evidence to suggest that a number of environmental factors influence the expression and progression of the autoimmune disease process that ultimately results in pancreatic beta-cell failure and insulin deficiency.

The following literature review provides a summary of the scope and incidence of IDDM. The major genetic determinants and autoimmune abnormalities associated with IDDM are addressed. An overview of the principal environmental influences thought to contribute to the etiology of IDDM are discussed with emphasis on infant feeding practices. A critical, in-depth review of the literature is provided on the potential protective effects of breast-feeding and the role of exogenous cow's milk proteins, including bovine serum albumin (BSA), in the immunopathogenesis of IDDM. Current recommendations for infant feeding practices will be evaluated in light of the composition of commercially available infant formulas and their potential contribution to the risk of IDDM.

OVERVIEW OF INSULIN DEPENDENT DIABETES MELLITUS

Incidence

In Northern Europe and the United States, IDDM is one of the most prevalent and costly severe chronic diseases of childhood (LaPorte, Matsushima, & Chang, 1995; Libman, Songer, & LaPorte, 1993; Rewers, LaPorte, King, & Tupmilehto, 1988).
The annual incidence of IDDM in the United States is 12 to 14 cases per 100,000 (Atkinson & MacLaren, 1994). Approximately 30,000 new cases of IDDM are diagnosed annually in the United States (LaPorte et al., 1995). Of the total number of new cases, it is estimated that over 13,171 individuals, age 0 to 19, will develop diabetes each year in the United States and that 123,032 currently have the disease (Libman et al., 1993). When considering cases of IDDM in both children age 0 to 19 and those either diagnosed in or progressing to adulthood, it is estimated that over 1.4 million Americans suffer from IDDM (Cerrato, 1993).

The age-specific incidence of IDDM is similar worldwide. The disease is rare prior to 9 months, has a peak incidence between 5 and 15 years of age, and declines thereafter (David, Leslie, & Elliott, 1994). Approximately 20 to 25% of new cases of IDDM are diagnosed in children less than 5 years old (Thai & Eisenbarth, 1993; Tuomilehto et al., 1995). The incidence of IDDM becomes two-fold and three-fold higher in the 5- to 9-year and 10- to 14-year age brackets, respectively, than in the 0 to 4 age group (Rewers et al., 1988). Incidence peaks are notable in both sexes during puberty (Dahlquist et al., 1985).

Minor differences in the incidence of IDDM by gender have been reported in several studies. Overall a slight male excess in disease incidence has been seen in Europe, which was particularly apparent in the areas with the highest incidence of disease. In contrast, a female excess has been seen in areas of low disease incidence (Green, Gale, & Patterson, 1992). Similarly, a slight excess of boys with IDDM was reported in Australia (Glatthaar et al., 1988). There appears to be a tendency for increased prevalence in males in countries of high risk and females in non-Caucasian populations of low risk (Rewers et al., 1988). The significance of these observations has not been established.

A 50-fold geographical variation exists between high risk countries such as Finland and low risk areas of China and Japan (LaPorte et al., 1995). The international variability in the incidence of IDDM is shown in Figure 1. A high incidence of IDDM is found in
individuals of Northern European descent, with the greatest frequency of occurrence reported in Finland of 40 cases per 100,000 diagnosed each year (Atkinson & MacLaren, 1994). While not as high as Finland, the Swedes report an incidence of 23.6 cases per 100,000, which shows an increasing incidence similar to that reported in other European countries (Dahlquist et al., 1985). A high incidence of IDDM has also been observed in the Liguria and Sardinia regions of Italy (Mazzella et al., 1994). The incidence of IDDM is as low as 1 to 2 cases per 100,000 in Japan (Atkinson & MacLaren, 1994; Rewers et al., 1988). Most active registries from Asian countries including China, Japan, and Korea report low incidence rates of less than 3 cases per 100,000 (LaPorte et al., 1995). A similar pattern is reported in Central and South America including Cuba, Chile, Mexico,
and Peru; full ascertainment from diabetes registries is questioned (LaPorte et al., 1995). The geographic influence does not appear to simply reflect a north-south gradient previously reported in Europe and throughout the world (Mazzella et al., 1994).

Racial and genetic influences are apparent. Diabetes has not been detected in the Aborigine population of Australia despite a prevalence in the Australian population of 12.3 per 100,000 (Glatthaar et al., 1988). Within the United States large racial and ethnic variation in IDDM incidence exists. An incidence of 3.3 per 100,000 is reported in the San Diego registry for African Americans compared to 20.6 per 100,000 for Whites in the Rochester, Minnesota registry. The incidence is higher in Hispanics of Puerto Rican descent than Mexican Americans. The incidence of IDDM in Mexico, itself, is 0.4 per 100,000 (Rewers et al., 1988). Approximately 40% of the variation in incidence of IDDM in the United States can be attributed to ethnic diversity (LaPorte et al., 1995).

Reports of a rising incidence of IDDM in the United States and Europe coupled to rapid rises during selective years in isolated areas pose some concern over an epidemic of IDDM (LaPorte et al., 1995; Libman et al., 1993). The incidence of IDDM has increased 50% over the past 20 years in Northern Europe (Schober, Schneider, Waldhor, & Tuomilehto, 1995). There has been a significant and consistent rise in IDDM since records have been available beginning in 1953. The incidence of IDDM in Finland has risen consistently since World War II, with the highest incidence reported in 1986. Between 1965 and 1992 the incidence of IDDM rose by 94% with an average annual increase of 2.8%. Of note, yearly fluctuations suggest epidemic peaks (Tuomilehto et al., 1995). The pattern of IDDM in Finland, Poland, and Italy points to a temporal variation consistent with a pandemic of IDDM resembling an infectious disease (Rewers et al., 1988). A recent study in Austria showed a pattern of rising incidence of the disease similar to that of the Northern European countries (Schober & Schneider, 1995).
The trend toward a rising incidence of IDDM could be attributed to improved diabetic registries (MacLaren & Atkinson, 1992). Others have suggested that a genetic bias exists that favors transmission of human leukocyte antigen (HLA) haplotypes that are diabetogenic. This genetic bias results in an increase in the pool of genetically susceptible individuals. Factors that could impact the size of the genetic pool include good survival of young-onset IDDM, an increased number of offspring of parents with IDDM, decreased perinatal mortality in diabetic pregnancies, and an increased number of woman with IDDM bearing children (Tuomilehto et al., 1995). In addition, an array of environmental determinants is currently being explored for their potential contribution to the etiology of IDDM given both the rising incidence and reports of disease peaks that are suggestive of an epidemic.

Pathophysiology

IDDM results from the autoimmune destruction of the insulin-producing beta-cells of the islets of Langerhans of the pancreas. It is generally accepted that some combination of genetic and environmental factors trigger an autoimmune sequence of events that ultimately lead to pancreatic beta-cell failure. The destructive autoimmune attack involves both cellular (T-cell) and humoral (B-cell) immune responses, cytokine release, and likely free radical accumulation (American Academy of Pediatrics, 1994). Nitric oxide may mediate the final toxic event. Histological abnormalities reveal islets lacking beta-cells, exocrine atrophy, and mild interstitial fibrosis (Atkinson & MacLaren, 1994). Insulinitis is a term used to describe the chronic inflammatory infiltrate of the islet cell. The infiltrate consists of CD8+ and CD4+ cells, B-lymphocytes, macrophages, and natural killer cells (Atkinson & MacLaren, 1994). Patients usually present with overt pronounced symptoms of hyperglycemia and ketosis resulting from a total lack of insulin production. This occurs
late in the pathogenesis of IDDM. The requirement for insulin to sustain life is a hallmark of the disease.

IDDM has a long prediabetic phase in which genetic, environmental, and autoimmune factors work in concert to trigger and sustain a process that results in destruction of the pancreatic beta-cells. Events leading to IDDM suggest a chronic autoimmune process that exists for years prior to onset of clinical disease (Atkinson & MacLaren, 1994). Humoral immune changes, increased fasting pro-insulin levels, decline in insulin secretion, and impaired glucose tolerance have been detected as much as 12 years prior to onset of clinical disease and provides evidence of a long diabetic prodrome (David et al., 1994).

Eisenbarth (1986) has ranked the progression of IDDM in six stages. Stage I is genetic predisposition. A hypothetical triggering event characterizes Stage II, which leads to active autoimmunity (Stage III). Initially, there is normal insulin secretion despite an active autoimmune process. Stage IV is characterized by progressive loss of glucose-stimulated insulin secretion in the presence of normal blood glucose. Gross abnormalities in insulin response to intravenous glucose have been observed prior to the onset of hyperglycemia. These findings are common in the later stages of the diabetic prodrome (Bingley, Bonifacio, & Gale, 1993). Marked loss of first-phase insulin secretion precedes the development of hyperglycemia in all subgroups of patients studied (Bingley et al., 1993; Eisenbarth, 1986; Thai & Eisenbarth, 1993). The selective loss of response to intravenous glucose likely parallels the reduction in beta-cell mass. Stage V is characterized by overt diabetes with hyperglycemia. Hyperglycemia induced by stress and asymptomatic glucosuria are early clinical observations of impaired glucose tolerance in children (Thai & Eisenbarth, 1993). Presence of C-peptide reflects minimal residual insulin secretion. Finally, complete beta-cell failure occurs (Stage VI). Fewer than 10% of beta-cells remain 2 to 4 months after initiation of insulin therapy (Bach, 1994).
Loss of first phase insulin response to intravenous glucose appears to represent the "point of no return" in which progression to clinical IDDM within 3 years is imminent. This change occurs in the last stages of the diabetic prodrome with loss of 80 to 90% of beta-cell function (Atkinson & MacLaren, 1994; Tarn et al., 1988). An inductive event at an early age is associated with more pronounced immunity to beta-cells (Atkinson & MacLaren, 1994). The variable age of disease presentation has been proposed to reflect different rates of disease progression (David et al., 1994).

Metabolically active beta-cells appear to be particularly subject to autoimmune attack and may be involved in the pathogenesis of IDDM. The incidence of IDDM in childhood is low in early infancy and peaks during puberty (Atkinson & MacLaren, 1994). The peak is reported to occur around 11 or 12 years of age. Increased metabolic demands on the beta-cell in puberty may exacerbate the pathogenesis of the disease (Atkinson & MacLaren, 1994). An increased need for insulin occurs during the pubertal growth spurt (Mazzella et al., 1994). It is generally accepted that puberty is associated with decreased insulin sensitivity. The relative insulin resistance is likely due to elevated levels of growth hormone that are known to induce insulin resistance. The resulting increased work load on the beta-cell may accelerate existing beta-cell destruction (Dahlquist, 1994) or increase substrate for autoimmune attack. A growth delay that occurs many months prior to clinical diagnosis has been attributed to subtle changes in glucose tolerance, insulin production, and sex hormone levels (David et al., 1994) and is a common early symptom of clinical disease.

IDDM is sometimes classified into two subgroups, epidemic and non-epidemic. Epidemic IDDM is characterized by seasonal incident peaks with short duration of symptoms. Rapid progress of the disease often follows an acute infection. Non-epidemic disease is spread throughout the year, has a more gradual onset of symptoms, and rarely follows an infection (Samuelsson, Johansson, & Ludvigsson, 1993).
hyperplasia, hirsutism, decreased hematocrit, and decreased creatinine clearance. Less common but more severe side effects of high dose long-term use include lymphoma and irreversible renal damage (Eisenbarth, 1986).

Exogenous insulin therapy has been utilized in an attempt to delay or prevent the onset of clinical IDDM. Prophylactic exogenous insulin has been administered to induce beta-cell "rest" (Scott & Marliss, 1991). Decreasing the metabolic activity of the beta-cells is thought to result in a decrease in the immunogenic target antigens (Atkinson & MacLaren, 1994) making islet cells less susceptible to immunologically mediated destruction or the direct effects of anti-insulin autoimmunity (Eisenbarth et al., 1994). Insulin administration may, therefore, induce tolerance to the beta-cells. In addition, intravenous insulin has been shown to enhance endogenous insulin production and may work by decreasing beta-cell damage caused by hyperglycemia. Insulin prophylaxis may decrease insulinitis, beta-cell damage, and the development of overt diabetes (Scott & Marliss, 1991).

High dose niacin has received attention as another possible mediator of remission to IDDM. High dose nicatinamide may induce remission in newly diagnosed patients with IDDM and delay disease onset in antibody positive non-diabetic children (Alberti, 1993). Nicatinamide has been demonstrated to prevent and cure spontaneous diabetes in non-obese diabetic mice, but not in the spontaneously diabetic biobreed rat (Hermitte, Atlan-Gepner, Payan, Mehelleb, & Viallettes, 1995). The proposed mechanism of action of nicatinamide is that it works in the pancreatic beta-cell by increasing the nicatinamide adenine dinucleotide pool required for poly (ADP-ribosyl) action and inhibiting the action of molecular poly (ADP-ribose) polymerase implicated in DNA repair (Alberti, 1993; Kolb, 1989). Nicatinamide may decrease progression of IDDM in individuals with high islet cell antibody titres and poor first phase insulin response. Nicatinamide may maintain insulin secretion in newly diagnosed diabetics; however, results have been inconsistent (Dorman,
McCarthy, O'Leary, & Koehler, 1995). The beneficial effects of niacin in the prevention of IDDM may not be seen when treatment follows substantial beta-cell loss evidenced by extremely impaired first-phase insulin response (Scott & Marliss, 1991).

Other experimental strategies aimed at delaying or preventing the onset of clinical IDDM have been evaluated in animal models. Administration of the islet cell antigen glutamic acid decarboxylase may increase tolerance to glutamic acid decarboxylase antibodies implicated in the pathogenesis of IDDM. High vitamin E intake and essential fatty acid deficiency have also been shown to decrease the incidence of IDDM in the diabetogenic biobreed rat (Hermitte et al., 1995). This could be related to the role of essential fatty acids in eicosanoid synthesis, inhibition of lipo-oxygenase by vitamin E, and the role of eicosanoids in mediating immune responses. Under preliminary experimental conditions, bacille Calmette-Guerin shows promise for inducing remission by generating regulatory cytokines, which may limit destruction of the islet cells (Atkinson & MacLaren, 1994).

While much of the research in prevention of IDDM has focused on non-diabetic siblings of those with IDDM, familial IDDM accounts for only a small fraction of cases. Sporadic occurrence accounts for 85-90% of cases of newly diagnosed IDDM (Alberti, 1993). In order to detect prediabetes, population screening would be necessary. This would be costly and complicated. Genetic markers are of limited use in predicting those who will develop IDDM. Islet cell antibodies appear to be one of the best long-term predictors of IDDM; however, random population screening would be expected to pick up only 1 diabetic in 4000 individuals screened in the general population (Tarn et al., 1988). Accurate identification of those at risk is needed.

The preventative strategies outlined above would need to be life-long and, at present, often come along too late because the disease has well progressed prior to diagnosis. At diagnosis, typically 90% of the beta-cells have already been destroyed with
few insulin-producing cells left to salvage (Cerrato, 1993). With apparent lack of primary prevention it would be beneficial if the disease process could be arrested, by secondary prevention strategies, when most beta-cells remain functional and normal glucose tolerance prevails (Tarn et al., 1988). Given the long prediabetic period prior to development of clinical symptoms of IDDM, the possibility of identifying individuals at increased risk and implementing prevention strategies before appreciable beta-cell damage occurs looks promising.

Treatment

IDDM is a chronic illness that requires ongoing medical care, nutrition therapy, and patient education to prevent acute decompensation due to diabetic ketoacidosis and reduce risk of chronic disease complications (American Diabetes Association, 1997). Treatment is aimed at lowering blood glucose levels to normal or near normal, improving the lipid profile, and normalizing metabolic status. The Diabetes Control and Complications Trial (DCCT; 1993) demonstrated conclusively that patients with IDDM can reduce risk of development or progression of retinopathy, neuropathy, and nephropathy by 50% to 75% with implementation of intensive treatment regimens. Intensive management includes: (a) aggressive insulin management with pump therapy or multiple daily injections, as needed; (b) nutrition intervention; (c) frequent blood glucose monitoring (e.g., preprandial and bedtime glucose, routine Hgb A1C); (d) regular exercise; and (e) patient education. Intensive management of IDDM is recommended and encouraged for all patients with IDDM. Despite this, the risks of devastating complications remains high (LaPorte et al., 1995).
Complications

In the pre-insulin era, prior to 1921, diabetic coma predominated as the cause of death, and life expectancy was usually less than one year following the onset of clinical symptoms. Insulin injections prevent death, but fail to preclude loss of sight, limbs, and kidney function associated with progressive degenerative microvascular disease and macrovascular complications, which may lead to premature death (Dosch, 1993; Rewers et al., 1988). The majority of deaths in individuals with IDDM now occur in middle and late adulthood (Portuese & Orchard, 1995). In the middle years, renal disease is the predominant cause of death. After 30 years, two thirds of IDDM deaths result from cardiovascular disease, which is further complicated by the presence of renal involvement (Portuese & Orchard, 1995).

Acute complications resulting from elevated peaks and low troughs in blood sugar still challenge the daily management of IDDM for many individuals. Poorly controlled diabetes, characterized by a glycosylated hemoglobin greater than or equal to 11% during the first 4 years of follow-up, was significantly (p < 0.001) more likely to result in development of complications such as microalbuminuria, proliferative retinopathy, distal symmetrical polyneuropathy, nephropathy and renal failure (Lloyd, Becker, Ellis, & Orchard, 1996). The DCCT (1993) has reported marked reduction in early complications with intensive treatment of IDDM. However, intensive treatment is not without risk. Weight gain and severe hypoglycemia are the most commonly reported problematic side effects of aggressive management of IDDM (Lloyd et al., 1996).

Mortality rates for individuals with IDDM remain high. Over 90% will develop disabling complications including heart disease, stroke, kidney failure, gangrene, and blindness (Strand, 1994). Childhood onset IDDM has a mortality rate 20 times higher than seen in age-matched controls (Portuese & Orchard, 1995). Greater than 15% will die...
before age 40 (Portuese & Orchard, 1995). Although greatly improved by insulin and intensive management, the long-term prognosis for individuals with IDDM remains poor.

Cost

In U.S. dollars the average cost to treat diabetes for someone diagnosed at age 7 through age 40 is $50,000. Cost after age 40 increase as complications progress (LaPort et al., 1995; Rewers et al., 1988). It has been estimated that the lifetime cost of an individual contracting IDDM in childhood may reach one million U.S. dollars (Akerblom, Dosch, et al., 1993).

Summary

The high incidence of IDDM in the northern tiers of the world, in particular northern Europe, makes it the number one chronic disease of childhood in these regions. IDDM is an autoimmune disease with a long latent period in which gradual destruction of the pancreatic beta-cells leads to absolute insulin deficiency and hyperglycemia. IDDM has serious implications in terms of life-long need for intensive disease management, debilitating life-threatening complications, and overall cost of the disease to the individual and health-care system. In the absence of primary prevention and only marginally effective secondary prevention, it is clear that further understanding of the complex interactions of genetic predisposition, autoimmunity, and environmental determinants is needed.

GENETIC DETERMINANTS OF INSULIN DEPENDENT DIABETES MELLITUS

Autoimmune destruction of the insulin-producing pancreatic beta-cells appears to occur only in those individuals with specific genetic markers of disease risk. Genetic
predisposition to IDDM has been mapped to 20 different chromosomal regions (Atkinson & MacLaren, 1994). Approximately 25% of the population carries at least one genetic marker of IDDM (Rennie, 1992). However, fewer than 5% of those possessing the identified "diabetes genes" will develop IDDM (Norris & Pietropaolo, 1994). While genetic predisposition appears to be required for development of IDDM, genetic influence alone does not confer overt clinical disease.

Familial Patterns of Disease Expression

The population risk of developing IDDM is 0.4% in the United States (Todd, 1990). Familial risk is considerably higher than the overall population risk. The incidence of IDDM in children is significantly higher when IDDM is present in relatives (i.e., parents, grandparents, parental siblings, cousins, and siblings; \( p < 0.05 \); Blom, Dahlquist, Nystrom, Sandstrom, & Wall, 1989). Risk for first degree relatives varies with the number of shared human leukocyte antigen (HLA) haplotypes with the proband. The reported risk is 10 to 16%, 2 to 9%, and less than 1% for those sharing two, one, and zero haplotypes, respectively (Deschamps, Bevessi, Khalil, Robert, & Hors, 1991; Tarn et al., 1988). Taken together, a sibling is 15 to 20 times more likely to develop IDDM before age 20 than an individual in the background population (Bingley et al., 1993). Ten to 12% of children newly diagnosed with IDDM have a first-degree relative with the disease (father, mother, or sibling; Bruining et al., 1989).

The incidence of IDDM is three-fold higher in offspring of fathers with IDDM than affected mothers (Warren, Krolewski, Gottlieb, & Kahn, 1984). The risk to offspring of a diabetic father developing IDDM is 4 to 6% compared to 2 to 3% for offspring of a diabetic mother. Age of diagnosis of clinical disease is correlated with the age of diagnosis of a diabetic father or sibling \( (p < 0.005) \). These observations were not seen in offspring of mothers with IDDM (Metcalfe & Baum, 1992).
Although IDDM is not inherited, the increased familial incidence of the disease clearly reflects a genetic bias. Shared environment risk for North American Caucasians ranges from 1 to 15% for first-degree relatives compared to less than 1% of individuals without relatives with IDDM (Dorman et al., 1995). Despite familial association, the vast majority of patients do not have a family member with IDDM and present with what has been referred to as "sporadic" IDDM (Bruining et al., 1989). These individuals do, however, possess an array of genetic markers consistent with predisposition to IDDM.

**Genetic Markers of Disease Risk**

The genetic predominance associated with a predisposition to IDDM has been mapped to the major histocompatibility complex (MHC) on chromosome 6 (Deschamps et al., 1991; Dosch, Karjalainen, Morkowski, Martin, & Robinson, 1992; Thai & Eisenbarth, 1993). MHC class molecules are located in the region of genes coding for highly polymorphic immune-system-recognition molecules termed human leukocyte antigen (HLA; Atkinson & MacLaren, 1994). These gene regions function to regulate immune recognition and response (Dosch et al., 1992).

MHC class I genes (HLA - A, B, and C) encode $\alpha$-chains, which combine with $\beta$-2-microglobulin to form transmembrane molecules (antigens). These are expressed on the surface of most nucleated cells and are responsible for cytotoxic T-cell activity (Deschamps et al., 1991). HLA class I molecules direct antigenic peptides to CD8$^+$ (cytotoxic and suppressor) T-lymphocytes (Atkinson & MacLaren, 1994).

Class II molecules are primarily expressed on cells of the immune system such as B-lymphocytes, activated T-cells, macrophages, monocytes, dendritic cells, and thymic cortical epithelium (Scott, 1994). Class II molecules present antigens to the T-cell receptor on T-helper cells (Bach, 1994; Lernmark, 1994). MHC class II molecules encode
complement components (Bf, C2, C4A, and C4B), 21-hydroxylase, tumor necrosis factors, and heat shock proteins (Deschamps et al., 1991). MHC class II molecules control CD4+ helper T-lymphocytes, which have been strongly implicated in the pathogenesis of IDDM (Todd, 1990). Foreign antigens are processed by these cells, bound to class II molecules. HLA class II proteins, HLA-DP, DQ, and DR, present antigenic peptides to CD4+ (helper and inducer) T-lymphocytes (Atkinson & MacLaren, 1994). Recognition of the HLA-peptide complex produces signals leading to the activation of T-helper cells and initiation of the immune response (Deschamps et al., 1991).

The peptide-binding groove of class II molecules is influenced by amino acid side-chains located within the groove. These amino acid side-chains likely contribute to the properties of class II molecules (Lernmark, 1994). The characteristics of the binding groove direct the ability of class II molecules to bind antigenic peptides. The ability to accept or reject an antigen may have significant implications in the susceptibility to IDDM and the contribution of specific exogenous antigens to disease pathogenesis.

Specific subclasses of HLA-DQ and HLA-DR class II proteins exhibit characteristics associated with increased risk or confer protection from IDDM. IDDM has been strongly associated with the class II alleles DR3 and DR4 (Alberti, 1993). DR3 or DR4 is present in approximately 90% of patients with IDDM compared to 40% of the non-diabetic population (Todd, 1990). DR3 positive individuals show slower disease progression in the presence of islet cell antibodies (Deschamps et al., 1991). DR4 alleles are associated with increased insulin autoantibodies (Thai & Eisenbarth, 1993). Susceptibility appears stronger in the D4 subregion compared to D3 (Deschamps et al., 1991). Progression to IDDM has been reported to be 3 to 4% with HLA-DR3 or DR4 alone and 16% for HLA DR3/DR4 heterozygotes. The presence of islet cell antibodies in the latter group has been associated with a 70% progression rate to overt clinical disease within 5 years. Those with both DR3 and DR4 haplotypes present with more severe disease and
Individuals homozygous for both DQB1*non-Asp-57 and DQA1*Arg-52 alleles have a high risk of IDDM, which can explain 62% of the incidence of IDDM in high-risk individuals (Deschamps et al., 1991; Dorman et al., 1995). The presence of aspartate at position 57 decreases susceptibility but does not appear to confer absolute protection (Todd, 1990). These findings point to a specific molecular basis of the genetic predisposition to IDDM.

Non-MHC genetic determinants have also been linked to IDDM. The insulin gene is associated with IDDM, particularly in HLA DR4 positive subjects (Bach, 1994; Todd, 1990). Susceptibility to IDDM has also been associated with genes coding for the transporter involved in antigen presentation (TAP), the TAP peptide transporter. In a recent review, TAP transport was reported to be defective in patients with IDDM (Atkinson & MacLaren, 1994). There are at least 10 other non-MHC predisposition loci (Bach, 1994). These other genes may be involved by coding for immunoregulatory cells such as cytokine genes and genes controlling beta-cell sensitivity to immune recognition (Bach, 1994).

Genetic Markers Conferring Disease Protection

In addition to the presence of aspartate at position 57 of the DQB chain, other MHC determinants are thought to provide disease resistance. Resistance to IDDM appears to be conferred by certain alleles of DQA1 and DQB1 (Todd, 1990). On the DQA1 locus, replacement of the usual A2 allele with the A3 allele has been observed in diabetics. These findings suggest that the A2 allele confers resistance to IDDM (Todd, 1990). Additionally, the DQB1*0602 haplotype has been reported to provide strong protection from diabetes in both familial and population based studies (Thai & Eisenbarth, 1993). DQ6, formed by the α-chain DQB1*0602 and the α-chain of DQA1*0102, appears to provide dominant protection. These determinants are present in 30% of the Swedish population despite an
otherwise high risk of IDDM (Lernmark, 1994). DQB1*0301 (DQ7) also appears to afford protection (Lernmark, 1994). DQ haplotypes may be primarily protective.

The DR2 (DRw15) haplotypes are inversely associated with IDDM in all races (Deschamps et al., 1991). This suggests that the DR2 (DRw15) haplotype provides disease resistance (Todd, 1990). Protection against IDDM appears to be provided by DR2 and the TAP2*0201 allele which codes for a transporter of antigenic peptides to MHC class I molecules (Bach, 1994). Defective peptide transport and capture of peptides by protective HLA molecules could prevent binding to predisposing HLA molecules, effective presentation to T-cells, and immunological tolerance (Bach, 1994). Lack of DR2 protection would leave one subject to autoimmunity triggered by an environmental antigen.

Use of Genetic Markers to Predict Risk of Insulin Dependent Diabetes Mellitus

Genetic resolution of MHC-IDDM association is complicated by disease heterogeneity in terms of age of onset and ethnic variables (Bach, 1994). Several genes determine predisposition to IDDM; however, nongenetic factors contribute equally to disease development (Deschamps et al., 1991). The positive predictive value of HLA genotyping is low and would provide a number of false positives if used to screen population groups (Lernmark, 1994). While HLA DR3 and DR4 are present in more than 90% of European patients with IDDM, they have a poor predictive value given that 40 to 55% of the background population share these markers (Tarn et al., 1988). Genetic population screening for IDDM risk is considered to be impractical, if not impossible. Genetic screening would pick up the 6% of the population heterozygous for HLA DR3 and DR4. Based on the current incidence of IDDM, 97.5% of this 6% would never develop overt clinical disease (Tuomilehto & Wolf, 1987). Additional determinants of risk and progression to clinical IDDM are needed.
AUTOIMMUNE PATHOGENESIS OF INSULIN DEPENDENT DIABETES MELLITUS

A long, latent period of subclinical autoimmunity exists in which selective destruction of the insulin producing pancreatic beta-cells precedes the development of IDDM (Norris et al., 1996). It is generally accepted that the initiation of autoimmunity begins in early childhood or infancy given that approximately 20 to 25% of cases of IDDM are diagnosed prior to age 5 (Thai & Eisenbarth, 1993; Toumialhehto et al., 1995). Sera conversion to beta-cell autoimmunity after age 5 is rare (Norris et al., 1996). It is now widely accepted that immunological and metabolic alterations occur long before symptomatic hyperglycemia (Thai & Eisenbarth, 1993). The autoimmune processes leading to beta-cell destruction are triggered by poorly understood factors. These immunological abnormalities occur in genetically predisposed individuals of whom only a small fraction will progress to clinical disease (Bruining et al., 1989). Environmental factors are likely to play a role in triggering and sustaining the complex autoimmune response, which eventually leads to beta-cell failure and insulin deficiency.

Tolerance

The main function of the immune system is to protect the host from foreign pathogens. In this capacity, the ability to discriminate self from nonself is critical (Cordle, 1994). An important feature of the normal healthy immune system is that B-cells and T-cells are physiologically tolerant to most self-antigens so that there is no pathological autoimmune response (Bach, 1994). The autoimmune pathogenesis of IDDM may evolve from acquired processes that overcome anergy or suppression resulting in IDDM (Rossini, Greiner, Friedman, & Mordes, 1993). The breakdown of self-tolerance that characterizes autoimmune diseases may occur through three major mechanisms: insufficient intrathymic
negative selection, bypass of peripheral anergy, or defective suppression (Bach, 1994). These three critical processes likely work in concert to prevent autoimmunity (Rossini et al., 1993). Breakdown of T-cell anergy could occur by deviant expression of MHC molecules and molecular mimicry (Bach, 1994). Defective suppressor T-cell function, which may be related to an imbalance in TH1/TH2, likely allows amplification of the autoimmune response that eventually destroys the beta-cells (Bach, 1994). The exact mechanisms underlying the autoimmune pathogenesis of IDDM are still unknown but likely involve loss of self-tolerance to islet antigens (Bach, 1994). Environmental insults may interfere with the regulatory balance and preclude self-tolerance.

**Immune System Abnormalities**

IDDM is associated with HLA genes known to be expressed in individuals with most other autoimmune diseases. Patients with IDDM suffer from other autoimmune diseases (e.g., rheumatoid arthritis, myasthenia gravis, pernicious anemia, and thyroiditis; Rossini et al., 1993). In addition, mononuclear cell infiltration of target tissues involved in disease pathogenesis is a common association of IDDM with other autoimmune diseases, most notably thyroiditis (Bach, 1994).

The immunopathogenesis of IDDM is characterized by infiltration of the islets of Langerhans by mononuclear cells and is termed insulinitis (Bach, 1994). Insulinitis is an inflammatory infiltrate which is confined to the islets. These infiltrates contain lymphocytes that express either CD8+ (suppressor/inducer) or CD4+ (helper/inducer) surface markers and MHC class II antigens. Increased expression of MHC class I molecules suggests lymphokine activity (e.g., gamma-interferon) by infiltrating cells (Rossini et al., 1993). IDDM is characterized by predominantly CD4+ polyclonal insulinitis (Miyazaki et al., 1995). Macrophages are seen in early pancreatic infiltrates. These are an important source of cytokines, which have been detected in inflamed islet
cells. Cytokines may mediate pancreatic beta-cell destruction and lysis (Todd, 1990). In the non-obese diabetic (NOD) mouse, insulinitis has been shown to be present well before the presentation of clinical diabetes. Insulinitis was seen in 50% of the mice at 40 days and 70% of the mice at 90 days. In humans, circulating monocytes are activated in newly diagnosed diabetics, both peripherally and at the local site of inflammation in the pancreas. Monocyte proliferation appears to reflect ongoing pathogenic immunological destruction of the pancreas (Josefson, Nielsen, Lorentzen, Damsbo, & Buschard, 1994). Insulinitis appears to be required for progression to frank clinical diabetes, but insufficient by itself to produce overt disease (Miyazaki et al., 1995).

The destruction of beta-cells is mediated by T-lymphocytes (Dotta, Anastasi, Tiberti, & Di Mario, 1994). T-cells are predominantly, if not exclusively, involved in creating islet cell lesions that lead to beta-cell atrophy. This appears to follow a stage of reversible inflammation (Bach, 1994). T-lymphocytes are required for initiation of both cell mediated and humoral immune responses. The presence of both CD4+ and CD8+ T-cells has been demonstrated to be necessary for beta-cell destruction (Todd, 1990). T-lymphocytes bind and react to only those antigens that associate with its autologous, specific MHC molecules (Dosch et al., 1992). In addition, increased circulating B-lymphocytes (CD5+) are seen in IDDM and are characteristic of other autoimmune diseases (Atkinson & MacLaren, 1994; Bach, 1994). The events that trigger the activation of auto-reactive lymphocytes in IDDM remain elusive (Krokowski et al., 1995).

Other nonspecific abnormalities in the immune system of individuals with IDDM include increased levels of T-cells expressing the interleukin-2 receptor (Rossini et al., 1993), lymphocytopenia, increased natural killer cell activity, and thymic abnormalities (Bach, 1994). Interleukin-1 inhibits insulin secretion and is cytotoxic at high concentrations (Atkinson & MacLaren, 1994). Interleukin-1B has been identified as a major beta-cell cytotoxic effector molecule (Fourth International Onnela Workshop, 1990).
Interleukin-6 is a powerful stimulator of the immune response and may contribute to both beta-cell destruction and insulinitis (Atkinson & MacLaren, 1994). Production of alpha-interferon is induced by viruses. Alpha-interferon stimulates the expression of HLA molecules and is chemotaxic to other lymphocytes. Patients dying of ketoacidosis exhibit high levels of alpha-interferon (Atkinson & MacLaren, 1994). Toxic free oxygen radicals (e.g., superoxide) and nitric oxide, produced during the abnormal autoimmune response, may be the final mediators of the cytotoxic destruction of the insulin producing pancreatic beta-cells (Atkinson & MacLaren, 1994; Fourth International Onnela Workshop, 1990).

**Autoantibodies**

Autoantibodies are found against a wide array of membrane and cytoplasmic constituents of beta-cells including insulin, proinsulin, and glutamic acid decarboxylase (GAD; Alberti, 1993; Bach, 1994). Other potential autoantigens include peripherin (a neuron cytoskeleton molecule) carboxypeptidase H, heat shock protein 65, 37kD protein, 38kD protein, 52kD antigen, the polar antigen, GT3 ganglioside, and other islet cell antibody-reactive gangliosides (GM2-1; Bach, 1994; Dotta et al., 1994). Autoantibodies have been detected against each of the following antigens in patients with IDDM: pancreatic sialoglycoconjugate, insulin, glutamic acid decarboxylase, bovine serum albumin, carboxypeptidase H, pancreatic sulfatide, lymphocyte surface, pancreatic cytokeratin, thymic hormone, HLA-DQ molecules, beta-cell glucose transporter, and a number of islet cell proteins (37- or 40kD, 38-kD, 52-kD, and 69kD proteins; Atkinson & MacLaren, 1994; Rossini et al., 1993). Monocytes taken from patients with IDDM show proliferation upon exposure to most of these antigens (Atkinson & MacLaren, 1994). Cellular staining of islet cell antibodies and insulin autoantibodies suggest multiple autoantigens (Reddy, Bibby, & Elliott, 1987). Islet cell antigens may be directly involved in the pathogenesis of IDDM or may function as targets for antibodies involved in
immunity to beta-cells and, therefore, do not directly contribute to disease development (Atkinson & MacLaren, 1994; Dotta et al., 1994). Of note, insulin autoantibodies and islet cell antibodies are absent in 15 to 20% of patients with recent onset diabetes (Bach, 1994).

**Insulin Autoantibodies**

To date, insulin is the only established beta-cell specific autoantigen in IDDM (Dotta et al., 1994). Insulin autoantibodies demonstrate both islet cell and beta-cell specific autoimmunity. Elevated insulin autoantibodies have been shown to be positively associated with DR4 genes and some DQA1 alleles. These observations clearly reflect the role of genetic influence in diabetic autoimmunity. Antibodies to insulin have been detected prior to other autoantibodies in prediabetes and have also been shown to correlate with age of onset of clinical disease (Eisenbarth et al., 1994). The highest frequency of insulin autoantibodies (100%) has been observed in children diagnosed before the age of 5. High titres of insulin autoantibodies may reflect the virulence of the autoimmune process and concordant level of beta-cell destruction (Thai & Eisenbarth, 1993). Insulin autoantibodies have been reported to be present in 50% of newly diagnosed cases of IDDM and are a highly predictive marker of disease risk (Atkinson & MacLaren, 1994). However, insulin autoantibodies appear to have little prognostic significance in the absence of islet cell antibodies (Bingley et al., 1993). Where both insulin and islet cell cytoplasmic autoantibodies are detected, there is a high risk of progression to clinical IDDM (Atkinson & MacLaren, 1994; Thai & Eisenbarth, 1993).

**Islet Cell Antibodies**

The presence of islet cell antibodies (ICAs) in newly diagnosed diabetics, which are absent or are detected in very low titres in the reference population, is a distinguishing characteristic of IDDM (Lipton & LaPorte, 1989). Islet cell antibodies bind primarily to the
cytoplasm of beta-cells, but can bind to the cytoplasm of other islet endocrine cells (Bach, 1994). Islet cell antibodies may help to identify molecules that can be a target of T-cells. T-cell autoantigens may have a pathogenic role in IDDM caused by a cell-mediated autoimmune response (Dotta et al., 1994). The presence of islet cell antibodies has been used as a marker of beta-cell damage and time course to progression to overt clinical IDDM. Impaired first phase insulin response to intravenous glucose has been observed around the time of presentation of detectable islet cell antibodies (Tuomilehto & Wolf, 1987). Islet cell surface antibodies may affect glucose stimulated insulin release (Lernmark, 1994). It is not clear whether islet cell antibodies play a direct role in disease process or serve as markers of tissue damage initiated by other etiological factors (Dorman et al., 1995).

The presence of islet cell antibodies prior to overt clinical symptoms of IDDM supports the long diabetic prodrome of disease pathogenesis. In the non-obese diabetic mouse, islet cell antibodies were detected well before the presentation of clinical diabetes. Islet cell antibodies were present in 40% at day 40 and 54% at day 90. The concordance rate was 50% at 90 days (Reddy et al., 1987). Islet-cell cytoplasmic autoantibodies have been reported to be detected at high levels in 70 to 80% of newly diagnosed patients with IDDM, 3 to 4% in non-diabetic relatives, and only 0.5% in the general population (Atkinson & MacLaren, 1994). Islet cell antibodies have been detected in non-diabetic siblings of those suffering from IDDM many years prior to the development of clinical symptoms in the sibling of the diabetic proband (Alberti, 1993). In a cohort of 4,015 non-diabetic relatives, 67.5% of those with positive islet cell antibody titres progressed to IDDM within 10 years (Riley et al., 1990). Similarly, Thai and Eisenbarth (1993) reported that greater than 50% of relatives exhibiting high islet cell antibody titres developed symptoms of clinical disease within 5 years. In a 10-year follow-up study of islet cell antibodies and development of IDDM in childhood, 4 of 8 subjects with islet cell antibodies progressed to overt clinical diabetes. Of the 4,806 children ages 5 to 19 who
tested negative for islet cell antibodies, 99.9% did not develop IDDM over the 10-year study period (Bruining et al., 1989).

The occurrence of islet cell antibodies in the general population of children is restricted to those who are genetically susceptible to IDDM (Tuomilehto & Wolf, 1987). High antibody titres to islet cells increase the risk of developing diabetes to 50%. Presence of two or more antibodies confirm a 90% risk of progression to clinical diabetes (Alberti, 1993). Complement fixing or high titre islet cell antibodies have been cited as predictable markers of impending IDDM. Fifty-four percent of subjects positive for complement fixing islet cell antibodies on three or more occasions were noted to develop frank IDDM. Approximately 76% of those positive for complement fixing islet cell antibodies can be expected to eventually develop IDDM (Tarn et al., 1988).

The highest antibody titres are seen at the onset of clinical IDDM. In fact, these antibodies occur years before the onset of clinical diagnosis. Following disease establishment, islet cell antibodies drop markedly (Lipton & LaPorte, 1989). Islet cell antibodies have been shown to be present in approximately 80% of patients newly diagnosed with IDDM, but were observed to show a progressive decline in the time leading up to diagnosis (Tarn et al., 1988). These may fall below the level of detection or below the baseline of nondiabetic controls within 2 to 10 years of diagnosis in a large percentage of patients (Lipton & LaPorte, 1989). Once IDDM is established, consistent with marked beta-cell destruction, islet cell antibody levels decrease as no antigen remains to support the immune response.

Use of Insulin Autoantibodies and Islet Cell Antibodies As Predictive Markers of Insulin Dependent Diabetes Mellitus

Islet-cell cytoplasmic autoantibodies and insulin autoantibodies have been used as metabolic markers to predict susceptibility to IDDM and pathogenesis of the disease.
Presence of islet cell antibodies are a potent indicator of risk for IDDM, at least in high risk individuals (Bingley et al., 1993; Lipton & LaPorte, 1989). Most cases of IDDM will develop in the islet cell antibody positive subgroup of first-degree relatives. It is likely that 60 to 70% of the islet cell antibody positive group will require insulin within 10 years with risk directly proportional to antibody titre (Bingley et al., 1993). However, it is not inevitable that the remainder will develop clinical disease (Bingley et al., 1993; Lipton & LaPorte, 1989). Islet cell antibodies alone have limited prognostic value in the general population. While it can be expected that 2.8% of school-age children will be positive for islet cell antibodies, progression to diabetes within 5 years would be expected to be seen in only 2 to 3% of the 2.8% positive for islet cell antibodies (Bingley et al., 1993). In islet cell antibody positive relatives, the presence of both elevated titres of insulin autoantibodies and loss of first phase insulin response is strongly predictive of rapid progression to clinical IDDM. Progression is seen within 3 to 4 years in 90% of these individuals (Bingley et al., 1993). In relatives positive for insulin autoantibodies, risk of developing IDDM becomes much higher in those also positive for islet cell antibodies (63% vs. 9%). With insulin autoantibody titres greater than 150nU/ml, 100% of relatives of a diabetic proband have been shown to advance to IDDM within 5 years (Dotta et al., 1994). Taken together, islet cell antibodies and insulin autoantibodies appear to be the best predictive markers of IDDM in high risk relatives (Bach, 1994).

While there is clearly an interrelationship between genetics, autoimmunity, and IDDM, only about 10% of the population have close relatives with IDDM. Lack of inexpensive, specific, and sensitive markers of IDDM is a limitation to early identification of all individuals at risk. To date, MHC typing is less powerful as an IDDM predictor than the presence of islet cell antibodies, complement fixing autoantibodies, insulin autoantibodies, or loss of first-phase insulin response (Scott & Marliss, 1991).
Immunological markers of IDDM, in combination with genetic screening, are currently limited to relatives of probands and to research settings. These markers can be utilized to detect individuals with early autoimmune lesions of IDDM and target evaluation of secondary preventative strategies.

Interaction Between Genetic Markers of Insulin Dependent Diabetes Mellitus and Autoimmunity

It is well accepted that there is a link between markers of the HLA-region of chromosome 6 and destruction of the pancreatic insulin producing beta-cells associated with IDDM (Wilkin & Armitage, 1986). The hallmark of genetic susceptibility to IDDM resides in an inherent defect in the ability to establish tolerance to beta-cell autoantigens (Atkinson & MacLaren, 1994). MHC class II molecules bind peptide fragments from foreign proteins and present these antigens to CD4+ helper/inducer T-cells. Defects in MHC class I proteins, which normally present fragments of endogenous proteins to the immune system, may result in impaired development and maintenance of normal self-tolerance and lead to diabetic autoimmunity (Rossini et al., 1993).

The ability of the antigen presenting cells (APCs) to bind and present antigens to the immune system is determined by HLA genes (McCarren, 1992). The proposed mechanism by which HLA-DR and DQ impact expression of IDDM is by affecting the degree of immune responsiveness to an autoantigen. Alternatively, these genes may present the antigen in a manner that does not promote normal tolerance (Atkinson & MacLaren, 1994). Interaction between the HLA molecule, the antigenic peptide, and the T-lymphocyte receptor causes lymphocyte proliferation, a process common to all immune responses (Atkinson & MacLaren, 1994).

Antigenic epitopes ranging from 13-25 residues are most likely to bind to MHC class II molecules (Papadopoulos, 1993). It appears that β-chain residue 57, which
occupies an outward facing cleft in the molecule, may be involved with antigen binding (Rossini et al., 1993). In "protective" MHC class II molecules, aspartate holds the number 57 position. In "susceptibility" molecules, arginine holds this position (Papadopoulos, 1993). Conformation and expression of DQ molecules impacts autoantigen binding (Todd, 1990). The variability in the size or shape of the antigen binding sites (cleft) of DQ molecules affects the ability of antigen-presenting cells to bind specific islet-cell autoantigens and establish immunological tolerance (MacLaren & Atkinson, 1992; Todd, 1990).

Genetic derangements that impact immune recognition and responsiveness are clearly involved in the pathogenesis of IDDM. It is generally accepted that genetic predisposition is, in fact, required to yield the abnormal autoimmune response (Bach, 1994). Diabetic autoimmunity is likely triggered by an exogenous environmental insult. In genetically susceptible individuals, this results in failure to establish self-tolerance and a deranged immune response that ultimately leads to the destruction of the insulin-producing pancreatic beta-cells.

Environmental Antigens in the Autoimmune Pathogenesis of Insulin Dependent Diabetes Mellitus

Research into the etiology and pathogenesis of IDDM has recently focused on the potential involvement of environmental antigens that may trigger the autoimmune process that leads to beta-cell failure and insulin deficiency in genetically susceptible individuals. Antigenicity is defined as the capacity of molecules (antigens) to interact with immunologically-specific antibodies. The antibody-antigen interaction is determined by the three-dimensional chemical structure of a relatively small segment of the molecules that contacts the antibody during binding (Cordle, Duska-McEwen, Janas, Malone, & Hirsch, 1994). An epitope is the three-dimensional site on an antigen to which an antibody will
bind (Siemensma, Weijer, & Bak, 1993). Antigen presentation is derived from antigen dose, route, and frequency of administration that ultimately leads to successful initiation of immunity (Cordle, 1994). White blood cells, known as antigen presenting cells (APC), engulf a foreign cell, digest it, and present fragments of the dead cell on the surface of the antigen presenting cell. This presentation of foreign matter to the immune system is followed by a T-cell-mediated response to the antigen and the synthesis of antibodies by the B-cells (McCarren, 1992). The ability to recognize a specific antigenic structure of proteins is genetically determined (Cordle, 1994).

Immunogenicity is the capability of molecules (immunogens) to elicit the production of specific antibodies and/or initiate specific cellular immune responses. Immunogenicity is effected by molecular weight, chemical complexity, "foreignness," dose, and mode of exposure (Cordle et al., 1994). Strong immunogens have multiple binding sites (Cordle, 1994). A number of potential dietary antigens have been identified that may be involved in the immunopathogenesis of IDDM. It is unlikely that only a single initiating antigen leads to islet cell damage (Dotta et al., 1994).

Several hypotheses have been put forward that describe potential interactions between genetic predisposition, environmental factors, and the immunopathogenesis of IDDM. Environmental factors such as food antigens and viruses may cause expression of an abnormal beta-cell surface protein. For instance, the beta-cell protein, p69, which is a recognized autoimmune target in IDDM, presents itself on the surface of the islet cells following stress and infection (McCarren, 1992). Expression of beta-cell p69 is mediated by gamma-interferon. The presence of p69 causes failure of the immune system to recognize the beta-cell as self or causes structural changes in the cell that impact insulin production (Rossini et al., 1993). Antibodies to p69 have been detected in 70% of newly diagnosed diabetics and 9% of first-degree relatives (Dotta et al., 1994). An alternative hypothesis proposed by Thai and Eisenbarth (1993) suggests that the autoimmune
derangements are initiated by somatic mutations similar to those seen in some genetic malignancies.

The immature immune system is capable of establishing the immune memory that sustains the diabetic autoimmune process culminating in the demise of the insulin-producing beta-cells (Dosch et al., 1992). The early and defective immunological response involves antigenic or molecular mimicry in which a specific MHC structure has the ability to bind to an antigen that is similar but not identical to some self-protein. Autoantibody production and abnormal T-cell suppressor function follow (Rossini et al., 1993). Molecular mimicry is a faulty process of immune recognition. The immune system sensitized to one protein, an antigen, sees the second similar protein as foreign and mounts an immune attack against the analogous protein, regardless of its endogenous host origin. The autoimmune response causes destruction of the tissues that express the self-protein (Dosch et al., 1992). In this case, it is the insulin producing pancreatic beta-cells.

Atkinson and MacLaren (1994) have recently proposed two elaborate immunological models for the pathogenesis of IDDM. Autoimmunity to beta-cells may be caused by an immunological response to a viral protein which shares an amino acid sequence with a beta-cell protein. This results in the production of cytotoxic CD8+ lymphocytes that react with the analogous self-protein on the pancreatic beta-cells (Atkinson & MacLaren, 1994). The second proposed mechanism involves exposure to an environmental insult. This in turn may generate cytokines and other mediators of the inflammatory response. These immune components induce expression of adhesion molecules in the vascular epithelium of islet cells. Enhanced adhesion and extravasation of circulating leukocytes, infiltrating macrophages, and lymphocytes result in beta-cell damage (Atkinson & MacLaren, 1994). In either case, the process would be perpetuated by release of inflammatory cytokines from the damaged islet cells leading to overexpression of HLA class I molecules on beta cells. Further destruction of islets could be
mediated by antibody-dependent cellular cytotoxicity, delayed hypersensitivity, complement activation, and elevated levels of cytokines (Atkinson & MacLaren, 1994).

Summary of the Autoimmune Pathogenesis of IDDM

IDDM is a chronic disease of insulin deficiency following a long diabetic prodrome in which multiple autoimmune abnormalities eventually lead to beta-cell failure. Failure to establish normal immune tolerance, production of autoantibodies against a number of beta-cell autoantigens and insulin, and a battery of other immunological insults mediated by T-lymphocytes and other immune regulators are characteristic features of the autoimmune pathogenesis of IDDM. Immunological intervention for IDDM will depend on early prediction, immunosuppression, and induction of immunological tolerance to self and mimicry antigens (Bach, 1994).

ENVIRONMENTAL INFLUENCES OF DISEASE EXPRESSION

The rising incidence of IDDM in Europe roughly reflects a two-fold increase in the number of reported cases in the past 20 years. This observation points to involvement of an environmental determinant in the etiology of IDDM (Green et al., 1992). A sharp peak in the incidence is consistent with a sudden outbreak of IDDM, considered to be indicative of an epidemic, lending support to the recognition of environmental factors as additional causative agents (Schober et al., 1995). There has been a rapid rise in the incidence of IDDM in several Scandinavian countries with pronounced geographical variation in reported cases, despite a population that is genetically homogenous (Blom et al., 1989). The major geographical variations in the worldwide incidence of IDDM are reported to be as high as 36- to 50-fold from region to region, with certain populations showing significant changes in incidence over time (Diabetes Epidemiology Research International, 1987). Migrants have been shown to take on the risk equivalent to their new country.
Pronounced differences in incidence rates between genetically similar populations, geographical location, and fluctuations over time point to the role of environmental factors. These environmental insults may directly damage the beta-cells or are involved in a complex autoimmune mechanism leading to beta-cell destruction.

Human leukocyte antigen (HLA) genes confer susceptibility but are not determining factors in that the majority of individuals possessing these "diabetes genes" do not develop IDDM (David et al., 1994). Over 95% of the population with high-risk histocompatibility antigens evade diabetes (Diabetes Epidemiology Research International, 1987). Additional support for the involvement of environmental events as a component of the complex pathogenesis of diabetes is provided by studies in twins. Concordance of IDDM in homozygotic twins has been reported to range from 35% to 50% (Atkinson & MacLaren, 1994; David et al., 1994; Tattershaw & Pike, 1972; Todd, 1990). Approximately 90% of siblings with identical histocompatibility antigens do not develop diabetes (Diabetes Epidemiology Research International, 1987; Todd, 1990). The average risk to siblings for developing diabetes is only 6% (Todd, 1990). Over 90% of children newly diagnosed with IDDM do not have a first-degree relative with the disease (LaPorte et al., 1995).

Environmental factors likely modulate the expression of predisposing or protective genes. The modulation hypothesis would suggest that the environmental factors are only important in the population at genetic risk with multiple and chronic exposure exerting positive and negative effects (Bach, 1994). Environmental factors may break tolerance to pancreatic beta-cell antigens and precipitate the onset of the autoimmune process leading to IDDM in genetically susceptible individuals (Baxter & Cooke, 1992). Environmental insults are likely to exert their effect early in life, in utero or during infancy (Kyvik, Green, Svendsen, & Mortensen, 1992). Environmental factors may operate over this relatively narrow window of early childhood inducing an autoimmune process that eventually
destroys the insulin-producing beta-cells. A long prodrome precedes the onset of clinical symptoms (David et al., 1994; Scott, 1994). Evidence to support an early environmental event is provided in the observation that 25% of IDDM is diagnosed prior to age 5. The decrease in incidence with increasing age is likely attributed to a decreased environmental effect. Later environmental events such as viruses and stress could precipitate disease presentation but not initiate disease process (David et al., 1994).

Primary worldwide determinants of IDDM are environmental, not purely immunogenic (Diabetes Epidemiology Research International, 1987). Investigators from this project have estimated that at least 60% of IDDM worldwide, and conceivably up to 95%, is environmentally determined and, therefore, is potentially avoidable. These environmental factors, if identified, are likely to be more effective and less risky in preventing IDDM than current immunological approaches (Diabetes Epidemiology Research International, 1987).

Demographic Patterns of Disease Prevalence

Geographical Variation

The geographic variation in incidence of IDDM in childhood is unique among chronic diseases. Reported incidence of IDDM based on geographic variation has been shown to correlate with the average yearly temperature of the environment, distance from the equator, prevalence of certain viruses, diet, life-style patterns, as well as ethnic characteristics of the population at risk (Diabetes Epidemiology Research International, 1988). Distribution of HLA-DQB non-Asp-47 and HLA-DQα Arg-52 alleles may, in part, explain the geographical variation in incidence of IDDM (Scott, 1994). Once genetic susceptibility is taken into account, there is a marked decrease in the impact of geographic differences on the incidence of IDDM (Scott & Marliss, 1991).
containing fish and many fresh fruits compared to the New Zealand diet where breastfeeding is less likely or of shorter duration, and the child is weaned to a diet high in animal proteins and vegetables (Scott & Marliss, 1991). Risk of IDDM closely parallels that of the host country rather than the pattern of risk of the country of native origin. The differences between ethnic groups may reflect socioeconomic status and life-style patterns in addition to genetic determinants of IDDM (Diabetes Epidemiology Research International, 1988).

Seasonal Variation

Onset of diabetes has been shown to be related to season (Mazzella et al., 1994). An increased number of cases are reported in the winter months, which could plausibly be related to the known increase in viral infections observed during this period. Seasonal variation in incidence rates, with peak incidence in autumn and winter months, has been reported in Sweden (Dahlquist et al., 1985; LaPorte et al., 1995). Seasonal variation in the United States is small but tends to take the pattern observed worldwide. A general decline in registered cases of IDDM occurs in the summer months (LaPorte et al., 1995). The seasonal pattern of cases of IDDM in the Marche region of Italy parallels other Mediterranean countries, again, with a greater frequency of cases diagnosed during the winter months (Cherubini et al., 1994). A colder environment has been indicated as a risk factor for IDDM due to increased need for insulin (Mazzella et al., 1994). Increased work load on the pancreatic beta-cell may increase vulnerability to lymphokines and accelerate beta-cell destruction (Dahlquist, 1994).

Psychological and Social Factors

The Swedish Childhood Diabetes Study examined social and perinatal determinants of IDDM (Blom et al., 1989). Most notable was a significant increase in the incidence of IDDM with increasing age of the mother over 35 years. There was a slight inverse
correlation with educational level of the mother. Other social factors that have been identified at greater frequency in IDDM compared to the background population include overcrowding, environmental deprivation, unemployment, and lack of home or car ownership (Dorman et al., 1995).

Severe psychological stress has profound effects on the immune system and may predispose to IDDM (Rossini et al., 1993). Acute stress has been shown to accelerate the onset of IDDM in non-obese diabetic mice (Bach, 1994). Physical, psychological, and social stress may influence onset of IDDM in humans, as well. In a case control study investigating multiple environmental risk factors, the most significant finding was the increased risk associated with social and psychological dysfunction in children and to a lesser extent, a physical or socially stressful event within 12 months preceding onset of clinical disease symptoms (Siemiatycki, Colle, Campbell, DeWar, & Belmonte, 1989). Life events such as accidents, pregnancy, and other personal problems frequently occurred within a year prior to diagnosis of IDDM (Dorman et al., 1995). In a Hungarian study, questionnaires were sent by mail to diabetic families to gain knowledge of family characteristics, social status, fetal and perinatal events, breast-feeding practices, infectious diseases, and stressful life events (Soltesz, Jeges, Dahlquist, & the Hungarian Childhood Diabetes Epidemiology Study Group, 1994). A higher number of diabetic children, in particular, those diagnosed between 10 and 14 years of age, reported a higher number of stressful life events ($p = 0.016$, odds ratio = 3.9). This reflected the greatest association with IDDM for any of the parameters studied.

Severe psychological stress increases insulin requirements due to increased cortisol and catecholamine release. The antagonistic effects of these hormones on insulin appear to accelerate or precipitate the onset of clinical disease symptoms (Blom et al., 1989; Dahlquist, 1994). In addition, increased glucose levels in response to stress may increase the metabolic activity of pancreatic beta cells and, therefore, make the beta-cell more
usceptibility to aetiology of the diabetic prodrome stress is exacted nonspecific to have autoimmune defects in the immune system.

Virus

The development of virus in infants and susceptibility is enormous process that ends to beta-cell failure and insulin secretory failure. Virus infection have been reported in an increase frequency during 3 to 12-months post to neonatal period with certain illnesses symptoms (Tailcatt, 1994; Semytich et al., 1989) in a questionnaire by nai to diabetic families and matched controls, a higher proportion of diabetic children reported nor-syndrome infection odds ratio = 2.54; Saltus, et al., 1994) in case control study, children with autoimmunity has been acoved in any environment, with increase exposure to viral infections, slope ted to diabetes and increased incidence of DMD Senitych et al., 1989) in a case-control study, children signifi cantly greater number of diabetic children reported a viral illness in the 3-month preceding the diagnosis of DMD. Given that the prodromal DMD, said in the presence of setted antibodies and increase in virus to response to various glucose, may be lasting as 10 year, a viral nait immediately preceding these diagnosis may precipitate clinical symptoms. The does nonnecessarily pose evidence that the virus has an etiological role in the pathogenesis of DMD (Gathara et al., 1988).

A tempo-relationships exist between the peak of certain virus at onset of DMD Rosini et al., 1993. Ribula: male, dimyrditis influenza, Yongloviu, and Costicite. Virus late be linked indirectly as viral or incidental incidence of DMD blovinge:psin (98, 994 Timmilho & Wol, 1987. Infectious agents non- seeded for an acute contributed disease pathogenesis (Kelt, 1989). With the expection of congenital viable, evidence strongly linking virus with DMD is cumulational.
Several mechanisms have been proposed that address the pathological role of viruses in IDDM. Viruses may exert direct cytolytic effects on the pancreatic beta-cells (Bach, 1994) or may exert their effects indirectly by altering normal immune tolerance and competence (Rossini et al., 1993). Viruses are associated with elevated lymphokines. The lymphokines, in turn, may cause an inflammatory response that is damaging the pancreatic beta-cells (Dahlquist, 1994). Defects in immune tolerance could occur in combination with a T-cell immune reaction to viral neoantigens on the cell surface. Viral induced T-cell activation may be necessary for development of diabetes, at least in some cases (Bach, 1994). The beta-cell protein p69, a known autoantigen, is detected on the surface of the beta-cell during times of stress such as a viral infection (McCarren, 1992). Molecular mimicry following a viral insult could occur where a nontolerized exogenous antigen cross-reacts with a tolerized antigen and breaks tolerance (Bach, 1994). Alternately, Dosch (1993) suggested that repeated viral infections eventually take their toll on the beta-cells. The combination of genetic, immunological, and multiple environmental events could explain why an insult in infancy results in the manifestation of clinical diabetes at different ages in different children.

Coxsackie virus, which has been associated with increased prevalence of IDDM, appears to exert its effects by the process of molecular mimicry described above. Coxsackie virus (PC-2 protein) has regions homologous to glutamic acid decarboxylase, a known beta-cell autoantigen (Bach, 1994). An immune system primed to attack Coxsackie virus may attack the glutamic acid decarboxylase beta-cell protein (McCarren, 1992; Rossini et al., 1993). In addition, interaction between genetic predisposition and environment has been demonstrated by an increase in the prevalence of Coxsackie B virus in DR3 positive versus DR3 negative patients with IDDM (Dorman et al., 1995). Similarly, cytomegalovirus may lead to expression of viral or host antigens on the beta-cell surface, resulting in the production of islet cell antibodies, or antibodies to cytomegalovirus
may recognize both host and viral antigens by the process of molecular mimicry (Dorman et al., 1995). Congenital cytomegalovirus is associated with the development of IDDM, usually by age 15 (Dahlquist, 1994).

Congenital rubella has a 13 to 40% prevalence of IDDM (Blom et al., 1989). The highest frequency of IDDM associated with congenital rubella is seen in those that are also HLA-DR3 and/or DR4 positive (Dorman et al., 1995). Rubella is known to multiply in the pancreas and cause lesions to the beta-cells (Tuomilehto & Wolf, 1987). A temporal relationship also exists between mumps and the development of IDDM. In a population of diabetics, over 50% were exposed to the mumps virus within 4 years preceding the diagnosis of IDDM (Dorman et al., 1995). An aggressive immunization program against rubella and mumps, the MMR (measles, mumps, and rubella) vaccine currently used in the United States, was implemented in Finland in 1982. Some have postulated that the continued rising incidence of IDDM, despite successful public health programs promoting breast-feeding, may be caused by the very immunization program targeted to reduce the risk of IDDM.

Viral infections may also serve a protective role. Viruses could promote the production of immunosuppressive cytokines (Bach, 1994). Todd (1990) has reported that infection and the resulting stimulation of the immune system may reduce susceptibility to IDDM in some cases. While the north-south gradient may expose to predisposing viral infections in the northern sector, increased incidence of Hepatitis A and cytomegalovirus in the southern tiers may be protective. This is supported by a similar north-south gradient observed for multiple sclerosis, another T-cell-mediated autoimmune disease. The mycobacteria Bacillus-Calmette-Guerin has been used as a vaccine in rodent models and has shown some therapeutic promise in preventing IDDM (Bach, 1994).

Currently, no concrete evidence exists for a direct pathogenic role of a virus in IDDM (Bach, 1994). There is only limited evidence associating viral infection or exposure
with the presence of islet cell antibodies (Lipton & LaPorte, 1989). Despite this, the viral contribution to IDDM remains central to pathogenesis of the disease (Bach, 1994).

Nutritional Factors

The role of diet has been both controversial and difficult to evaluate. The long latent period prior to the onset of clinical disease symptoms makes it difficult to determine which dietary components may be diabetogenic (Scott, 1994). A single nutrient approach to studying the effects of diet on IDDM, particularly in epidemiological studies, may be too simplistic (Scott, 1994). Dietary factors may serve as catalysts for induction of IDDM (Coleman, Kuzava, & Leiter, 1990).

Diet has been demonstrated to modify the incidence of diabetes, and impact age of onset of IDDM and the degree of associated insulinitis in experimental animal studies (Hoorfar, Bushard, & Dagnaes-Hansen, 1993). As much as 85% of diabetes in the spontaneously diabetic biobreed rat appears to be dependent on dietary exposures (Scott, 1994). Elliott and Martin (1984) were the first to demonstrate that dietary proteins, in particular wheat and milk proteins, fed during the weaning period could impact progression to diabetes in biobreed rats. The incidence of IDDM in rats fed a synthetic diet containing hydrolyzed proteins was significantly lower than in rats fed a diet supplemented with gluten or milk (15%, 35%, and 52% incidence of IDDM, respectively). Intact protein was concluded to be necessary for full expression of genetic susceptibility to IDDM in the biobreed rat (Elliott & Martin, 1984). In non-obese diabetic mice, the window of vulnerability to diabetogenic dietary constituents occurs between weaning and 70 days of age (Hoorfar et al., 1992). The impact of nutritional factors may be limited to a relatively narrow, early phase of the postnatal period.

These observations have been applied to numerous human epidemiological, experimental, and case control studies that have attempted to evaluate the role of infant
protein intake was previously low (Tuomilehto & Wolf, 1987). The protective effect of feeding a casein hydrolysate in the prevention of IDDM in the biobreed rat and non-obese diabetic mouse would appear to be the lack of intact protein and, therefore, diabetogenic antigens (Elliott, Reddy, Bibby, & Kida, 1988; Hoorfar et al., 1993).

A great deal of research has been directed toward reaching an understanding of the role of various cow's milk proteins in the pathogenesis of IDDM. At least some milk proteins appear to function as diabetogenic antigens. The role of specific milk proteins in the pathogenesis of IDDM is discussed under the section entitled "Infant Feeding Practices: Breast-feeding and Early Introduction of Cow's-Milk-Based Infant Formula in the Pathogenesis of Insulin Dependent Diabetes Mellitus."

Wheat

Animal studies have shown that cereal-based rodent diets are the most diabetogenic (Hoorfar et al., 1992; Scott & Marliss, 1991). Preliminary findings from Australia suggest that high consumption of cereals is associated with increased risk of IDDM (Virtanen & Aro, 1994). Long-term ad libitum feeding of wheat flour has consistently been associated with a diabetes incidence of 40 to 65% in the biobreed rat and non-obese diabetic mouse strains (Elliott & Martin, 1984; Hoorfar et al., 1993; Scott et al., 1989). Foods rich in carbohydrate are typically rich in wheat gliadin. The increased incidence of IDDM observed in children with higher carbohydrate intakes may support the hypothesis that wheat gliadin is harmful to the beta-cell (Dahlquist et al., 1990). Celiac disease and IDDM have been shown to coexist. Both diseases are associated with HLA DR3 and DQ. The potential impact of the gliadin fraction of gluten, which is responsible for celiac disease, in the pathogenesis of IDDM was first proposed by Scott and colleagues (1989). Provision of wheat gluten to biobreed rats resulted in a significantly greater incidence of IDDM than in the casein-fed control group. In children diagnosed with IDDM, over half
had increased levels of IgG anti-gliadin antibodies despite the absence of clinical symptoms of celiac disease (Scott et al., 1989).

**Soy**

Experimental animal studies have linked soy feeding to IDDM. Long-term ad libitum feeding with soy bean meal in non-obese diabetic mice has been associated with a relatively high incidence of diabetes (45%) (Hoorfar et al., 1993). In the biobreed rat, soy administration resulted in at least a 50% incidence of IDDM with up to an 80% frequency of insulinitis (Scott, 1994).

Soy formulas are commonly used in children with allergies to intact cow's milk protein with or without alterations in gastrointestinal function. Approximately 8 to 25% of children with hypersensitivity to cow's-milk-based proteins are allergic to soy products (Kleinman, Bahna, Powell, & Sampson, 1991). Viral infections may precipitate the use of soy formulas due to alterations in the mucosal lining and absorption (Fort et al., 1986). Children given soy milk may exhibit increased gut permeability to antigens due to preexisting viral infection, diarrhea, lactose intolerance, or milk allergy. Soy protein is at least as antigenic as milk protein and should be used with caution as prophylaxis against possible dietary antigen/antibody-related disease (Eastham, Luchauco, Grady, & Walker, 1978). These factors may contribute to an autoimmune response to a foreign protein, such as bovine serum albumin, implicated in the pathogenesis of IDDM. Diabetic children have been reported to be twice as likely to have received soy formula in infancy as compared to control children (Fort et al., 1986). Soy formula is clearly not a viable alternative to polymeric cow's-milk-based infant formulas for prophylaxis against IDDM.
Fatty Acids

Dietary lipids have long been recognized as potent immune system regulators. Cell membrane composition can be altered by the degree of saturation of dietary fats potentially resulting in changes in membrane fluidity and function. Effects of varying the polyunsaturated to saturated fat ratio (P/S) of the diet include altered membrane phospholipid content, B-lymphocytes, resistance to tolerance, suppressor activity, and T-cell dependent immune responses (Scott & Marliss, 1991). Omega-3 polyunsaturated fatty acids are potent immunosuppressants. They function as a competitive inhibitor for cyclo-oxygenase, resulting in a decrease in thromboxane synthesis. Supplementation of diets with omega-3 polyunsaturated fatty acids has been shown to suppress the synthesis of several cytokines (interleukin-1α, interleukin-1β, and tumor necrosis factor). Interleukin-1β has been reported to be toxic to beta-cells (Rossini et al., 1993). In addition, studies using fish oil supplements have shown a reduction in the production of monokines, including interleukin-1 and tumor necrosis factor (Scott & Marliss, 1991). Diets low in essential fatty acids and protein may be protective against IDDM by interfering with the anti-islet immune response (Bach, 1994).

Nitroso Compounds

Exposure to dietary nitrates, nitrites, and nitrosamines may increase the risk of developing IDDM (Blom et al., 1989; Tuomilehto et al., 1995). Streptozocin (STZ) and the rodenticide Vacor, both N-nitroso compounds, are experimental diabetogenic agents (Siemiatycki et al., 1989). Streptozocin is thought to act as a hapten in vivo, resulting in T-cell activation, lymphokine secretion, and subtoxic effects potentially exerted directly on the beta-cell (Scott & Marliss, 1991). N-nitrosothiazolidine (NTHZ) and 3-nitrosothiazolidine-4-carboxylic acid (NTCA) have been shown to increase plasma glucose levels in mice following a single injection under experimental conditions (Scott, 1994). Alloxan
can also induce IDDM (Bach, 1994). Nitrosamine compounds are toxic to beta-cells (Dahlquist, 1994) and have been associated with a higher risk of IDDM in several studies (Virtanen & Aro, 1994).

These findings have raised some questions as to the diabetogenicity of cured meat products. In the Icelandic population, increased incidence of IDDM in boys born in October may be correlated with periconceptual intake of smoked cured mutton during the Christmas holidays. This hypothesis proposes that periconceptual intake of nitrates may impact the beta-cells in utero with a long latency period of approximately 15 years prior to expression of overt clinical disease (Tuomilehto & Wolf, 1987). In Finnish children a high dietary intake of nitrite, but not nitrate, was associated with greater risk of developing IDDM (Virtanen & Aro, 1994). The incidence of IDDM has been correlated with the nitrate content of drinking water (Dahlquist, 1994). A dose response relationship has also been observed between frequency of intake of foods containing nitrosamine and the risk of children developing IDDM (Dahlquist et al., 1990). Historically there has been a low prevalence of IDDM in primitive societies that do not use food preservation such as nitrosamines (David et al., 1994); however, the results have been mixed. A case control study of environmental risk factors for IDDM showed no increased risk associated with intake of meats preserved with nitrates (Siemiatycki et al., 1989).

**Vitamin C**

While there has been some concern that degraded vitamin C may accelerate beta-cell damage, the antioxidant effects of vitamin C may, in fact, be protective. Ascorbic acid may scavenge free radicals that have been implicated in beta-cell damage (Glatthaar et al., 1988). Free radical scavengers could partially suppress the cytotoxic activity of macrophages toward islet cells (Scott & Marliss, 1991). Descriptive epidemiology of the childhood diabetic population in Australia showed that the greatest difference between cases and
controls was the more frequently reported intake of vitamin C in controls (Glatthaar et al., 1988). Others have reported no significant differences in vitamin C intake in diabetic children compared to controls (Dahlquist et al., 1990).

Summary of the Role of Environment in the Etiology of IDDM

The large variation in the incidence of IDDM among population groups with similar genetic characteristics points to the influence of environmental factors which initiate and sustain the diabetic autoimmunity or confer protection from the disease. The search to identify etiological environmental factors contributing to IDDM continues. Clearly stress, viruses, and nutritional practices play a role in the pathogenesis of IDDM. The potential for interaction of several environmental factors with genetic predeterminants of risk cannot be overlooked in the attempt to unravel the complex autoimmune pathogenesis of IDDM.

INFANT FEEDING PRACTICES: BREAST-FEEDING AND EARLY INTRODUCTION OF COW’S-MILK-BASED INFANT FORMULA IN THE PATHOGENESIS OF INSULIN DEPENDENT DIABETES MELLITUS

Infant feeding practices resulting in early exposure to a number of food antigens has been a major area of focus of research in the pathogenesis of IDDM over the past 20 years. The proposed theory suggests that exposure to environmental events occurs over a limited period in early childhood. The variable age of presentation of IDDM reflects different rates of disease progression rather than different ages of critical environmental exposure (David et al., 1994). Sensitization to a dietary antigen could occur either prior to gut closure and/or following an acute gastrointestinal infection.

Cow’s milk carries the reputation of being one of the most allergenic foods given to infants. Unfortunately, cow’s-milk-based infant formulas are often introduced early in life
as an alternative to breast-feeding. While cow's-milk-based formulas are the mainstay of nutrition for bottle-fed infants and provide supplemental feedings for infants not exclusively breast-fed, early exposure to dietary antigens remains an area of concern. Specific milk proteins, in particular bovine serum albumin (BSA), have been implicated in the pathogenesis of IDDM.

The impact of breast-feeding, duration of breast-feeding, and early introduction of cow's-milk-based infant formulas on the pathogenesis of IDDM will be reviewed in detail in the following sections. An in-depth review of the literature supporting and refuting the role of BSA in the pathogenesis of IDDM is provided. Specific attention is given to the mechanism by which BSA contributes to IDDM and its interaction with predisposing genetic characteristics in the autoimmune pathogenesis of IDDM.

Physiology of the Gastrointestinal Tract in Infancy

The gastrointestinal tract is not fully developed at birth even in term infants. Age dependent changes in gut permeability to macromolecules (e.g., cow's milk proteins), maturation of gastrointestinal proteases, slow maturation of the intestinal barrier, and gut-based tolerance to foreign proteins occur over the first year of life (Akerblom, Savilahti, et al., 1993). Notable immaturity of the gastrointestinal tract is observed with increasing prematurity.

Digestion and absorption of dietary protein occur in three phases. The initial phase, which occurs in the stomach and gut lumen, involves breakdown of intact protein to free amino acids and short peptides. The second phase occurs at the brush border of the enterocyte where brush border peptidases attack peptides. Free amino acids and peptides are transported into the mucosal cell by four major amino acid transporters and more than one peptide transporter. The final stage occurs in the cytoplasm of the enterocyte where dipeptides and tripeptides are broken down to free amino acids by cytoplasmic enzymes.
IgA locally in the small intestine. Circulating antibodies to ingested food proteins can be detected in almost all infants during the first 3 months of life due to a systemic immunological response to proteins absorbed from the small intestine (Eastham et al., 1978). Normally the immune system learns to tolerate foreign proteins that enter the body through the gastrointestinal tract or mounts only a mild ineffective assault against them (Rennie, 1992). This is reported to be of little pathological significance (Eastham et al., 1978). Slow maturation of the intestinal immunological barrier against dietary macromolecules, in particular potential antigens, occurs in infancy (Akerblom, Savilahti, et al., 1993; Fourth International Onnela Workshop, 1990). In addition, development of host non-immunologic and immunologic defense mechanisms further protects against uptake of antigenic macromolecules (Fourth International Onnela Workshop, 1990).

**Gastroenteritis**

Functional and structural alterations in the mucosal barrier may occur due to malnutrition, acute gastroenteritis, cow's milk allergy, and enzyme deficiencies and result in increased absorption of macromolecules (Fourth International Onnela Workshop, 1990). Gut permeability can be increased temporarily due to diarrhea (Akerblom, Savilahti, et al., 1993). Transient changes in the gut mucous associated with episodes of gastroenteritis permit passage of intact dietary proteins that may function as antigens raising antibodies to a number of dietary constituents. Acute gastroenteritis in children less than 3 years of age results in increased protein absorption, which may trigger food allergy, in particular, allergy to cow's milk proteins. Human α-lactalbumin serum concentrations in children during the acute phase of rotaviral gastroenteritis have been shown to be similar to those of healthy children, but rise significantly \((p < 0.001)\) during the 5- to 8-week period of convalescence (Holm, Andersson, Gothfors, & Lindberg, 1992). Damage to the intestinal epithelium by infections (e.g., retroviral insults) may lead to an excess passage of intact
cow's milk protein into the blood stream and increase antibody production in response to BSA (Norris & Pietropaola, 1994). Siemiatycki and colleagues (1989) have reported a higher incidence of recent gastrointestinal infections in newly diagnosed cases of IDDM.

**Cow's Milk Allergy**

An apparent feature of gastrointestinal syndromes accompanied by diarrhea during the first year of life is a considerable overlap between characteristics, symptoms, findings, and etiology. Nonspecific vomiting, colic, and diarrhea can occur in infants without immune-mediated reactions to dietary antigens and are thought to occur due to increased intestinal permeability. Gastrointestinal blood loss, protein losing nephropathy, enterocolitis, colitis, and malabsorptive syndrome result from allergic reactions to dietary antigens that occur in infancy. Each of these symptoms has been at one time attributed to intake of cow's milk protein (Kleinman et al., 1991).

Cow's milk allergy is estimated to affect 0.4% to 7.5% of all infants (Bahma, 1987; Kleinman et al., 1991). Infants present with malabsorptive syndrome characterized by protracted diarrhea, carbohydrate and fat malabsorption, small intestine villous atrophy, and patchy enteropathy (Kleinman et al., 1991). Enterocolitis is usually seen in infants between the ages of 1 week and 3 months. Infants present with occult blood, leukocytes, and eosinophils in stool specimens. This is accompanied by carbohydrate intolerance and watery diarrhea. Peripheral polymorphonuclear leukocytosis is often seen. This results commonly from immune-mediated allergic reaction to cow's milk, but has been observed with other intact protein antigens (Kleinman et al., 1991). Elemental, hydrolyzed formulas are indicated for use in both the management of malabsorption and to reduce exposure to intact antigenic proteins. However, it appears that some residual antigenicity remains even after extensive fragmentation of the protein (Seban, Konijn, & Freier, 1977). Despite these findings showing increased antigen permeability with diarrhea and milk allergy, a positive
history of allergy or gastroenteritis has not been reported to be associated with elevated antibody titres to cow's milk proteins in diabetics, sibling-controls, or population controls (Virtanen et al., 1994).

**Lactose Intolerance**

Chronic diarrhea and alterations in the gastrointestinal mucosa resulting in lactase deficiency may alter gut permeability to peptides. While lactose intolerance has not been directly linked to the pathogenesis of IDDM, it is interesting to note a fairly high incidence of late onset lactose intolerance is observed in Finnish children, a population previously noted to have the highest worldwide incidence of IDDM. Prevalence of lactose intolerance of late-onset is reported to be 4% in 7- to 11-year-olds, 9% in 12- to 15-year-olds, and 15% in 16- to 20-year-olds (Fourth International Onnela Workshop, 1990). In populations where genetic lactose intolerance is prevalent (e.g., Japanese, Australian Aborigines, and Native Americans), milk consumption is avoided, and the incidence of IDDM is far less than would be expected by genetic profile and the presence of lactose intolerance (Strand, 1994). However, in Finland and other areas where milk consumption is high and lactose intolerance has been reported, evaluation of the prevalence of lactose intolerance in diabetic children compared to non-diabetic children may be warranted.

**Summary of the Physiology of the Gastrointestinal Tract in Infancy**

Antigen permeability appears to occur in infancy prior to gut closure, following an episode of acute gastroenteritis or due to gastrointestinal damage associated with milk allergy or intolerance. Decreased mucosal IgA and immaturity of the immunological tolerance mechanism may result in an immunological response to a foreign dietary antigen. Abnormal immunological responses to the exogenous antigen could trigger diabetic autoimmunity and subsequent pancreatic beta-cell destruction leading to insulin deficiency.
the duration of breast-feeding and the age of introduction of supplementary formula feedings increased between 1972 and 1982. Breast-feeding practices were shown to correlate inversely with the incidence of IDDM. In addition, international patterns of breast-feeding prevalence at 3 months of age are inversely related to the prevalence of IDDM between countries ($r = -0.53; p < 0.05$).

**Case-Control Studies**

Case-control data lend support to cross-sectional national comparison studies that suggest a relationship between breast-feeding practices and prevalence of IDDM. Children who developed diabetes have been reported to be completely or partially breast-fed for a significantly shorter period of time than their healthy siblings and the background population. In addition, fewer diabetic children had ever received breast milk (Borsch-Johnson et al., 1984). Similarly, Metcalfe and Baum (1992) reported a significant difference ($p < 0.01$) in breast-feeding incidence and duration between a large sample of index cases of IDDM compared to the reference population obtained from breast-feeding statistics in the British Isles. In comparison, a descriptive epidemiological report of IDDM in Australian children revealed that only a marginally significant excess of diabetic children had not been breast-fed at all or failed to be breast-fed beyond 1 week. However, there is a relatively low frequency of breast-feeding in the Australian population as compared to the Scandinavian population (Glatthaar et al., 1988). Similarly, Samuelsson and colleagues (1993) report a marginal role, if any, of breast-feeding in the prevention of IDDM, again in a population with low breast-feeding rates. The relationship of infant feeding practices to childhood diabetes suggest that a longer duration of breast-feeding is associated with a lower risk for developing diabetes (Verge, Howard, Irwig, Simpson, & Silink, 1992).

Breast-feeding duration of less than 3 months has been associated with an increased risk of IDDM in a number of retrospective, case-control studies. A negative relationship
has been reported between breast-feeding at 3 months of age and risk of developing IDDM (p < 0.05; Scott, 1990; Verge et al., 1992; Virtanen, Rasanen, Aro, Lindstrom, Sippola, et al., 1991; Virtanen, Rasanen, Aro, Lindstrom, Tuomilehto, et al., 1991; Virtanen et al., 1991; Virtanen, Rasanen, Aro, Lindstrom, Tuomilehto, et al., 1992). Risk of developing diabetes was reported to be reduced in children who were breast-fed during infancy for at least the first 2 to 3 months (odds ratio 0.60 and 0.63 respectively) (Virtanen, et al., 1992). In Finnish children presenting with overt clinical diabetes prior to age 7, a significantly smaller portion of diabetic children were breast-fed compared to control children at each interval evaluated: 3 months (75% vs. 89%, p < 0.04) and 4 months (43% vs. 61%, p < 0.04). Given the infant feeding practices of Finnish children and the incidence of diabetes diagnosed prior to age 7, it would appear that the proportion of children exclusively breast-fed is limited and may be an associated factor of disease risk (Virtanen, Rasanen, Aro, Lindstrom, Sippola, et al., 1991; Virtanen, Rasanen, Aro, Lindstrom, Tuomilehto, et al., 1991). In addition, exclusive breast feeding for 3 months or more has been demonstrated to have a significant protective effect against the development of IDDM (Verge et al., 1992), while breast-feeding of less than 3 months has been cited as a risk factor for IDDM (Blom et al., 1989).

In populations having a higher frequency and duration of breast-feeding, the impact of a longer duration of breast-feeding on IDDM could be evaluated. In a retrospective case-control study of 268 children selected from the Colorado IDDM Registry and the Barbara Davis Center for Childhood Diabetes, a significantly smaller portion of children with diabetes (30%) had been breast-fed. In addition, the group that was breast-fed for a shorter duration also had an increased incidence of IDDM. A greater decrease in this risk of IDDM was seen in those infants breast-fed for 12 months or more (Mayer et al., 1988). The incidence and duration of breast-feeding was higher in this population than other populations reporting both association and lack of correlation between breast-feeding and incidence of IDDM.
Early exposure to cow's milk in the diet and/or short duration of breast-feeding may lead to increased risk of development of IDDM (Verge et al., 1992). Blom and colleagues (1989) evaluated the effect of both partial and exclusive breast-feeding and the associated risk of IDDM. Even though, breast-feeding of less than 3 months was cited as a risk factor for developing IDDM, no significant differences in the time of introduction of infant formula was noted between diabetic and non-diabetic children. Here, breast-feeding appeared to have a protective effect independent of age of introduction of cow's-milk-based infant formula.

It appears that the benefits of breast-feeding in the prevention of IDDM may be more strongly related to diabetes that develops early in childhood. In a multivariate analysis of children by age-group, breast-feeding duration of less than 3 months was a significant risk determinant for diabetes in the 0 to 4 age group (Dahlquist et al., 1991). The impact of short duration of breast-feeding did not carry over to the older age brackets. Similar findings were reported by Blom and colleagues (1989). In children 0 to 6 years of age, the duration of breast-feeding was significantly shorter for the diabetic children compared to controls ($p = 0.03$). The median duration of breast-feeding for diabetic children was 5 months, 1 month shorter than the control children. The impact of breast-feeding was not significant in the 7 to 14 year age group (Blom et al., 1989). In children with IDDM, diabetes developed at a later age in those that were breast-fed as infants compared to those receiving breast-milk substitutes.

**Meta-analysis of Breast-Feeding and Incidence of Insulin Dependent Diabetes Mellitus**

Meta-analysis can be used to combine the results of clinical trials and observational studies and obtain a summary risk estimate suitable for discussions of causality and development of health policy (Kostraba, 1994). In a recent meta-analysis by Gerstein
(1994), decreased incidence and duration of breast-feeding was consistently related to IDDM in ecological and time-series studies in which the prevalence of IDDM was compared to the rate of breast-feeding in different populations over a specified period of time. Case-control studies, in which neonatal feeding histories of patients with IDDM were compared to control subjects, showed that patients with diabetes were more likely to have been breast-fed in infancy for an interval of less than 3 months. A weak, but significant association between infant feeding practices and the risk of IDDM was reported (odds ratio 1.5) (Gerstein, 1994). In a separate meta-analysis, reviewing similar studies, the amount of IDDM that could be explained by breast-feeding practices if these practices were causal was reported to be 14% and ranged from 2 to 26% according to breast-feeding prevalence reported in each study (Mayer et al., 1988). Similarly, Scott (1994) reported that as much as 14% of IDDM may be attributed to a lack of breast-feeding. Children with IDDM were less likely to have been breast-fed or were breast-fed for a shorter period of time.

**Lack of Association Between Breast-Feeding and Risk of Developing Insulin Dependent Diabetes Mellitus**

In contrast to studies supporting a protective effect of breast-feeding, others have reported little if any association between breast-feeding incidence and duration and later development of IDDM in childhood. A case-control study of 268 children developing IDDM prior to age 15 in the United Kingdom found that short duration of breast-feeding, defined as partial or exclusive breast-feeding of less than 3 months, had no influence on the incidence of IDDM (Bodington, McNally, & Burden, 1994). Similarly, in another comparison of breast-feeding rates in diabetic probands with non-diabetic siblings and pair-matched controls, no difference in breast-feeding rates was observed between the three groups (Fort et al., 1986). Breast-feeding incidence was only 18% in all three groups (Fort et al., 1986), which was notably low compared with national and regional data.
As previously noted, where the prevalence and duration of breast-feeding is low, it is difficult to determine the impact, if any, of breast-feeding on the incidence of IDDM.

Studies relating breast-feeding practices to incidence of IDDM have been criticized because much of the data has been based on parental recall many years after the fact (Ellis & Atkinson, 1996). Only two studies on breast-feeding reported in the literature have used data on the duration of breast-feeding that was collected during infancy, rather than after the diagnosis of diabetes (Kyvik et al., 1992; Nigro, Campea, De Novellis, & Orsini, 1985). Kyvik and colleagues (1992) failed to find evidence to support the hypothesis that increased duration of breast-feeding reduces subsequent risk of developing IDDM. Nigro and colleagues (1985), found that diabetic children had in fact been breast-fed slightly longer than the background population. These studies do not support the results reported in retrospective studies that relied on long-term recall to establish the history of infant feeding practices (Kostraba, 1994). Accuracy of recall in reporting infant feeding practices retrospectively and the potential of bias due to a known diagnosis of IDDM may impact results.

Abrams, Schrans, Vertommen, and De Leeuw (1994) looked specifically at those diagnosed with IDDM between 15 to 40 years of age for a relationship between infant feeding practices and autoimmune aggression (i.e., islet cell antibodies, insulin autoantibodies, and C-peptide concentrations). While a small, but significant protective effect of breast-feeding has been observed in children who develop diabetes between the ages of 0 and 15 (Gerstein, 1994), there were no significant differences related to the mode of infant feeding in autoimmune aggression, C-peptide levels, and age of diagnosis of IDDM (Abrams et al., 1994). However, in those with clinical diabetes it would seem unlikely that these general pathological findings would differ regardless of infant feeding practices. These results are not surprising given that the studies previously reporting a
which could have direct effects or alter normal gastrointestinal function (Scott, 1994). Human colostrum protects the infant against early gastrointestinal infections (Samuelsson et al., 1993). Breast milk compensates for the deficiency of local gut immunoglobin production during early infancy, further protecting the infant from gastrointestinal infections (Akerblom, Dosch, et al., 1993). Improved anti-inflammatory response, decreased free radical production, and increased free radical scavengers delivered to breast-fed infants may decrease the intracellular immune response and beta-cell destruction (Mayer et al., 1988). In addition, mothers who breast-feed longer are less likely to smoke or to have children in daycare (ESPGAN Committee on Nutrition et al., 1993). Both of these factors may decrease risk of IDDM by protecting against exposure to viral infections independent of breast-milk composition.

**Decreased Exposure to Exogenous Food Antigens**

An alternative explanation is that chemicals or proteins present in infant formula may be directly or indirectly damaging to the beta-cells (Borsch-Johnson et al., 1984). Mothers who breast-feed longer are more likely to delay introduction of solid foods (ESPGAN Committee on Nutrition et al., 1993), a number of which have been cited as potential antigenic triggers of IDDM. Breast-fed infants are less likely to receive solids prior to 3 months of age and are exposed to a less diverse diet in terms of food antigens (Kostraba, 1994). Introduction of solid foods prior to 3 months of age is associated with a highly significant 2.5 fold increased risk of IDDM (Kostraba et al., 1992).

**Growth Patterns in Breast-Fed and Formula-Fed Infants**

Breast-feeding may exert its protective effect by limiting weight gain in infancy as compared to formula feeding (Samuelsson et al., 1993). Children who are exclusively breast-fed have a lower energy intake. This results in a lower rate of growth after 3 months of age (Fomon, Thomas, Filer, Ziegler, & Leonard, 1971; Hithcock, Gracey, & Gilmour,
Increased weight gain in infancy due to supplemental formula feeding could increase insulin demand and serve as a contributing environmental factor in the pathogenesis of IDDM (Virtanen & Aro, 1994).

The DARLING Study (Davis Area Research on Lactation, Infant Nutrition, and Growth) found that growth differences were related to intake and were higher in the formula-fed infants than in the breast-fed infants between 3 and 9 months of age. Intake was consistently and significantly higher in formula-fed than in breast-fed infants. Once solids were introduced, total calorie and protein intake remained significantly higher in the formula-fed infants; however, the percent of total calories contributed by either breast milk or formula did not differ significantly. Breast-fed infants, however, exhibited a greater percentage gain in lean body mass (Heinig, Nommsen, Peerson, Lonnerdal, & Dewey, 1993).

In a similar study conducted by Johansson, Samuelsson, and Ludvigsson (1994), growth patterns were evaluated in 297 children who had been diagnosed with IDDM and compared to reference children. Birth weight in children who developed diabetes was less than reference children; however, rate of growth and weight gain at 6, 9, 18, and 30 months was significantly greater in the diabetic children ($p < 0.02$). There was also a correlation between formula feeding and weight gain, which has also been observed in the general population. Weight gain of children who had not been breast-fed was significantly greater for both diabetic and control children. Children developing diabetes in the 0 to 4 age category had the lowest birth weights but showed rapid weight gain in infancy (Johansson et al., 1994). In contrast, Metcalfe and Baum (1992) reported that children who developed diabetes were more likely to be heavier at birth compared to the national reference data from the British Isles. Among those exclusively breast-fed for greater than 2 months, diabetic children gained significantly more weight (Johansson et al., 1994). These findings support earlier work conducted by Baum, Ounsted, and Smith (1975) reporting
Breast-Feeding Patterns of Women with Insulin Dependent Diabetes Mellitus

Women with IDDM discontinue breast-feeding and introduce formula significantly earlier than non-diabetic mothers. The mean duration of breast-feeding was reported to be 22 days in diabetic mothers compared to 52 days in non-diabetic women (Ferris et al., 1993). These observations are further supported by a Finnish pilot study of the feasibility of avoidance of intact cow's milk protein during infancy. Only 1 of 20 infants of mothers with IDDM exclusively breast-fed for 6 months (Akerblom, Savilahti, et al., 1993). Unfortunately, mothers with IDDM are more likely to supplement formula earlier than non-diabetic mothers despite intentions to breast-feed at a similar rate (Gerstein, Simpson, Atkinson, Taylor, & VanderMeulen, 1995). This is notable in that the risk of IDDM is significantly higher in first degree relatives. The interaction of genetic predisposition with lack of breast-feeding, supplementation with cow's-milk based-infant formulas, and early introduction of solids may pose increased risk for the infant of contracting IDDM in childhood.

Comment on Breast-Feeding Studies

Exclusive breast-feeding appears to be limited. Only two of the studies reviewed above reported on exclusive breast-feeding (Virtanen, Rasanen, Aro, Lindstrom, Sippola, et al., 1991; Virtanen, Rasanen, Aro, Lindstrom, Tuomilehto, et al., 1991; Virtanen et al., 1992). In a recent feasibility study of mothers already having one diabetic child, 87% indicated that their next child would be breast-fed. Of those planning to breast-feed, only 5.6% reported plans to exclusively breast-feed for more than 2 months (Gerstein et al., 1995). One apparent confounding factor in most studies is that they fail to consider duration of exclusive breast-feeding rather than duration of breast-feeding despite introduction of supplemental feedings. It is only exclusive breast-feeding that protects
from cow's milk and other foreign dietary proteins (Akerblom, Savilahti, et al., 1993). Introduction of cow's-milk-based formula, as full or supplemental feedings, provides dietary antigens that have been associated with an increased risk of developing IDDM later in childhood. An increased caloric load and insulin response may prime the pancreas making the beta-cells targets for autoimmune attack and destruction. Most studies failed to take into account age of introduction of supplemental formula feedings, the type of formula introduced, and other foods consumed by the infant within the first year of life independent of breast-feeding practices. Therefore, the likelihood of contamination and confounding of study results with cow's-milk-based formula exists. Enough cow's milk could have been provided as supplemental feedings to breast-fed infants to elicit the autoimmune response leading to IDDM. History of cow's milk exposure prior to 3 to 4 months of age with supplemental feedings may have a greater influence on the pathogenesis of IDDM than the total duration of breast-feeding. The duration of breast-feeding may not be as important in the etiology of IDDM as the age of introduction of breast-milk substitutes, in particular cow's-milk-based infant formula.

Cow's Milk and Cow's-Milk-Based Infant Formula

Considerable attention, in both the scientific and lay literature, has been focused on the potential contribution of cow's milk to the autoimmune pathogenesis of IDDM. The process of autoimmunity induced by milk proteins is thought to result from formation of immunological memory cells and increased antibody production in response to cow's milk proteins followed by a cross-reactive immune response with a protein on the pancreatic beta-cells (Virtanen et al., 1994). Through this mechanism, known as molecular mimicry, the ability to produce insulin fails resulting in clinical IDDM. Exposure to cow's milk proteins in early infancy, prior to gut closure, to an immature immune system may promote IDDM in genetically predisposed individuals.
The protein content of cow's milk differs from human milk. Cow's milk is higher in protein, and this difference is largely reflected in the casein content. Beta-lactoglobulin present in cow's milk is essentially absent from human milk and has been extensively associated with milk allergy in infants. In addition, bovine serum albumin (BSA), a minor whey protein, shows slight variations in amino acid sequence and major variations in antigenicity from human serum albumin (Martin, Trink, Daneman, Dosch, & Robinson, 1991).

Cow's milk is considered to be a strong antigen, and it would be expected that infant formulas containing intact cow's milk proteins exhibit antigenic characteristics. Infant formulas containing intact cow's milk protein (e.g., Enfamil, Similac, Gerber, and SMA) and partially hydrolyzed whey protein fractions (e.g., Good Start) have in fact been demonstrated to be strong immunogens (Cordle et al., 1994). This has been shown to be true of the partially hydrolyzed product despite a significant reduction in antigen load (80-85%). Antigenicity of infant formulas has been demonstrated in neonates where absorption of intact antigens is largely attributed to increased permeability of the immature intestine and a relative deficiency in secretory IgA (Eastham et al., 1978). While increased absorption of intact protein in infancy was once thought to be of little pathological significance (Eastham et al., 1978), recent evidence points to the potential involvement of milk protein antigens in the autoimmune pathogenesis of IDDM.

**Animal Studies: Cow's Milk Protein and Insulin Dependent Diabetes Mellitus**

Animal studies in the spontaneously diabetic biobreed (BB) rat and non-obese diabetic (NOD) mouse have revealed a significant impact of dietary cow's milk proteins on the incidence of diabetes. BB rats fed skim milk powder had a 52% incidence of IDDM compared to animals fed either casein or hydrolyzed lactalbumin having an incidence of
diabetes of only 7% and 2%, respectively (Scott, Elliot, & Kolb, 1989). BB rats fed a diet devoid of intact protein, containing synthetic amino acids, had a 15% incidence of IDDM as compared to an incidence of over 50% of animals receiving intact cow's milk protein (Elliott & Martin, 1984). Elimination of intact cow's milk protein from the diet of the BB rat significantly reduced the incidence of IDDM (Martin et al., 1991).

Greater than 70% of non-obese diabetic mice (NOD) exhibit diabetes by 30 weeks. Coleman and colleagues (1990) were able to eliminate IDDM in NOD mice by feeding a hypoallergenic infant formula containing casein hydrolysates as the protein source (Pregestimil). Animals remained free of diabetes throughout the 1-year study period. Similarly, Pregestimil was effective in preventing spontaneous IDDM in female NOD mice, and cyclophosphamide induced diabetes in male NOD mice (Hermitte et al., 1995). Pregestimil was effective in preventing early appearance of widespread insulinitis, a major feature of diabetes in NOD mice (Hermitte et al., 1995; Coleman et al., 1990). A significant reduction in the number of beta-cells invaded by macrophages was observed. However, the protective effect of the protein hydrolysate could not be explained by alterations in immune response directly attributed to Pregestimil (Hermitte et al., 1995).

These early animal studies suggest that intact cow's milk protein may in some way contribute to the expression of IDDM. Elimination of intact cow's milk protein and/or other potential diabetogenic agents present in the natural diet may confer protection (Elliott et al., 1988). Avoidance of the intact milk proteins was most effective in reducing incidence of IDDM when done during the pre-weaning period. The protective effect of the hydrolyzed protein diet fed during the pre-weaning period appeared to carry over even when dairy products were reintroduced into the diet at an later age.
Epidemiological Population-Based Studies of Milk Consumption

Patterns and Disease Prevalence

IDDM is geographically related to cow’s milk consumption and infant feeding practices (Gerstein, 1994). A significant positive correlation exists between the consumption of unfermented cow’s-milk-protein and the incidence of IDDM (Scott, 1990). Data gathered on the incidence of IDDM from countries with established diabetes registries were correlated with data on cow’s milk consumption obtained from the International Dairy Federation (Dahl-Jorgensen et al., 1991). A direct linear relationship was observed in the incidence of IDDM and cow’s milk consumption ($r = 0.96$), Figure 2. In Japan, where milk protein consumption is estimated to be below 5 grams per day, approximately 1 person per 100,000 will develop IDDM. Of countries extensively studied, this reflects the lowest incidence of IDDM and milk consumption worldwide. Americans consume 19 grams of milk protein per day, and the incidence of IDDM rises to 15 cases per 100,000. The highest prevalence of IDDM is reported in Finland of 28 cases per 100,000 where milk consumption is estimated to provide 30 grams of protein per day (Cerrato, 1993).

A correlation between milk protein consumption and incidence of IDDM from region to region within the same country having a genetically homogenous population has been demonstrated by Fava, Leslie, and Pozzilli (1994). The incidence of IDDM in children 0 to 14 was reported to vary significantly across regions within Italy and was highly correlated with fluid milk consumption ($r = 0.84$, $p < 0.004$). The highest incidence of both IDDM and cow’s milk consumption was seen in Sardinia, an area with a diabetes incidence similar to the Northern Scandinavian countries of Europe and higher than any other region of Italy.

Polynesians have a slightly higher incidence of IDDM than the Japanese, but are a low-risk population. The traditional Polynesian diet is high in fruits and fish. Following migration to New Zealand, Polynesians exhibit an incidence of IDDM consistent with the
population of New Zealand. Once weaned, the child is given the traditional local diet high in cow's milk and meat (Cerrato, 1993). This suggests that milk consumption impacts the expression of diabetes independent of ethnic background. It has been estimated that 74-94% of the geographic variation in disease incidence might be explained by differences in milk consumption (Dahl-Jorgensen, Joner, & Hansses, 1991; Gerstein, 1994).

**Case Control Studies of the Impact of Early Introduction of Cow's Milk Proteins and Risk of Insulin Dependent Diabetes Mellitus**

Early introduction of cow's milk and cow's-milk-based infant formula has been associated with an increased risk of IDDM. This is particularly true when cow's milk proteins are introduced prior to 3 months of age (Kostraba et al., 1992; Verge et al., 1992).
It can be assumed that infants who are not breast-fed or who are breast-fed for only a short period are very likely to receive cow's-milk-based infant formula as an alternative to breast milk. The first study to independently take into account both the duration of breast-feeding and the age of introduction of supplementary milk feeding and its impact on prevalence of IDDM suggested a stronger link with introduction of artificial breast milk substitutes as compared to an independent protective effect of breast-feeding. Children who were introduced to supplementary milk feedings prior to 2 months and 3 months of age had an increased risk of IDDM (odds ratio 1.54 and 1.52, respectively). Finnish children diagnosed with diabetes prior to age 7 were reported to be significantly more likely to have received supplemental milk feeding in infancy compared to control children at each interval evaluated: 2 months (15% vs. 5%, $p < 0.005$), 3 months (25% vs. 12%, $p < 0.05$), and 4 months (38% vs. 21%, $p < 0.03$). In a follow-up study, early introduction of dairy products was associated with an increased risk of IDDM in Finnish children with age of introduction of dairy products identified as the most important risk determinant of IDDM (Virtanen et al., 1993). The protective effect of breast-feeding has previously been reported to be related to the age of exposure to breast milk substitutes (Kostraba et al., 1992). Early introduction of dairy products is an independent risk factor for IDDM regardless of breast-feeding practices. This appears to be particularly true when cow's milk proteins are introduced prior to 3 months of age. Here intact milk proteins are likely to cross the mucosal barrier prior to gut closure and development of gut immunity. This, in turn, may precipitate the complex process of autoimmunity leading to IDDM.

Meta-Analysis of the Effect of Early Introduction of Cow's Milk Proteins and the Incidence of IDDM

In a recent meta-analysis of breast-feeding and use of artificial breast milk substitutes most studies have shown a weak, but significant association between infant
feeding practices and the risk of IDDM (odds ratio 1.5; Gerstein, 1994). Children with IDDM had been consistently exposed to cow's milk earlier than were healthy non-diabetic individuals. Introduction of cow's milk prior to 3 months of age was associated with an odds ratio of 1.63 for risk of IDDM. It was estimated that the risk of IDDM could be reduced by as much as 30-40% if cow's milk were eliminated from the diet for the first three months of life. Gerstein (1994) suggested that the impact of cow's milk protein in the pathogenesis of IDDM may have a much stronger link to IDDM than reported if genetically predisposed individuals were matched as controls. Only two of the studies included in the meta-analysis used family members as the control group, but no further genetic screening was performed.

**Genetic Predisposition, Cow's Milk Exposure, and Disease Risk**

The impact of genetic contribution was convincingly demonstrated by Kostraba and colleagues (1993). The effect of early introduction of cow's milk was evaluated in a population predisposed to IDDM based on HLA region genetic markers. Genetic risk was determined as high and low by the HLA-B1 marker where susceptibility is conferred by the absence of aspartate at position 57 of the HLA-DQB chain. Children exposed to cow's milk prior to 3 months of age had an increased risk of developing IDDM (odds ratio = 2.9) compared to genetically matched controls. Similarly, genetic predisposition alone accounted for a substantial increased risk (odds ratio = 3.2). The interaction between genetic predisposition and the environmental influence of early cow's milk exposure where both risk factors were present, resulted in an impressive 11-fold increased risk of IDDM compared to those having neither risk factor (odds ratio = 11.3; Kostraba et al., 1993). Clearly the impact of genetic predisposition to IDDM cannot be overlooked when attempting to evaluate the role of environmental factors in the immunopathogenesis of IDDM.
Immunological Response to Cow's Milk Proteins

Introduction of cow's milk to the infants' diet soon after birth results in systemic cow's milk protein antibody production (Saukkonen et al., 1994). The pattern of antibody response to cow's milk proteins was reported as early as 1971 by Kletter, Gery, Freier, and Davies. Neonates produce antibodies to cow's milk proteins. The peak rise in IgG antibodies occurred at three 3 months of age, followed by an IgA peak at 7 months of age, and then gradually tapered off (Kletter et al., 1971). IgG antibodies were markedly higher than antibodies in other classes. The IgM response was small. These observations were again independently reported by Eastham and colleagues (1978). Infants initiated on cow's-milk-based formula at birth had a steady increase in cow's milk protein antibodies during the first 3 months of life. Antibody titres tended to plateau at 3 months and show a downward trend by 1 year of age. The decreased immunological response after the period of infancy may be attributed to a change in the permeability of the gut to protein, decreased milk consumption following 1 year of age, or the development of a state of tolerance toward milk protein (Kletter et al., 1971).

The presence of cow's milk protein antibodies was followed for a period of 28 months in healthy infants in order to compare the impact of breast-feeding and bottle-feeding practices (Tainio et al., 1988). Low levels of IgG cow's milk antibodies were detected at birth in all infants. Small amounts of BSA and other cow's milk proteins have been detected in human breast milk. IgG levels, in exclusively breast-fed infants, remained low over the 9-month period. The greatest rise in IgG antibody titres was seen in infants exposed to formula prior to 30 days of age. This observation may be due to decreased mucosal barrier protection allowing absorption of greater quantities of intact antigens. Mucosal secretory IgA in the developed gut combines with luminal proteins inhibiting their absorption. The secretory immune system is not developed fully in
neonates (Walker, 1985). Mean IgG levels peaked in all infants at 4 to 5 months following initial exposure to formula. A second peak was observed that corresponded to initiation of whole cow’s milk, which may have stronger antigenicity than processed formulas (Tainio et al., 1988). IgA and IgM antibodies were absent at birth and were not a differential marker of cow’s milk exposure (Tainio et al., 1988). Proliferation of blood mononuclear cells, in response to lactoglobulin, has been reported to be prominent in the infants at 9 months exposed to intact cow’s-milk-based infant formula (Virtanen & Aro, 1994).

The Nationwide Childhood Diabetes in Finland study revealed that early introduction of dairy products and high milk consumption was positively associated with higher antibody titres. β-lactoglobulin antibody titres were shown to be inversely related to the duration of breast-feeding and positively correlated with the incidence of IDDM (Virtanen et al., 1994). These findings are consistent with work previously reported by Dahlquist, Savilahti, and Landin-Olsson (1992), who found a significant risk associated with age of introduction of formula and levels of β-lactoglobulin IgG and cow’s milk protein IgA. Insulin autoantibodies, BSA IgA antibodies (odds ratio 1.59), β-lactoglobulin (odds ratio 1.44), IgM antibodies to cow’s milk formula (odds ratio 0.62), but not islet cell antibodies, were affected by the age of introduction of dairy products. High IgA antibodies, in particular to BSA and β-lactoglobulin, have been associated with increased risk of IDDM (Dahlquist et al., 1992; Virtanen et al., 1994). Cow’s milk protein antibodies of several types including anti-BSA and anti-β-lactoglobulin have been shown to be correlated with each other and to islet cell antibodies (Dahlquist et al., 1992). This lends further support to earlier findings of elevated antibody titres to both β-lactoglobulin and to BSA found in new onset diabetic humans and animal models (Martin et al., 1991).

Children with early onset IDDM, 0 to 5 years, have increased levels and frequency of antibodies to a variety of milk proteins (Savilahti et al., 1988). Supranormal and
significantly higher levels of IgA antibodies to cow's milk were detected in diabetics ($p = 0.0002$) while IgG levels were similar in both diabetics and controls. Both IgG and IgA antibodies to β-lactoglobulin were higher than age matched controls. The authors implied that these results might be attributed to increased cow's milk consumption, increased immunological reactivity to cow's milk proteins, or greater than normal gastrointestinal permeability to cow's milk proteins. Interestingly, immune response to cow's milk did not correlate with the presence or absence of HLA-DR antigens (Sanilahti et al., 1988). In a subsequent study by the same research group, diabetic children less than 3 years of age had significantly elevated median levels of IgG ($p = 0.030$) and IgA antibodies ($p = 0.002$) to cow's milk and IgG antibodies to β-lactoglobulin ($p = 0.03$; Savilahti et al., 1993). In the youngest age group, the antibody levels were six to seven times higher than the reference population. Diabetic children 3.0 to 6.9 and 7.0 to 14.9 years of age also exhibited significantly elevated levels of IgA antibodies to cow's milk and β-lactoglobulin, although the observed values were somewhat closer to the control population than in the youngest age group. IgG and IgA β-lactoglobulin antibodies were significantly associated with risk of IDDM at a young age independent of islet cell antibody status and early weaning to cow's milk formula. An abnormally strong humoral immune response to cow's milk-proteins is particularly apparent in the youngest patients diagnosed with IDDM. However, the authors concluded that these findings do not suggest a direct autoimmune process of pancreatic beta-cell destruction due to marked overlap in antibody titres between diabetics, siblings, and controls. Further, antibody titres did not fall as IDDM progressed in this population despite progressive destruction of the pancreatic beta-cells (Savilahti et al., 1993).

In summary, the immunological studies reviewed suggest a contribution of milk proteins to the pathogenesis of IDDM. Immunological responses to milk proteins appear to occur with early exposure to intact protein prior to gut closure and full development of gut
immunity. Exacerbated immunological responses to cow's milk protein in patients newly diagnosed with IDDM, increased risk of IDDM with early introduction of cow's-milk-based infant formula, and correlation of geographical patterns of milk consumption with prevalence of IDDM lend support to the involvement of bovine proteins in the autoimmune pathogenesis of IDDM. The effects of infant feeding practices on the autoimmune pathogenesis of IDDM appear to be most pronounced in cases diagnosed prior to age 5.

Conflicting Reports on the Role of Cow's Milk Protein in the Pathogenesis of Insulin Dependent Diabetes Mellitus

A review of several animal and human studies provides some evidence that does not support the hypothesis that milk proteins are involved in the pathogenesis of IDDM. One animal study has been cited repeatedly that reported that an addition of 10% skim milk powder to rat chow containing 10% casein did not increase the incidence of IDDM in NOD mice. However, the authors noted that it may have been difficult to discriminate the additional contribution of skim milk powder to an already diabetogenic mixed diet (Coleman et al., 1990). All other animal studies have shown cow's milk protein to be a potent contributor to risk of IDDM.

Epidemiological studies showing a positive correlation between cow's milk intake and incidence of IDDM have been criticized for failing to account for genetic bias or the age of exposure to cow's milk proteins (Ellis & Atkinson, 1996). Sardinia, a large Mediterranean island, has a high diabetes incidence second only to Finland. The Sardinian pathological profile is similar to that of Finland since both are characterized by high levels of islet cell antibodies. While the incidence of IDDM correlates with the consumption of dairy products, the actual prevalence of IDDM was cited as being three times higher than could be predicted by milk consumption alone (Muntoni, Loddo, Stabilini, Stabilini, & Muntani, 1994).
relatively small study group, particularly of young diabetic patients less than 3 years of age (Savilahti, Saukkonen, Akerblom, Virtanen, & Dahlquist, 1994). This may, in part, explain the lack of significant increases in the level of IgG antibodies to β-lactoglobulin and a relation to infant feeding practices.

Savilahti and colleagues (1994) argued that antibodies to milk proteins may not be directly associated with the disease process, but rather provide an early and potent immunization of individuals prone to develop IDDM. Increased antibodies to cow’s milk proteins, including BSA, could result from a nonspecific immune response at the onset of IDDM. Alternatively, reports of a recent episode of gastroenteritis prior to the onset of clinical symptoms of IDDM may result in an elevation of cow’s milk and other food antibodies reported in newly diagnosed diabetics (Kostraba, 1994), but not necessarily contribute to the pathogenesis of the disease. Determining whether these antibodies to cow’s milk antigens, in particular BSA and the ABBOS peptide, are present prior to beta-cell damage and clinical onset of disease may help to clarify this issue. It is also unclear if certain HLA genes are required for the formation of antibodies to cow’s milk proteins (Kostraba, 1994).

The most recent study to address the issue of association between early exposure to cow’s milk, and in this case beta-cell autoimmunity rather than clinical IDDM, used a cross-sectional design with retrospective analysis in genetically predisposed individuals (Norris et al., 1996). Of 253 children from families with IDDM, only 18 exhibited beta-cell autoimmunity. Only 3 of these had been exposed to cow’s milk prior to 3 months of age. There were no significant differences between children up to 7 years of age exhibiting beta-cell autoimmunity and control children in response to cow’s milk exposure prior to either 3 or 6 months of age. In fact, there were no differences seen with any other dietary antigens including cereals, fruits, vegetables, or meat proteins. This is in conflict to all animal studies that have shown that intact proteins from a number of sources appear to be
required for full expression of IDDM. Of note, children who were previously identified as having diabetes related antibodies were excluded from the study. Since the autoimmune diabetic prodrome is known to be long and is proposed to be initiated in infancy, it would seem most of the high-risk children who were likely to develop IDDM prior to age 5 were excluded from the study. In addition, the lack of a positive contribution of early infant feeding practices and IDDM may be due to the intermediate marker of beta-cell autoimmunity, in which less than half of positive subjects will progress to IDDM within 5 years. Progression to IDDM, even in beta-cell autoimmune positive subjects, may require a second environmental factor (Norriss et al., 1996). In addition, as previously noted, genetic predisposition may be required for early exposure to cow’s milk to contribute to the etiology of diabetes (Kostraba, 1993).

**Summary of Early Exposure to Cow’s Milk Proteins**

Supplementary feeding prior to 3 to 4 months of age is more likely to show a significant effect on the incidence of IDDM if formula feeding is limited to intact cow’s-milk-based products (Dahlquist et al., 1992; Kostraba et al., 1992). Infant formulas are likely to be heterogeneous in terms of ingredients, processing, and treatment. Studies using age as a marker of gut maturity may also be subject to error (Kostraba, 1993) since gut closure is variable over the first year of life. Components of the infant diet are not independent in terms of their contribution to risk of IDDM. This has been demonstrated by a significant correlation between the duration of breast-feeding and age of exposure to cow’s milk protein ($p < 0.01$; Norris et al., 1996). Gerstein (1994) concluded from a comprehensive meta-analysis of breast-feeding incidence and duration and the age of introduction of cow’s milk protein that there was a 1.5-fold increase in the incidence of IDDM with a history of early exposure to cow’s milk and short duration of breast-feeding. Similar findings were reported by (Norris & Scott, 1996). In addition, elevated risk of
IDDM was consistently associated with age of first exposure to breast-milk substitutes when this occurs prior to 6 months of age (Norris & Scott, 1996). The impact of early introduction of cow's milk proteins in the autoimmune pathogenesis of IDDM is likely to be most notable in those developing IDDM in early childhood.

There is mixed but substantial evidence to suggest that early introduction of cow's milk protein may be linked to the complex autoimmune pathology of IDDM. A large, prospective, randomized trial has been initiated in the Scandinavian countries to determine whether providing a diet of breast milk and/or a diet devoid of intact cow's milk protein will decrease the incidence of IDDM in those who are genetically at risk (Akerblom, Dosch, et al., 1993). Evaluation of the interaction of interrelated environmental factors (e.g., breast-feeding and introduction of infant formula) and genetic predisposition should provide insight into this complex issue.

**Bovine Serum Albumin**

The whey protein, bovine serum albumin (BSA), has been implicated as the most likely dietary factor involved in the pathogenesis of IDDM. Serum albumin is present in a higher concentration in cow's milk compared to rat and human milk. BSA consists of 608 amino acids and is immunologically distinct from human serum albumin showing little or no cross-reactivity (American Academy of Pediatrics, 1994). There are several sequences of BSA that differ from human serum albumin. The peptide sequence, ABBOS, containing 17 amino acids in the region of the BSA molecule from residues 152 to 168 (FKADEKKFWGKLYEIAVR) accounts for the major sequence differences between human, cow, rat, and mouse albumin (Bach, 1994; Dosch et al., 1992; Glerum, Robinson, & Martin, 1989). Where the deviation between the amino acid sequences of human and bovine serum albumin is the greatest, lies the area most likely to act as an antigenic epitope (Glerum et al., 1989).
It is proposed that an antigen-driven, selective immune response may initiate the autoimmune process implicated in the pathogenesis of IDDM (Dosch et al., 1992). The ABBOS peptide has been identified as a possible trigger for IDDM based on a cross-reactive immune response between the peptide and a beta-cell membrane antigen (Karjalainen, Martin et al., 1992; Karjalainen, Saukkonen, Savilahti, & Dosch, 1992).

Antibodies to BSA have been shown to recognize islet cell surface proteins, in particular ICA p69. Immaturity of the gastrointestinal immune system and selective resistance of BSA to enzymatic hydrolysis by trypsin may allow passage of intact BSA and/or the ABBOS epitope into the blood stream conferring an abnormal immunological response that results in pancreatic beta-cell destruction and insulin deficiency.

**Proposed Mechanism of BSA-Induced Autoimmunity in Insulin Dependent Diabetes Mellitus**

The molecular basis for the immunological response to BSA has been attributed to the observation that the beta-cell autoantigen ICA 69 and BSA share three regions of short sequence homology. One region lies between amino acids 38 and 47 (iKAtgKKede) and amino acids 152 and 161 of the BSA ABBOS peptide (fKAdeKKfwg; Figure 3; Eisenbarth, Gianani, Pugliese, Verge, & Pietropaolo, 1994; Pietropaolo et al., 1993).

Anti-BSA antibodies have been shown to cross-react with a rat islet beta-cell protein of 67-69,000 molecular weight. Immunization of animals with the ABBOS peptide results in production of antibodies that are able to react with the p69 beta-cell surface protein (Fourth International Onnela Workshop, 1990; Karjalainen, Saukkonen, et al., 1992).

Specifically, the N-terminus of the beta-cell protein p69 has an amino acid sequence homologous to the ABBOS peptide. T-cells that are reactive to a synthetic analog of this region have been detected in preliminary evaluation of eight of eight children recently diagnosed with IDDM (Cheung et al., 1994). A cow's-milk-induced anti-islet T-cell
response could precipitate beta-cell destruction through molecular mimicry due to the ABBOS sequence common to both BSA and a 69-kD beta cell protein (Figure 3; Bach, 1994; Karjalainen, Martin, et al., 1992). The 69 kD beta-cell autoantigen (p69) has been reported to be identical to islet cell antibody ICA 69 (Miyazaki et al., 1995). If the ABBOS peptide were to be introduced through a leaky immature gut in early infancy, it could induce an immune reaction that could be boosted by ICA 69, induce beta-cell damage, and ultimately set in motion the complex sequence of events leading to IDDM (Dahlquist, 1994; Karjalainen, Martin, et al., 1992).

Beta-cell p69 does not appear to be a native protein but rather is induced in islet cells by gamma-interferon stimulation, which functions to mediate inflammatory responses (Glerum et al., 1989; Karjalainen, Martin, et al., 1992). It is suggested that during the prediabetic phase, the body releases gamma-interferon upon exposure to viruses. The gamma-interferon results in the presentation, or studding, of the beta-cell surface with p69. Primed by exposure to the ABBOS epitope of BSA, the immune system now sees the p69 protein as foreign and ultimately mounts a response that destroys the islet cells (Rennie, 1992). This immunological response, known as molecular mimicry, underlies the hypothesis by which BSA triggers the immunopathogenesis of IDDM.

Interaction of Genetic Predeterminants of Insulin Dependent Diabetes Mellitus, Exposure to BSA, and Islet Cell Autoimmunity

Immunological consequences of oral exposure to BSA are impacted by genetic factors that predispose individuals to IDDM (Saukkonen et al., 1994). A region of BSA is homologous to the β-subunits of MHC class II proteins Ia, DQ, and DR. The overlapping region lies between amino acids 157 to 175 (Glerum et al., 1989; Martin et al., 1991). A molecular basis for BSA binding to MHC class II proteins and affecting antigen presentation has been proposed by Dosch and colleagues (1992). Cells containing MHC
class II antigens appear to produce antibodies and cytotoxic T-lymphocytes against the region of BSA which shares a common epitope with the MHC class II protein. Isolation of islet cells from the BB rat using rat anti-BSA antiserum revealed a cross-reacting protein (Mr = 69,000). The structure of the beta unit of the islet cell protein was found to have a region of homology in the DQ and DR region with BSA. Further analysis of the amino acid homology in relation to the combined DR/DQ human allotype was strongly correlated with BSA and the incidence of IDDM. Individuals with poor DR and DQ homology appear to be protected against the anti-BSA autoimmune response (Glerum et al., 1989).
Antigen presentation of ABBOS by diabetes associated MHC-class II molecules and a delay in oral (or mucosal) tolerance underlies the BSA hypothesis. Environmental antigens are presented to T-cells in conjunction with HLA class II antigens. DR antigens play a major role in antigen presentation within the intestinal epithelial cells. It is here that the primary immune reaction to oral BSA takes place (Saukkonen et al., 1994). The single highest marker of diabetes risk is DQ\(\beta\) non-aspartate 57, which also marks susceptibility to IgA deficiency, a regulatory abnormality in mucosal immunity (Karjalainen, Saukkonen, et al., 1992).

Human albumin has been noted to have an aspartic acid residue at position 57 of the DQ\(\beta\) chain while BSA has an alanine at the 57 position. The ABBOS epitope appears to show sufficient homology to the available human DQ\(\beta\) structure to fit into the peptide-binding groove of the MHC class II molecule. The antigenic epitope appears only to fit when the key aspartate 57 residue is replaced with either alanine or serine. Non-aspartate-57 of the DQ\(\beta\) chain is a highly predictive marker of genetic predisposition to IDDM and has been reported to be present in 96% of patients with IDDM (Dosch et al., 1992). In humans, DR and DQ allotypes have been shown to be strongly correlated with the incidence of IDDM (Robinson et al., 1993). Diabetes associated DR alleles show strong affinity for BSA and its peptides (Saukkonen et al., 1994).

Presentation of the ABBOS peptide is particularly likely to occur in the intestine where surface epithelial cells express DR antigens. The predisposition to IDDM is based on the identity of MHC class II proteins, interaction between and self-recognition of the islet beta-cell membrane antigen peptide, and the strength of the immune response to the ABBOS peptide presented by DR rather than DQ. Non-aspartate-57 DQ individuals (homozygous), at high risk of developing IDDM, would have poor tolerance to ABBOS due to poor presentation and failure to arrest induction of self recognition and tolerance. Strong tolerance is conferred by the presence of Asp 57 on DQ. This model not only
provides a role of ABBOS in the pathogenesis of IDDM, but also provides evidence for and interaction between cow's milk exposure and the genetic factors contributing high susceptibility to IDDM. The presence of both DR and DQ allotypes and the lack of aspartate at position 57 on the DQβ chain confer high risk of IDDM (Robinson et al., 1993).

The theory is that if islet cell autoantigen p69 is displayed on the beta-cell prior to exposure to milk proteins, specifically ABBOS, the infant's immune system will see the islet cell protein p69 as self. However, if the exogenous ABBOS epitope is presented to the immune systems first, the infant is likely to establish an immune response to this foreign peptide (McCarren, 1992). Through the process of molecular mimicry, the sensitization to ABBOS now makes the beta-cell vulnerable to attack by the host immune defense mechanisms. In this model, exposure to dietary BSA in infancy would mediate a T-cell response prior to establishment of p69 self-tolerance (Cheung et al., 1994). BSA-induced autoimmunity is boosted by gamma-interferon-induced expression of MHC class II antigens on the surface of the beta-cell supporting a complex series of events that ultimately leads to beta-cell destruction.

**Bovine Serum Albumin Triggered Islet Cell Autoimmunity: A Hypothesis**

A hypothesis has been proposed by Karjalainen, Martin, and colleagues (1992) that relies on an elaborate sequence of events that ultimately results in the destruction of the beta-cells and concomitant clinical diabetes. The ABBOS peptide crosses the immature gastrointestinal tract intact. This peptide is immunogenic in individuals genetically prone to develop IDDM. Those individuals with the diabetes-associated HLA Class II (DR/DQ) haplotypes are able to bind and present this antigen. Exposure leads to immunological sensitization and development of memory cells for ABBOS. Since ABBOS and p69 share a common epitope, p69 should be able to boost and sustain ABBOS-specific immune
memory following gut closure. The cross-reactive beta-cell p69 maintains the antibody response until the destruction of the islet cell is complete and p69 is no longer available. Elevated gamma-interferon, associated with infections, would induce expression of p69 on the surface of the beta-cell (Dosch, 1993; Karjalainen, Martin, et al., 1992; Karjalainen, Saukkonen, et al., 1992). Presence of p69 activates beta-cells, making them transiently subject to immune attack. The ABBOS-mediated immune response could cause destruction of the beta-cells by cross-reactivity with p69 and function as the possible molecular mimicry epitope (Karjalainen, Saukkonen, et al., 1992). The long preclinical course could be explained by the short-lived, but recurrent, nature of episodic expression of p69 on the pancreatic beta-cells (Karjalainen, Martin, et al., 1992). Induction of p69 expression by gamma interferon by variable and etiologically unrelated infections would impact progression to overt clinical disease. Recruitment of additional pathogenic (p69-specific) and disease-associated islet cell antibody-specific lymphocytes accelerates beta-cell destruction (Akerblom, Dosch, et al., 1993). The long, latent period of IDDM of 8 to 10 years suggests the need for multiple viral events to ultimately produce destruction of 90% of islet cells associated with clinical disease. This hypothesis is enticing as it takes into account genetic predisposition, autoimmune mechanisms, and the environmental influence of both cow’s milk protein and exposure to viral infections.

A Review of the Critical Evidence Implicating Bovine Serum Albumin as a Causative Agent in the Pathogenesis of Insulin Dependent Diabetes Mellitus

Animal Studies

Early experimental studies attempting to establish a link between BSA and IDDM were conducted by Elliott and Martin using the spontaneously diabetic BB rat model (1984). A significant increase in antibodies to BSA was found at the time of diagnosis in
all rats progressing to IDDM. Rats that became diabetic were shown to have the highest titres of anti-BSA-antibodies at 90 days compared to those with only a subclinical picture. Peak anti-BSA-antibody titres were observed at 120 days (Elliott & Martin, 1984). Sera from normal and diabetic Sprague Dawley rats has been shown to contain anti-albumin antibodies which bind to albumin contaminated rat islet preparations (Marshall & Gotfredsen, 1986). BSA-IgM antibodies have also been detected at significantly higher levels in diabetic versus nondiabetic rats (Scott et al., 1989).

NOD mice have also been used to study the potential contribution of BSA in the autoimmune pathogenesis of IDDM due to their known exaggerated autoimmune responses to exogenous antigens (Beppu, Winter, Atkinson, Fujita, & Takahashi, 1986). Beppu and colleagues (1987) found abnormal immunological responses to BSA in NOD mice, which included high levels of BSA binding and the presence of anti-BSA antibodies. Mice developing diabetes exhibited the highest BSA binding.

While critics of the BSA hypothesis have argued that the presence of BSA antibodies prior to the clinical onset of IDDM has not been established in humans, anti-BSA antibodies have been detected prior to diagnosis of diabetes in rodent models. These findings suggest a possible role of BSA in the pathogenesis of IDDM (Beppu et al., 1986). Anti-BSA antibody titres have shown to be inversely proportional to the age of disease presentation in biobreed rats (Martin et al., 1991). Withholding dietary BSA has been shown to prevent IDDM in experimental animals (Karjalainen, Saukkonen, et al., 1992). Immunization to BSA may be the result of defective immuno-regulation or tolerance to ingested antigens (MacLaren & Atkinson, 1992). This is supported by the observation that induction of tolerance in early life with BSA has been shown to prevent the development of IDDM in diabetic prone rodents (Akerblom, Dosch, et al., 1993; Karjalainen, Saukkonen, et al., 1992). Tolerance induced by BSA in laboratory animals provides evidence that BSA is involved in the pathogenesis of IDDM (Dosch et al., 1992).
Demonstrated Autoimmunity to Bovine Serum Albumin in Newly Diagnosed Diabetics

**Anti-BSA antibodies.** The pioneering work establishing the presence of BSA autoimmunity in patients with IDDM was conducted by Karjalainen, Martin, and colleagues (1992). Serum concentrations of antibodies against BSA were found to be significantly higher in newly diagnosed diabetics than in control children. Elevated serum concentrations of IgG and IgA anti-BSA antibodies were detected in 44 (100%) of newly diagnosed diabetics by particle concentration fluorooimmunoassay (PCFIA) compared to only 4% of control subjects. A significant note regarding methodology was reported. PCFIA had both a high specificity and sensitivity compared to enzyme-linked immunosorbant assays (ELISA). Only 25% of diabetic children in the same population tested positive for anti-BSA antibodies using ELISA with as many of 10% of the control children sera positive (Karjalainen, Martin, et al., 1992).

Saukkonen and colleagues (1994) detected significantly elevated levels of IgG and IgA immunoglobulin antibodies to BSA using ELISA in newly diagnosed diabetic children less than 7 years of age than in unrelated control children \((p = 0.0001; \ p = 0.0009,\) respectively). In contrast to earlier reports by Karjalainen, Martin and colleagues (1992), similar elevations in levels of IgG and IgA antibodies to BSA were obtained using PCFIA.

Elevated IgG antibodies to BSA have consistently been found to be significantly higher in newly diagnosed diabetic children compared to the nondiabetic, normoglycemic control population in several independent studies (Dosch et al., 1992; Luhder et al., 1994; Pardini et al., 1996; Pigny, Morteux, Racadot, Stuckens, & Boersma 1995). Sheard (1993) has reported the mean anti-BSA antibody titres to as much as seven-fold higher in children with diabetes compared to healthy controls. Significantly higher titres of IgG and IgA class antibodies to BSA have been found in newly diagnosed diabetics across all age groups as compared to the reference population (Saukkonen et al., 1994). These findings are supported by the work of Krokowski and colleagues (1995) who reported that levels of
anti-BSA antibody were significantly higher in those with adult onset IDDM than in healthy control subjects ($p < 0.01$).

**Evidence of the presence of anti-BSA antibodies prior to disease onset.** Early studies criticizing the BSA hypothesis in the pathogenesis of IDDM point to the lack of evidence in human studies confirming the presence of BSA autoimmunity prior to the onset of clinical symptoms. Saukkonen and colleagues (1994) followed siblings of diabetic probands for 10 years following diagnosis. Siblings who developed IDDM during the follow-up period were significantly more likely to have had positive anti-BSA antibody titres during the study period than those siblings who remained disease free (60% and 34% respectively; $p = 0.04$). Krokowski and colleagues (1995) found two out of five diabetic patients had significant anti-BSA antibody titres prior to clinical onset of the disease.

**Presence of antibodies directed specifically against the ABBOS peptide.** Elevated serum concentrations of IgG anti-BSA antibodies were detected in 100% of patients with IDDM. The bulk of these antibodies were reported to be directed against the ABBOS epitope. Low levels of anti-BSA antibody, significantly lower than in newly diagnosed diabetics, were found in all control subjects, and only 2.5% of this population expressed ABBOS-specific IgG antibodies (Karjalainen, Martin, et al., 1992). At the time of diagnosis, diabetic children have been shown to have higher mean anti-ABBOS-antibody levels of IgG, IgM, and IgA isotypes ($p < 0.001$). By 3 months after initial diagnosis of IDDM, these begin to fall. ABBOS antibodies were shown to correlate with anti-BSA antibody levels for all isotypes at the time of diagnosis and at the 3-month follow-up ($p < 0.0001$). These data suggest a heavy bias towards in vivo selection of ABBOS-specific clones (Karjalainen, Saukkonen, et al., 1992).

A direct link between the ABBOS epitope and pancreatic beta-cells was elucidated with first polyclonal and then monoclonal anti-ABBOS antibodies. The anti-ABBOS antibodies were shown to recognize an epitope on p69 an interferon-inducible beta-cell

T-cell response to BSA. IDDM is a T-cell mediated disease and early invasion of insulin producing beta-cells by macrophages and T-cells is critical to the pathogenesis and progression of the disease. Specific T-cell response to BSA are consistent with an autoimmune process where exposure to BSA has failed to induce normal tolerance. BSA reactive T-cells have been reported to be present in 68 of 78 children at the time of diagnosis of IDDM (Miyazaki et al., 1995). T-cell recognition was demonstrated to islet cell antigen p69, specifically T-cell epitope p69 (Tep69) located near the N-terminus of the antigen. Tep69 has a homologous region to BSA, which was recognized by isolated lymphocytes from patients with diabetes but not by lymphocyte extractions from non-diabetic patients. BSA causes T-cell proliferation. The p69 protein induces early activation of T-cells but insufficient interleukin-2 production to warrant anergy. Normally, the high affinity self-peptide results in anergy, but the low-affinity mimicry antigen (i.e., BSA) causes T-cell proliferation (Miyazaki et al., 1995). These findings are consistent with the hypothesis proposed by Karjalainen, Saukkonen, and colleagues (1992) suggesting that the autoimmunity induced by BSA results from molecular mimicry.

Cheung and colleagues (1994) were able to detect BSA-specific T-cell activity and were further able to map the response to the ABBOS epitope (pre-BSA position 152-169). ABBOS-sensitive T-cells were found in 28 of 31 children with new onset IDDM, but were absent in controls. The magnitude of proliferative responses to BSA and ABBOS were similar. There were no cumulative antigenic responses of BSA and ABBOS further suggesting that the ABBOS epitope is the immunologically active peptide on BSA. The
decline in responsiveness to BSA and ABBOS with no patient remaining positive for one and negative for the other. The presence of BSA ABBOS peptide specific T-cells strengthens the immunological link between BSA and IDDM (Cheung et al., 1994).

**Early onset IDDM.** The frequency and titres of IgG and IgA antibodies to BSA in newly diagnosed diabetics compared to nondiabetic controls has been demonstrated to be significant across all age groups (Krowkowski et al., 1995; Pardini et al., 1996; Saukkonen et al., 1994). However, Saukkonen and colleagues (1994) found the difference in antibody titres to be greatest in the youngest age group where children ranged from 0.8 to 2.9 years of age. Further, with adult onset IDDM incidence and titres of anti-BSA antibodies were found to be lower than previously reported in children (Krokowski et al., 1995). These findings may not be surprising given that the likelihood of BSA contributing to the pathogenesis of IDDM is more relevant to those who develop IDDM at a younger age. Anti-BSA antibody titres have been shown to be inversely correlated with age of diagnosis (Pardini et al., 1996). Higher insulin antibody titres have also been observed in juvenile onset versus adult onset IDDM (Krokowski et al., 1995). In addition, levels of anti-BSA antibodies prior to diagnosis of diabetes in the BB rat model have been shown to be inversely proportional to the age of disease presentation (Martin et al., 1991). These findings are consistent with a review of earlier epidemiological studies reporting that the association of IDDM and early cow's milk exposure decreases with increasing age of diagnosis of the disease (Gerstein, 1994). IDDM-associated BSA autoimmunity appears to be most remarkable in the youngest patients.

**Mechanism of BSA induced autoimmunity.** Elevated concentrations of IgG and IgA isotypes to BSA are implicated to be consistent with an antigen driven response (Karjalainen, Martin, et al., 1992). Anti-BSA antibodies were shown to cross-react with islet antigens demonstrated by the observed binding of anti-BSA antibodies from newly diagnosed patients with IDDM to ICA p69 (Karjalainen, Saukkonen, et al., 1992).
Savilahti and colleagues (1993) suggested that the levels of BSA and ABBOS antibodies are reinforced by an ongoing autoimmune process in the pancreatic islet cells. They suggested that there is a specific response to BSA and not a general immune response to all cow's milk proteins in patients with IDDM given that antibodies to other cow's milk proteins were not detected or not detected at significant levels (Karjalainen, Saukkonen, et al., 1992; Sheard, 1993). Mononuclear cells from 90% of children with newly diagnosed IDDM have been reported to show a significant proliferative response to BSA but not to several other albumins (p < 0.0001; Cheung et al., 1994).

**Association between anti-BSA antibodies and other immunological markers of disease risk.** Luhder and colleagues (1994) have reported increased levels of anti-BSA antibodies in newly diagnosed diabetics over controls (p < 0.01). However, there was no association between anti-BSA antibodies and islet cell reactive antibodies, cytoplasmic islet cell antibodies, and glutamate decarboxylase autoantibodies, all of which were detected at significantly higher levels in diabetics. These findings are consistent with earlier reports that failed to show an association between anti-BSA and ABBOS antibody titres with either islet cell antibodies or insulin autoantibodies (Karjalainen, Martin, et al., 1992). Given the lack of correlation with BSA antibodies and other known markers of IDDM, Luhder and colleagues (1994) proposed a slightly different alternative mechanism of action for elevations in anti-BSA antibodies. They suggested that the humoral immune response to BSA in IDDM may reflect a nonspecific defect of the immune system.

Anti-bovine serum albumin antibodies were measured in sera by particle concentration fluoroimmunoassays and compared with the presence of islet cell antibodies and human leucocyte antigen (HLA)-DGB genotypes (Levy-Marchal, Karjalainen, Dubois, Czernichow, & Dosch, 1995). Sera from French children who were newly diagnosed with IDDM were compared to nondiabetic children with positive islet cell antibodies, and to islet cell negative healthy control children. Elevated anti-BSA antibodies were found in 74% of
diabetic children compared to 5.5% of controls. Approximately two thirds of anti-BSA positive diabetic children were also positive for islet cell antibodies. These findings are in contrast to those reported by Luhder and colleagues (1994), who failed to show correlation between anti-BSA antibodies and islet cell antibodies. Elevated IgG anti-BSA antibodies were associated with IDDM in the French population, which lends support to the hypothesis that BSA plays a role in diabetic autoimmunity (Levy-Marchal et al., 1995).

**Genetic markers and BSA induced autoimmunity.** The pioneering research of Karjalainen, Saukkonen, and colleagues (1992) revealed no correlation between anti-BSA-antibodies and any diabetes associated combination of HLA haplotypes. A similar disassociation has been reported between islet cell antibodies and HLA haplotypes. This lack of association of immunological responses to milk proteins with HLA genetic markers has previously been observed (Karjalainen, Martin, et al., 1992). The authors suggested that HLA haplotypes are likely required during the initiation of the immune response specific to BSA and ABBOS, but they do not appear to determine its intensity.

Krokowski and colleagues (1995) were the first to report genetic interactions between the BSA antigen and known HLA markers of IDDM. Anti-BSA antibody titres were shown to be higher in HLA-DR3 and/or DR4 positive patients compared with DR3 and/or DR4 positive control subjects ($p < 0.001$). Further, the interaction of autoimmunity to BSA and genetic predisposition is highlighted by the observation that antibody titres to BSA were four-fold higher in DR3 and/or DR4 positive patients than DR3 and/or DR4 negative patients. A stronger link appears to exist with DR3 positivity found in 9 of 10 anti-BSA positive diabetics (Krokowski et al., 1995). This high level of significance may again point to the need to use genetically predisposed individuals as the control group when trying to isolate the specific impact of BSA on the autoimmune pathogenesis of IDDM.
Predictive and Diagnostic Value of Anti-Bovine Serum Albumin Antibodies

While anti-BSA antibodies are detected more frequently in patients with IDDM, particularly newly diagnosed cases, anti-BSA antibodies do not appear to be sensitive or specific biological markers of IDDM (Pardini et al., 1996), and do not reflect an independent risk factor for diabetes (Saukkonen et al., 1995). No patient with IDDM was found to be positive for anti-BSA antibodies in the absence of other antibody markers of IDDM. Presence of anti-BSA antibodies has no additional diagnostic value in IDDM (Luhder et al., 1994). There was no correlation with the presence of islet cell antibodies and there was overlap with control subjects (Krokowski et al., 1995). There is no evidence that members of the general population positive for anti-BSA antibodies would have the potential to develop IDDM in the absence of genetic predisposition. Anti-BSA antibodies do not appear to be a relevant predictive marker of IDDM.

Conflicts and Controversies Surrounding the BSA Hypothesis

Controversy still surrounds the role of BSA, if any, as a causative agent in the pathogenesis of IDDM. In contrast to previous reports that have shown that BSA can induce tolerance in laboratory animals (Akerblom, Savilahti, et al., 1993; Dosch et al., 1992; Karjalainen, Saukkonen, et al., 1992), Petersen and colleagues (1994) failed to demonstrate that neonatal tolerance could be induced by BSA in NOD mice. No correlation was found between the BSA antibodies and the development of IDDM (Petersen et al., 1994). In both autoimmune spontaneously diabetic female NOD mice and cyclosphophamide-induced male NOD mice, casein hydrolysate has been previously reported to be almost 100% effective in preventing IDDM. When BSA was added to the casein hydrolysate, animals did not develop IDDM (Hermitte et al., 1995), suggesting that BSA is not a causative factor in the pathogenesis of IDDM. However, these reports are in
contrast to other animal studies that have shown that most cases of spontaneous diabetes in rodents are food induced (Scott, 1996), and can be induced with cow's milk protein (Elliott & Martin, 1984).

Other arguments point to the lack of evidence confirming specificity of immunity to BSA in patients with IDDM. Poccecco, Nicoloso, Tonini, Presani, and Marinoni (1991) observed elevated levels of IgA antibodies to cow's milk in 10 of 20 newly diagnosed diabetes. However, there were no significant differences between diabetic and control subjects with regard to duration of breast-feeding, history of cow's milk intolerance, presence of islet cell antibodies, and other autoantibodies (Poccecco et al., 1991).

In contrast to several epidemiological, ecological, time-series, and experimental studies, Atkinson, Koa, and MacLaren (1994) report a lack of B-cell and T-cell immunity to BSA. Ellis and Atkinson (1996) have argued against the BSA hypothesis as their research has shown similar incidence and titres of antibodies to BSA in children with IDDM and healthy control subjects (Atkinson et al., 1993). A similar lack of a significant Ig and IgG immune response to BSA was reported by Pigny and colleagues (1995). Of interest, slightly higher levels of BSA and anti-BSA antibodies have been detected in individuals with other autoimmune diseases (e.g., rheumatoid arthritis, multiple sclerosis, and systemic lupus erythematosi; Atkinson et al., 1993; Strand, 1994). In general, individuals with autoimmune diseases had antibodies with higher affinities for all antigens, therefore, antibodies to BSA in patients with IDDM have been suggested to reflect a non-specific defect in immunological tolerance associated with a predisposition to autoimmunity rather than specifically to beta-cells (Atkinson et al., 1993, 1994; Norris & Peitropaolo, 1994). Specific ABBOS sensitized T-cells have been detected, only, in children with IDDM but not with systemic lupus erythematosi (SLE) or juvenile rheumatoid arthritis (JRA) (Cheung et al., 1994).
Specificity of peripheral-blood mononuclear cell responses, supposedly stimulated in response to BSA and ABBOS, has not been established (Ellis & Atkinson, 1996). While chronic mononuclear-cell infiltration of the pancreatic islet cells has been cited as the pathological hallmark of IDDM, it is not certain whether this is the mechanism by which BSA or ABBOS exert their effects. Atkinson and colleagues (1993) found no activity to peripheral blood mononuclear cells in response to either BSA or ABBOS in patients with new onset IDDM or those at increased risk. Since other recognized islet cell antigens have been shown to stimulate peripheral blood mononuclear cells, the research by Atkinson and colleagues (1993) raised questions regarding the role of BSA and ABBOS autoimmunity in the pathogenesis of IDDM. Gut immaturity and exposure to rotavirus or other infections may allow excessive permeability of intact proteins resulting in the production of antibodies to BSA and other cow's milk proteins. Despite these findings, a high incidence of IDDM has not been reported in this population (Atkinson et al., 1993).

Critics of the work of Karjalainen, Saukkonen, and colleagues (1992), while acknowledging some merit to the scheme of BSA ABBOS peptide induced molecular mimicry with the ICA 69 islet cell protein, cast doubt on the hypothesis due to a lack of proof that the process occurs only in hosts with diabetes associated HLA class II (DR/DQ) haplotypes capable of binding and presenting the antigen and that early exposure to cow's milk initiates the process. Despite reports of uniform recognition frequencies for anti-ABBOS antibodies in patients with IDDM, only a small fraction of patients with IDDM have antibodies that react with islet-cell p69. The 100% sensitivity for anti-BSA antibodies in newly diagnosed diabetics reported by Karjalainen's and Martin's group (1992) is criticized as being remarkably high given that the "gold standard" for islet cell antibody detection is only 75%. In addition, there is no evidence that anti-BSA antibodies are present prior to beta-cell damage or clinical onset of diabetes (Norris & Peitropaulo, 1994). If BSA is indeed a principal culprit in the pathogenesis of IDDM, antibodies would likely
evaluation of the presence of islet cell antibodies, glutamic acid decarboxylase antibodies, and BSA/ABBOS antibodies (Akerblom, Dosch, et al., 1993).

Summary of Infant Feeding Practices in the Pathogenesis of IDDM

Infant feeding practices have received attention in the search to identify an environmental dietary antigenic trigger of the autoimmune process that leads to beta-cell destruction and IDDM. The infant's immature gastrointestinal mucosal barrier and immature immune system make the infant vulnerable to potential dietary antigens. Failure to establish normal tolerance is a hallmark of any autoimmune disease, including IDDM. Through a process of molecular mimicry it is postulated that a dietary antigen could cross-react with a protein on the surface of the beta-cell, in this case ICA 69, which is induced by gamma interferon associated with viral infections. The long diabetic prodrome is associated with a battery of abnormal immunological responses that ultimately result in beta-cell failure and frank insulin deficiency.

Breast-feeding likely exerts its protective effect by enhancing immunological function in the newborn, helping to prevent contraction of viral infections, and providing optimal nutrition to the infant without exposure to foreign antigens present in cow's-milk-based infant formulas and other foods. There is substantial, but not totally convincing, evidence from epidemiological, population, and case-control studies that early introduction of cow's milk protein, in particular BSA, is associated with IDDM. Observations of specific immunological responses to BSA and cow's milk protein, autoimmune abnormalities, and in some cases, associated genetic markers of predisposition to IDDM further support a role of cow's milk proteins in the autoimmune pathogenesis of IDDM. The association of IDDM and cow's milk exposure decreases with increasing age of disease diagnosis (Gerstein, 1994). IDDM developing in the first one to four years of life may be
more likely to be related to breast-feeding frequency and duration and timing of introduction of cow’s milk proteins than IDDM developing in later childhood and adolescence.

It still remains unclear why BSA, one exogenous substance absorbed intact that comes in contact with the immune system, is non-antigenic in normal controls but has been shown repeatedly to be antigenic in diabetic patients (Yokota et al., 1990). While antibodies to BSA are detected at some frequency in even normal children, they appear to disappear in adulthood. Unfortunately, conflicting reports exist that both support and undermine the BSA autoantigen hypothesis. The discrepancy in results linking BSA immunity to IDDM may be due to differences in study populations, specificity and sensitivity of antibody assay techniques, and the cutoff points for positive determinants. There appears to be a large overlap of antibody titres in IDDM and controls (Pardini et al., 1996). This may be partially explained by the heterogeneous pathophysiology of IDDM. Genetic protection may override environmental factors such as early exposure to cow’s milk protein, in particular BSA, despite elevated anti-BSA antibody titres. Food-induced diabetes would require both initiation and maintenance of diabetogenesis that depends on frequent exposure to common dietary antigens and occurs only in genetically predisposed individuals. The BSA hypothesis remains controversial.

IMPLICATIONS FOR FEEDING INFANTS

If the BSA hypothesis holds true, infant feeding practices, in particular introduction of cow’s milk and cow's-milk-based infant formulas as breast-milk substitutes, need to be reevaluated for their potential contribution to the pathogenesis of IDDM in genetically predisposed individuals. The following sections provide a brief history of infant formula, trends in breast-feeding and bottle-feeding, and implications of breast-feeding and bottle-feeding in developing nations. The nutritional composition of breast milk compared to cow’s milk is provided along with recommendations for introduction of whole cow’s milk
into the diet. An in-depth summary of the characteristics of cow's-milk-based infant formulas, with special attention to protein composition and BSA content, is also provided.

Infant Formula: A Historical Perspective

In the early part of the nineteenth century, few infants survived who were not breast-fed (Fomon, 1974). When a mother died in childbirth or was unable to nurse the infant, a wet nurse was usually employed. In the southern regions of the United States, use of "negro" wet nurses to nurse White infants was common. Breast-feeding remained the most common means of feeding infants well into the twentieth century (Fomon, 1974).

Increased feasibility and success of bottle-feeding emerged in the late 1800s and early 1900s. One of the most important factors contributing to success of bottle-feeding during this period was chlorination of water and the development of safer water supplies. However, bacterial contamination of milk remained a problem until the early part of the twentieth century when the kitchen icebox became popular (Fomon, 1974). Advances in controlled heating and acidification of milk resulted in decreased bacterial growth and contamination (Fomon, 1974). Reduction in curd tension, which results in increased digestibility and nutritional quality of cow's milk, was accomplished by acidification, boiling, dilution, modification of mineral content, treatment with enzymes, and homogenation (Fomon, 1974). By the turn of the century, a product similar to today's sweetened condensed milk was widely used for infant feeding (Fomon, 1974). However, problems arose due to the high carbohydrate content, high caloric density, high osmolality, and low water content of these preparations. By the 1920s, commercial canned evaporated milk became widely used as an infant feeding alternative and was recognized as being readily digestible and well tolerated by the young infant. Evaporated milk or fluid whole cow's milk with added carbohydrate became the mainstay of formula-feeding until the
1950s (Fomon, 1974). Ascorbic acid and Vitamin D deficiencies were seen, but poorly understood until the latter half of this century.

By the 1950s commercial infant formula, derived from cow's milk with added carbohydrate and often vitamins, was provided in both powdered and concentrated liquid form. Concentrated liquid formulas held the majority of the infant formula market for the next 25 years. However, the lowest income groups continued to use evaporated milk (Fomon, 1974) with or without modifications in carbohydrate content and vitamin supplementation.

Increased safety and convenience of infant formula from the late 1940s well into the 1970s, accompanied by failure of investigators to provide evidence of definite superiority of breast-feeding over formula-feeding during this period, resulted in physicians becoming unenthusiastic about promoting breast-feeding (Fomon, 1974). Hill (1968, p. 161) observed that "formula-feeding has become so simple, safe, and uniformly successful that breast-feeding no longer seems worth the bother." It was recognized that where good hygiene was possible, there was little difference between formula and human milk (Minchin, 1987). During this period, hospital nurseries adopted use of commercial formula services. By the late 1960s, sterile ready-to-serve formulas with disposable nipples were provided to hospitals and to new mothers free of charge by major manufacturers. Today, it can be assumed that the bulk of infants not breast-fed receive commercial infant formula.

Trends in Breast-Feeding and Bottle-Feeding

The advent of readily available infant formulas that were seen even by medical professionals as nutritionally sound, and in some cases superior, breast milk alternatives had a dramatic impact on breast-feeding incidence following World War II in North America and most of Europe. In the United States 65% of newborns were breast-fed in
By 1958, only 25% of infants born in the United States were breast-fed at 7 days (Martinez & Nalezienski, 1979). Reported in Fomon (1974), similar trends have been observed in many northern European countries including the United Kingdom, Norway, and Sweden. A small decline in the prevalence of breast-feeding occurred between 1955 and the early 1970s according to both the Ross Laboratorones Mothers Survey and National Surveys of Family Growth (Ryan et al., 1991). Breast-feeding prevalence at 1 week reached a peak in 1982, which was two-fold higher than in 1955. Sixty-two percent of mothers were breast-feeding at hospital discharge and 30% continued to breast-feed at 6 months postpartum (Department of Health and Human Services, 1990a). A gradual decrease in breast-feeding rates was apparent throughout the remainder of the 1980s (Ryan et al., 1991). Breast-feeding trends in the United States are reported in Figure 4.

Breast-feeding rates are highest among women who are White, married, have had at least some college education, and who live in the western United States (Department of Health and Human Services, 1990a). A lower incidence of breast-feeding is reported in women less than 25 years of age, those in lower socioeconomic groups, Black women, and those living in the southeastern United States (Department of Health and Human Services, 1990a; Ryan et al., 1991). Breast-feeding incidence is unfortunately lowest in those infants who could benefit most from the immunological advantages of human milk (Spisak & Gross, 1991).

Health experts worldwide now agree that breast-feeding is the best way to nurture infants and should be practiced whenever possible (Spisak & Gross, 1991). Breast-feeding provides optimal nutrition for normal growth and development, protection against disease, decreased risk of allergies, and ultimately, decreased health care cost for infants. Breast milk provides a number of immunologically protective factors and likely unknown agents that cannot be mimicked in infant formulas (Cunningham, Jeiliffe, & Jeiliffe, 1991; Minchin, 1987), despite attempts to "humanize" these preparations. Even in industrialized

nations, breast-feeding's protection against otitis media and respiratory infections (e.g., respiratory syncytial virus) cannot be ignored. In addition, breast-feeding has been reported to prevent bacteremia and meningitis associated with *Haemophilus influenzae* infections and reduce septicemia and death due to neonatal necrotizing enterocolitis (Cunningham et al., 1991). Significantly lower rates of diarrhea, otitis media, juvenile diabetes, ulcerative colitis, lymphomas, and Sudden Infant Death Syndrome occur among breast-fed infants and children in the United States (U.S. Committee for UNICEF and Wellstart International, 1996). It is still not entirely clear how breast-feeding exerts its protection against various diseases, or for that matter, how introduction of infant formula
may precipitate disease. In addition, Lucas, Morley, Cole, Lister, and Leeson-Payne (1992) found that premature infants fed breast milk had an 8.3 point IQ advantage over formula-fed infants at 7.5-8 years of age after adjusting for maternal educational level and social class ($p < 0.0001$). It appears possible that early feeding practices may affect mental development as well as physical health and growth.

The renewed emphasis on breast-feeding and health has prompted a number of policy statements regarding breast-feeding practices in the United States. In the U.S. Department of Health and Human Services publication, *Promoting Health/Preventing Disease: Objectives for the Nation*, the goal for breast-feeding for the year 1990 was to increase the proportion of women who breast-feed their babies at hospital discharge to 75% and 35% at 6 months of age (1980). Breast-feeding promotion in the United States became a nutrition priority area by 1990. The breast-feeding objective was updated in *Healthy People 2000: National Health Promotion and Disease Prevention Objectives* to "increase to at least 75% the proportion of women who breast-feed their babies in the early postpartum period and to at least 50% the proportion who continue breast-feeding until their babies are 5 to 6 months old" (Department of Health and Human Services, 1990b, p. 379). If these initiatives are successful, increased rates of breast-feeding are likely to have a profound impact on the infant formula industry. The most recent data from the Ross Laboratories Mothers Survey (1996), "Updated Breast-feeding Trends 1986-1994," reveal that rate and duration of breast-feeding continue to lag behind the national objective.

Infant Feeding Practices in Developing Countries: An International Perspective

Many of the problems that contributed to unsuccessful bottle feeding at the turn of the century in the United States and Northern Europe remain critical factors, contributing to the high infant mortality and morbidity of bottle fed infants, in developing nations today.
The availability of clean water, free of pathogenic microorganisms, remains a paramount issue in the success of bottle feeding. It is well recognized that worldwide nutrition relief efforts failing to recognize and implement concurrent programs in waste-water treatment, safe drinking water, and food safety and sanitation have met with only marginal success. Breast-feeding can circumvent many of these issues.

Breast-feeding has been shown to prevent infantile diarrhea in all settings and improve infant survival rates in poor countries (Cunningham et al., 1991). Most deaths related to bottle feeding worldwide can be attributed to diarrheal disease with mortality rates reported to be as high as 14-fold that of breast-fed infants. The risk of dying from a lower respiratory tract infection in the urban environment of developing nations is four-fold higher in bottle-fed infants (Cunningham et al., 1991). The high prevalence of diarrheal and respiratory disease and associated mortality seen with bottle-feeding in developing countries often overshadows less common diseases that are now recognized in developed countries to be associated with either a lack of breast-feeding or the introduction of infant formula. The negative impact of formula-feeding on initiation, success, and duration of breast-feeding cannot be overlooked. In addition, breast-feeding serves an important role in child spacing (Minchin, 1985), which has been associated with decreased mortality in children less than age 5.

Due to the striking negative impact of infant formula and bottle-feeding on morbidity and mortality in developing countries, the formula industry has come under close scrutiny by a number of breast-feeding advocacy groups, including LaLeche League International, United Nation's Children's Fund (UNICEF), Wellstart International, and the World Health Organization (WHO). To address these concerns, the World Health Organization has developed a code for marketing breast-milk substitutes. WHO acknowledges that breast-milk substitutes should be available through commercial and non-commercial distribution systems, but marketing or distribution of these products in ways
that interfere with the protection and promotion of breast-feeding is in violation of the
WHO code (Minchin, 1985). Current marketing of infant formula and free distribution of
infant formulas to new mothers in the United States are clearly in violation of the code.

Health care practices have been shown to play a major role in breast-feeding
initiation and success. In 1991 UNICEF and WHO established the Baby-Friendly Hospital
Initiative (BFHI). A major emphasis of the Baby-Friendly Hospital Initiative is to target
the national ministry of health, hospitals, physicians, nurses, midwives, and other
influential health professionals and to emphasize the critical need for all to promote the
superiority of breast-feeding over bottle feeding. The BFHI is a global program to
encourage and recognize hospital and birthing centers that offer an optimal level of care for
breast-feeding. Ten steps to successful breast-feeding are outlined to facilitate the role of
the hospital or birthing center in providing women the choice and opportunity to breast-
feed. The BFHI has been undertaken in over 170 countries and 4,000 hospitals in both
developing and industrialized nations (U.S. Committee for UNICEF and Wellstart

Nutritional Composition of Human Milk

The macronutrient composition of human milk is provided in Table 1. The casein-
to-whey ratio of human milk is reported to range from 60% whey and 40% casein up to
80% whey and 20% casein (Jost et al., 1987). Lactalbumin is the prominent protein in
human milk and is of high biological value. Human milk contains a relatively small amount
of casein and forms a soft, flocculent curd with increased digestibility compared to cow's
milk protein (Fomon, 1974). Lactose is the major carbohydrate in human milk. Fifty
percent of the calories in human milk are provided by fat in the form of triglycerides. The
fatty acid composition of human milk reflects maternal intake. Linoleic acid contributes 4%
of the total calories in human milk. The concentration of water-soluble vitamins in human
Table 1

Nutrient Composition of Human Milk, Cow's Milk, and Cow's-Milk-Based Infant Formula per Liter

<table>
<thead>
<tr>
<th>Milk/formula</th>
<th>Calories</th>
<th>Protein (gms)</th>
<th>Fat (gms)</th>
<th>CHO (gms)</th>
<th>Protein source</th>
<th>Casein:whey</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human milk</td>
<td>750</td>
<td>11</td>
<td>45</td>
<td>70</td>
<td>Lactalbumin, casein</td>
<td>40:60</td>
</tr>
<tr>
<td>Cow's milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skim</td>
<td>357</td>
<td>35</td>
<td>2</td>
<td>50</td>
<td>Casein, whey</td>
<td>80:20</td>
</tr>
<tr>
<td>2%</td>
<td>503</td>
<td>34</td>
<td>20</td>
<td>49</td>
<td>Casein, whey</td>
<td>80:20</td>
</tr>
<tr>
<td>Whole</td>
<td>624</td>
<td>33</td>
<td>34</td>
<td>47</td>
<td>Casein, whey</td>
<td>80:20</td>
</tr>
<tr>
<td>Standard infant formulas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Similac</td>
<td>676</td>
<td>15</td>
<td>36.3</td>
<td>72.3</td>
<td>Nonfat milk (casein)</td>
<td>80:20</td>
</tr>
<tr>
<td>Enfamil</td>
<td>676</td>
<td>15</td>
<td>33</td>
<td>69</td>
<td>Nonfat milk, reduced mineral whey</td>
<td>40:60</td>
</tr>
<tr>
<td>SMA</td>
<td>676</td>
<td>15</td>
<td>36</td>
<td>72</td>
<td>Nonfat milk, demineralized whey</td>
<td>40:60</td>
</tr>
<tr>
<td>Bonamil</td>
<td>676</td>
<td>15</td>
<td>38</td>
<td>71</td>
<td>Nonfat milk (casein)</td>
<td>80:20</td>
</tr>
<tr>
<td>Gerber</td>
<td>670</td>
<td>15</td>
<td>36</td>
<td>71</td>
<td>Nonfat milk (casein)</td>
<td>80:20</td>
</tr>
</tbody>
</table>

(table continues)
<table>
<thead>
<tr>
<th>Milk/formula</th>
<th>Calories</th>
<th>Protein (gms)</th>
<th>Fat (gms)</th>
<th>CHO (gms)</th>
<th>Protein source</th>
<th>Casein: whey</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolyzed infant formulas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good Start</td>
<td>670</td>
<td>16</td>
<td>34</td>
<td>74</td>
<td>Hydrolyzed, demineralized whey</td>
<td>100% whey</td>
</tr>
<tr>
<td>Nutramagen</td>
<td>670</td>
<td>19</td>
<td>26</td>
<td>90</td>
<td>Casein hydrolsate</td>
<td>100% casein</td>
</tr>
<tr>
<td>Pregestimil</td>
<td>670</td>
<td>19</td>
<td>27</td>
<td>90</td>
<td>Casein hydrolsate</td>
<td>100% casein</td>
</tr>
<tr>
<td>Alimentum</td>
<td>676</td>
<td>19</td>
<td>38</td>
<td>69</td>
<td>Casein hydrolsate</td>
<td>100% casein</td>
</tr>
</tbody>
</table>
milk generally reflects maternal intake, and for this reason it is recommended that lactating mothers take a daily multivitamin supplement. While breast milk is considered complete nutrition for the infant, it is now recognized that supplementing the infant with an initial injection of vitamin K at birth and vitamin D and iron by 4 to 6 months optimizes the nutritional status (Fomon, 1993).

In addition to nutrients, human milk contains a number of immunologically active substances that are thought to protect infants from infection. Lactoferrin may slow bacterial growth by decreasing availability of iron to the infective organism. Lysozymes may destroy bacterial cell membranes (Department of Health and Human Services, 1988). The presence of secretory IgA plus colonization and acidification of the infant’s gastrointestinal tract with Lactobacilli may provide additional protection.

Cow's Milk

It is well recognized that unmodified whole cow’s milk is an inappropriate feeding for young infants. Casein accounts for the major fraction of protein in cow’s milk with a casein:whey ratio of 80% casein to 20% whey (Jost et al., 1987). The tough, hard casein curd is difficult for young infants to digest. Use of whole cow's milk in early infancy may cause occult bleeding from the gastrointestinal tract and subsequent anemia. In addition, cow's milk contains lipids, butterfat, that are less digestible than those found in human milk. Increased fecal fat excretion has been observed with butterfat but is thought to be of little clinical significance (Fomon, 1993). Cow’s milk has a high renal solute load due to the high concentration of minerals and protein. The renal solute load may exceed the excretory capacity of the immature kidney (Department of Health and Human Services, 1988). The amino acid profile of cow’s milk varies from that of human milk. Cow's milk is higher in tyrosine and phenylalanine and lower in taurine and cystine than human milk (Department of Health and Human Services, 1988).
Introduction of cow's milk (whole, 2%, and skim) has been a controversial issue in infant feeding for the past 20 years. It can be assumed that cow's milk was the most likely alternative breast milk substitute prior to the advent of canned condensed and evaporated milks. Use of whole cow's milk is generally not recommended the first year of life (American Academy of Pediatrics, Committee on Nutrition, 1992) and is not used as a primary infant feeding in developed industrialized nations. Two percent and nonfat milk are deficient in energy, essential fatty acids, and certain vitamins, and they contain excessive protein and minerals per calorie for infants less than one year of age. Use of low fat or skim milk is not recommended for infants and toddlers less than 2 years of age (Mahan & Escott-Stump, 1996). The macronutrient composition of cow's milk is provided in Table 1.

Characteristics of Commercial Infant Formulas

A wide variety of commercial infant formulas is heavily marketed and available as breast-milk alternatives. While there are some standards for infant formula, it should be recognized that infant formulas are not homogeneous, vary widely in composition, and should not be considered to be a consistent product (Minchin, 1987). Revisions in formula composition are ongoing and new products are introduced to the market and old products withdrawn on a sporadic basis. Modifications of infant formula composition are often based on optimization of a product to meet the needs of the infant, but are also based on market demand. Most infant formulas are derived from intact cow's milk proteins. Soy-based formulas and specialized products are available to treat children with allergies, inborn errors of metabolism, and other conditions.

Current infant formulas for healthy term infants are designed to simulate human milk. Most formulas are derived from nonfat cow's milk, vegetable oils, and added carbohydrate. Butterfat has largely been replaced with vegetable oils to enhance fat
absorption (Fomon, 1974). Carbohydrate is added, in the form of sucrose, corn syrup solids, starch, glucose, lactose, fructose, or a combination (Fomon, 1974) in order to provide a calorie-to-nitrogen ratio consistent with human milk. Lactose has largely replaced corn syrup solids as the preferred carbohydrate additive in standard infant formulas. Demineralized whey has been added to provide a casein-to-whey ratio consistent with that present in human milk. Cow's milk may be diluted to a more favorable mineral and solute composition, one that parallels that of human milk. Vitamins are added to meet recommended nutrition requirements of infants.

The unique protein composition of the major cow's-milk-based infant formulas currently marketed in the United States is reviewed below. Attention is focused on modifications in protein composition that are likely to affect the BSA composition of these preparations. The macronutrient composition of cow's-milk-based polymeric and hydrolyzed infant formulas is provided in Table 1. Formula preparations vary in their casein-to-whey ratio, degree of thermal processing, and extent of enzymatic hydrolysis. These factors are likely to affect the BSA content of the formula.

### Polymeric Cow's-Milk-Based Infant Formulas

Standard polymeric infant formulas currently marketed in the United States include two high-whey products and three high-casein products. Demineralized whey, which contributes 12% protein and 75% lactose by weight, is added to cow's-milk-based infant formulas in order to raise the whey protein fraction and increase the non-protein calorie to nitrogen ratio. Several commercial formulas now contain a protein mix of 60% whey and 40% casein, which closely parallels the casein-to-whey ratio of human milk. These include Enfamil, SMA (also marketed under private label), specialized formulas designed for premature infants, and newly modified Similac. Whey increases the proportion of soluble protein, limits excessive curd formation, and increases the digestibility of the protein. In
addition, the high-whey products have an amino acid profile that is more consistent with that of human milk. A higher whey fraction has also been shown to have the benefit of reducing metabolic acidosis in low-birth-weight infants (Jost et al., 1987).

The high-whey formulas are, however, likely to contain significantly more BSA, a soluble protein, than the more traditional high-casein cow's-milk-based formulas. This may be of particular concern in the premature infant with an immature gastrointestinal tract. Formulas designed for premature infants contain the highest fractions of whey in their nutrient mix. The well-known allergenicity and antigenicity of whey proteins, β-lactoglobulin, α-lactalbumin, and serum albumin, have been documented (Asselin, Amiot, Gauthier, Mourad, & Hebert, 1988; Jost et al., 1987; Pahud, Monti, & Jost, 1985).

Three polymeric cow's milk products currently marketed in the United States have a casein:whey ratio of 80% casein to 20% whey. Casein-based formula preparations are generally well tolerated by the neonate. Incomplete protein digestion, in particular impacting casein, is considered a normal physiological characteristic of digestion in infancy (Mason, 1962) and has not been shown to pose significant problems. While casein is considered to be less digestible than whey, a variety of processing techniques has been employed by the formula industry to address the issue of casein curd formation. Acidification, dilution, boiling, homogenation, modification of mineral content, and treatment with enzymes are utilized to decrease curd tension and increase digestibility of milk protein (Fomon, 1974). Growth in infants on high-casein formulas has not been reported to be different from those receiving whey-based preparations. High-casein formulations would be expected to contain smaller amounts of BSA than other polymeric formulas and may be a preferable choice given the potential role of BSA in the pathogenesis of IDDM. While not established, this would be particularly true if the impact of BSA followed a standard dose response curve.
Polymeric infant formulas are marketed in several forms. These include spray-dried powder, ready-to-serve bottled or canned liquid, and canned liquid concentrate. The generally held thought is that the three forms of a given product are nutritional equivalents. However, differences in temperature and duration of thermal processing may impact protein structure and BSA composition. Moderate heating such as pasteurization or ultra heat treatment produces little detectable change in caseins; however, the whey proteins are more sensitive to denaturation by heat. Extensive heating results in almost complete loss of their unique three-dimensional structure, reduces solubility, and decreases antigenicity (McLaughlan, Anderson, Widdowson, & Coombs, 1981). The effect of heat treatment on immunogenicity and allergenicity of milk proteins has been varied (Asselin et al., 1988).

BSA has been shown to be denatured by heat, but at temperatures and for a duration in excess of manufacturing standards. Extensive heat treatment of BSA denatures the protein and disrupts conformational epitopes, but the impact on sequential epitopes, such as ABBOS, has not been demonstrated. Degree of denaturation, measured by immuno-diffusion, has been demonstrated to be complete following heat treatment at 90 degrees Celsius for 10 to 30 minutes or after heat treatment at 125 degrees Celsius for 60 to 180 seconds (Jost et al., 1987). The lower temperature would require extensive heating and cool-down periods, which would be undesirable in the industrial setting. The ultra high temperature range presents the danger of lysine blockage in which lysine complexes with lactose (Jost et al., 1987) via the Maillard reaction, forming a nutritionally inferior derivative. Scorching of milk at these high temperatures and agglutination of proteins may alter palatability, digestibility, and antigenicity of the proteins.

Hydrolyzed Infant Formulas

Enzymatically hydrolyzed casein and whey formulas have been available for over 40 years for use in infants with defects in protein absorption and allergic reactions to
cow's-milk-protein-based infant formulas (American Academy of Pediatrics, Committee on Nutrition, 1989). Protein hydrolysates have been shown to have substantially lower immunogenicity than the parent proteins. Antibodies specific for the intact parent proteins show little cross-reactivity with the tryptic hydrolysates (Cordle, Mahmoud, & Moore, 1991). Enzymatic hydrolysis results in destruction of stable structural epitopes and cleavage of sequential epitopes (Mahmoud, Malone, & Cordle, 1992; Rugo, Wall, & Wahn, 1992). Casein-based hydrolysates are characterized by 70% free amino acids and peptides of up to five to eight amino acid residues (Siemensma et al., 1993). Casein hydrolysates have been demonstrated to have non-antigenic peptides of less than 1,200 molecular weight and are generally thought to be superior to whey protein hydrolysates for use in hypoallergenic infant formulas (American Academy of Pediatrics, Committee on Nutrition, 1989). Extensively hydrolyzed casein formulas include Pregestimil, Nutramagen, and Alimentum. These products are considered truly hypoallergenic and do not contain peptides exceeding a molecular weight of 1,200 (Knights, 1985).

Whey protein hydrolysates range from 40% to 60% free amino acids with peptides up to 10 to 12 amino acid residues (Siemensma et al., 1993). In addition, partially hydrolyzed whey products are available that contain only 10% free amino acids with peptides up to 15 amino acids in length (Lahl & Braun, 1994; Siemensma et al., 1993). The cutoff point for antigenicity appears to be peptides of 8 to 10 amino acid residues (Siemensma et al., 1993). While marketed as potentially better than intact cow's-milk-based formulas, some of these partially hydrolyzed products do not meet the American Academy of Pediatrics standards for hypoallergenic formulas (American Academy of Pediatrics, Committee on Nutrition, 1989). Antigenicity and allergenicity of cow's milk hydrolysates have been reported to vary significantly with the degree of hydrolysis (Cordle, 1994; Oldaeus, Bjorksten, Einarsson, & Kjellman, 1991). Whey hydrolysates containing a high percentage of larger peptides retain a high capacity to induce positive skin
tests, provocative responses, and to bind human IgE antibodies in children known to be allergic to cow's milk. Where peptides greater than 1,500 and up to 20,000 daltons exist, considerably allergenic activity remains (Rugo et al., 1992).

Theoretically BSA and the active ABBOS epitope could remain intact in partially hydrolyzed infant formulas. Of the major proteins present in milk, BSA has been shown to be one of the most resistant to hydrolysis by trypsin and other proteases (Jost et al., 1987). BSA is strongly disulfide-bonded, shielding the primary structure from enzymatic cleavage. Antigenic peptide fragments of BSA have been isolated and identified in tryptic, chymotryptic, and peptic hydrolysates of BSA (Habeeb & Atassi, 1976; Pahud et al., 1985; Wahn, Peters, & Siraganian, 1981). While SDS-PAGE showed disappearance of the major whey proteins following only 1 hour of enzymatic hydrolysis, a faint band in the region of BSA could still be detected following 4 hours of treatment (Pahud et al., 1985). Good Start, marketed as Beba HA in Europe, has a protein base of 100% partially hydrolyzed whey. Good Start has been demonstrated to contain a number of unresolved and non-degraded or partially degraded whey proteins in the range of 5 to 20 kilodaltons. Positive skin prick tests following administration of Good Start were observed in 24% of children with cow's milk hypersensitivity (Oldaeus et al., 1991). Depending on the extent of protein hydrolysis and the whey concentration of these hydrolyzed formula preparations, antigenic epitopes of BSA, including the ABBOS peptide, may still be present.

**BSA in Cow’s Milk and Cow’s-Milk-Based Infant Formula**

The reported BSA content of cow's milk varies by four-fold, ranging from 0.1 to 0.4 grams per liter (Robin et al., 1993; Swaisgood, 1982; Whitney, 1988). Quantitative data on the BSA content of cow's-milk-based infant formulas are lacking. Much of the attention on detection of specific milk proteins in formula has focused on evaluating the degree of residual antigenicity in extensively hydrolyzed formulas designed for use in
infant with hypersensitivity to cow's milk proteins. Specifically, BSA has not been shown to the present in Nutramagen or Alimentum by polyacrylamide gel electrophoresis (PAGE) using silver stain (Cordle, 1994; Oldaeus et al., 1991; Sampson, Bern-Broadbent, Yang, & Scanlon, 1991). Reports regarding the presence of intact BSA in the partially hydrolyzed product, Good Start, again using PAGE with silver stain, are mixed (Cordle et al., 1994; Oldaeus et al., 1991). Serum antibody production to BSA in guinea-pigs has been shown to be greatly reduced in formula (SMA) provided in liquid concentrate form compared to antibody production following feeding of the corresponding SMA powder (Heppel, Cant, & Kilshaw, 1984).

Monte, Johnston, and Roll (1994) detected BSA in three of four standard infant formulas using radial immunodiffusion. BSA was not detected in those subjected to ultra high temperature sterilization or in the one hydrolyzed product tested. Immunodiffusion methods have previously been reported to be 10,000-fold less sensitive than ELISA and are likely to underestimate antigenicity (Leary, 1992). Radial immunodiffusion is sensitive down to 40 mg BSA/Liter, which is about 10% of that found in cow's milk (Strand, 1994) and may not be sensitive enough to detect BSA in high-casein products or to detect residual BSA in ultra high temperature sterilized or hydrolyzed preparations. Monte and colleagues (1994) used centrifuged samples representing the defatted soluble fractions of infant formulas. Therefore, quantitative data could not be extrapolated back to the original formula of milk. In addition, the casein-to-whey ratio of polymeric standard infant formulas has not been addressed.

Research Implications

The extensively hydrolyzed casein-based formula preparations are considered to be free of BSA and, based on molecular weight of remaining peptides, should also be devoid of the ABBOS epitope. There are mixed reports in the literature as to whether partially
hydrolyzed whey formulas contain intact BSA or the ABBOS epitope. Based on reports of high residual antigenicity and the presence of higher molecular weight peptide fragments, it appears likely that BSA or the ABBOS peptide may be present. Standard polymeric infant formulas containing whey certainly contain BSA and the ABBOS epitope with amounts likely proportional to the whey protein concentration. The impact of various methods and degrees of heat treatment used in the preparation of infant formulas on the antigenicity of BSA has not been established. No comprehensive quantitative information is available describing the BSA content of infant formulas.

Additional knowledge of the BSA content of existing infant formulas is needed in order to further assess the role of BSA and ABBOS in the pathogenesis of IDDM and to make sound nutritional recommendations for those infants not exclusively breast-fed. The present study was undertaken to develop a simple and reliable quantitative analysis of BSA content of cow's milk and cow's-milk-based infant formulas. Both direct and indirect ELISAs were evaluated as a method for quantitating BSA. A sensitive direct ELISA was developed to specifically detect and quantitate the amount of BSA present in cow's milk and infant formula preparations. Use of a polyclonal antibody to BSA allowed for detection of BSA and antigenic epitopes of BSA. The information obtained provides a consistent relative quantitative measure of the contribution of cow's milk and cow's-milk-based infant formula to the BSA antigen load in infants' diets.

Thermal and enzymatic processing of infant formula is utilized in the industrial setting to reduce allergenicity of these preparations. These processes affect other proteins, in addition to BSA, resulting in a substantially modified product or protein base having potential nutritional implications. There are several inherent drawbacks of heat treatment of infant formula to eliminate BSA. Heat treatment of milk and cow's-milk-based infant formula has been reported to affect the nutritional quality of these preparations and even if effective, may not provide the optimal means of destroying or removing BSA and the
ABBOS epitope from commercial formulas. Heating may cause lysine blockage in which lysine complexes with lactose in the Maillard reaction. Lysine blockage needs to be assessed and controlled in order to maintain the nutritional quality of the formula (Jost et al., 1987; Kilshaw, Heppell, & Ford, 1982). Lysine is an essential amino acid and is required in relatively large amounts by the infant for normal growth and development. The Maillard reaction has been reported to result in a 24% decrease in lysine availability in heat-treated commercial infant formula (Heppel et al., 1984). Another product formed during heat treatment is lactulose, a poorly absorbable derivative of lactose. Osmotic activity of lactulose and fermentation in the intestine can contribute to diarrhea, particularly in infants recovering from gastroenteritis (Jost et al., 1987). Extensive heat treatment of skim milk has also been shown to destroy all vitamin B12, 60% of the thiamin, 70% of the ascorbic acid, 30% of the folate with considerable loss of B6, while retaining its capacity to sensitize guinea-pigs to beta-lactoglobulin and the caseins (Kilshaw et al., 1982). Extensive heat treatment may also result in scorching and associated flavor alterations. In addition, enzymatically hydrolyzed infant formulas require fortification with specific amino acids destroyed in processing and may not be well accepted by the infant due to flavor and texture alterations. The cost of extensively hydrolyzed elemental preparations is up to three times as much as standard, cow's-milk-based infant formulas.

The objective of the second component of the study was to develop an alternative technology to produce a BSA-free polymeric protein base without extensive heat treatment or enzymatic hydrolysis. Affinity chromatography was employed to develop an immunomagnetic capture system to selectively extract BSA from cow's milk and whey protein isolates that are used in the manufacture of commercial infant formula. Both monoclonal and polyclonal antibodies were evaluated for capture efficiency. This methodology could be used in combination with molecular sieving or other alternative
means of removing or destroying BSA that may be less than 100% effective. An overview of immunocapture methodology is provided in the following section.

OVERVIEW OF ENZYME-LINKED IMMUNOSORBANT ASSAYS AND IMMUNOAFFINITY CHROMATOGRAPHY

Enzyme-Linked Immunosorbant Assays

Immunoassays can be performed to estimate the amount of a substance, an analyte, in an unknown or test specimen. Enzyme-linked immunosorbant assay (ELISA) technology was introduced in the early 1970s as an alternative to radio immunoassays. The major difference is that the endpoint is measured by an enzyme-driven colorimetric reaction rather than radioactivity (Makarananda & Neal, 1992). ELISA is a useful technique to provide specific and sensitive examination of a substance. Monoclonal or polyclonal antibodies to the substance can be used to capture and detect the analyte. The hallmark of ELISA methodology involves an enzyme-labelled detector antibody which, upon reacting with a substrate, elicits a colored reaction product that is proportional to the amount of analyte present in the sample. ELISAs have comparable sensitivity to radioimmunoassays without the limitations of short half-life and problems with disposal of radioactive materials (Kemeny & Chantler, 1988).

Common uses of immunoassays involve detection and quantitation of antigens, serum antibodies, and a variety of other proteins. Common classifications of ELISA methodology include the competitive ELISA, direct ELISA, indirect ELISA, and class capture for immunoglobulin subclasses. The focus of the following discussion will be limited to direct and indirect ELISA techniques for detection and quantitation of antigens.
Assay Components

Solid Supports

A number of solid supports are available for ELISA. These include plastic, nitrocellulose, agarose, glass, cellulose, polyacrylamide, dextran, and beads (Carpenter, 1992). Plastic 96-well microtitre plates formulated from polystyrene or polyvinyl chloride are most commonly used. Microtitre plates are typically coated with antigen or antibody in concentrations ranging from 1 to 50 µg/ml diluted in carbonate coating buffer (pH 9.6). Antigens and antibodies are bound via hydrophobic interactions (Carpenter, 1992). Adsorption is dependent on surface attributes of the plastic, concentration and characteristics of the antigen, time and temperature (Carpenter, 1992). These factors must be optimized in each assay system. The limitation of most microtitre plates is the capacity to bind antigen (Kemeny & Chantler, 1988).

Capture Antibodies

The most important requirement for capture antibodies, once bound to the solid phase, is a high capacity to bind relevant antigens (Kemeny & Chantler, 1988). If the detection antibody is highly specific for the analyte in a sandwich procedure, some low-level binding to irrelevant antigens by the primary antibody may be tolerated. Most polyclonal antibodies bind well to the solid phase (Kemeny & Chantler, 1988). Monoclonal antibodies are more likely to be problematic, both in terms of their affinity for the test antigen and stability upon adsorption to the microtitre plate (Butler, 1988).

In most cases it is preferable to use immunoglobulin fraction, rather than whole serum or ascites, for coating wells. Use of immunoglobulin fraction prevents competitive binding and interference by other proteins present in serum or ascites fluid (Kemeny & Chantler, 1988). Antibody abundance of polyclonal antibodies is essential to the assay.
Polyclonal antibodies of antiserum having low antibody concentration may require affinity purification. However, selection of a polyclonal antibody with high antibody abundance may be most desirable as it avoids the work of affinity purification and potential problems of ligand leakage that can occur during purification (Butler, 1988).

**Antibody Conjugates**

The quality and efficacy of detector antibodies used for enzyme labeling and conjugation of the antibody with the enzyme are critical determinants of assay performance (Kemeny & Chantler, 1988). Each ELISA requires an antibody conjugated with an enzyme that can react with a chromogenic substrate (Makarananda & Neal, 1992). The enzyme, which is attached to an antibody, reacts with a substrate eliciting a colored reaction product. The amount of color is proportional to the concentration of the analyte and can be monitored visually or via a spectrophotometer depending on the desired sensitivity needs of the specific assay. A number of multichannel photometers are available for reading 96-well microtitre plates.

A number of factors may affect the choice of enzyme including purity, specific activity, sensitivity of substrate detection, ease of conjugation, efficacy, and stability (Kemeny & Chantler, 1988). The most popular enzymes used for ELISAs are alkaline phosphatase (AP), horseradish peroxidase (HRP), and β-galactosidase (Butler, 1994). These enzymes have been extensively used in ELISA, and product detection systems are available that are suitable for most uses (Kemeny & Chantler, 1988). These enzymes may be conjugated with whole polyclonal antibodies, immunoglobulin G (IgG) fractions, affinity purified polyclonal antibodies, and monoclonal antibodies (Carpenter, 1992). Affinity purified antibodies generally yield the best results with high detectability of analyte and low background interference (Kemeny & Chantler, 1988).
Enzyme Substrates

When selecting the substrate, attention should be given to sensitivity, stability, background absorbance, toxicity, and cost. Since many of these compounds are potentially carcinogenic, toxicity may be a major concern (Carpenter, 1992). HRP reduces hydrogen peroxide and oxidizes a second substrate, which collectively produces the colored reaction product. The most common substrates used with HRP are o-phenylenediamine (OPD), 5-aminosalicylic acid, 2, 2-azinodi-(3-ethylbenz-0-thiazoline-6-sulfonate, and 3, 3', 5, 5'-tetramethylbenzidine (TMB). OPM and TMB have been reported to be the most sensitive for detection of low levels of enzyme with TMB having the advantage of being nonmutagenic and noncarcinogenic (Kemeny & Chantler, 1988). The most common substrates used for photometric measurement of antibodies labeled with alkaline phosphotase and β-galactosidase are p-nitrophenyl phosphate (pNPP) and O-nitrophenyl-β-D-galactopyranoside (ONGP; Butler, 1994).

General Overview of Selected ELISA Procedures

Direct ELISA

The direct ELISA is often called a sandwich ELISA because the analyte to be measured is layered between molecules, which recognize either the same epitope or different epitopes on the analyte (Butler, 1988). The two-antibody sandwich ELISA is the most useful method to detect and quantitate antigen (Harlow & Lane, 1988). The sandwich ELISA is suited only to the measurement of multivalent molecules such as proteins, antigens, and polysaccharides (Butler, 1988). This method requires either affinity purified polyclonal antibodies or two monoclonal antibodies that bind to two different sites on the antigen (Harlow & Lane, 1988).

In a sandwich ELISA, one antibody, the capture antibody, is bound to the solid phase. Antigen in the sample is allowed to bind the capture antibody. The analyte to be
measured, usually the antigen, is captured by the antibody-coated matrix on the solid phase. The enzyme-labelled secondary antibody is added, completing the sandwich (Carpenter, 1992). It is preferable, but not essential, that the capture and detection antibodies are from the same species in order to minimize species cross-reactions (Kemeny & Chantler, 1988). Sample antigen is detected and quantitated by measuring the amount of secondary, labelled antibody that binds to the captured antigen (Harlow & Lane, 1988). Reacting the enzyme with substrate yields color that is proportional to the amount of secondary antibody and, therefore, antigen (Makarananda & Neal, 1992). The direct ELISA procedure for quantitation of immunoglobin or antigen is shown in Figure 5.

Indirect ELISA

With indirect ELISA either antibody or antigen can be bound to the solid phase. Typically, antigen is bound to the solid phase, which can be used as a target for antibodies in the test sample (Kemeny & Chantler, 1988). The specific antibodies present in the sample bind to the antigen-coated solid phase during a second incubation step. Unbound material is removed by washing. Bound antibody can be detected by a secondary enzyme-labelled anti-immunoglobin (Carpenter, 1992; Kemeny & Chantler, 1988). Detection of the antibody may be limited by the ability to bind antigen to the solid phase (Kemeny & Chantler, 1988). After washing, the assay is quantitated by measuring the amount of antibody bound to the antigen (Harlow & Lane, 1988).

The indirect ELISA can also be used to assess the amount of antigen in a sample bound to the solid phase. The antigen is attached to the solid support during incubation, and the labelled antibody is allowed to bind (Harlow & Lane, 1988). When the sample containing antigen is bound to the solid phase, an excess of labelled antibody, to allow full saturation between antigen and antibody, is required to detect and quantitate the sample antigen. After washing, the assay is quantitated by measuring the amount of antibody
Figure 5. Direct (two-site) ELISA. Antibody is added to the microtitre plate and incubated. Unadsorbed antibody is removed by washing with PBS-T prior to addition of the sample containing immunoglobin or antigen. Test sample is incubated with the capture antibody. Unbound sample is removed by washing. Enzyme labelled antibody is added and incubated with the captured immunoglobin or antigen. The enzyme chromagenic substrate is added as the final step.

Figure adapted from Kemeny & Chantler (1988).
bound to the antigen (Harlow & Lane, 1988). In some cases, a primary antibody that is labeled with an enzyme is commercially available for the specific antigen in question. If not, a secondary labelled anti-immunoglobulin is required. The indirect ELISA procedure is described in Figure 6.

Quantitation

Quantitative immunoassays can provide an absolute amount in weight per unit volume (e.g., nanograms to micrograms per milliliter) using a precalibrated standard or a relative comparison of the amount of an analyte by dilution analysis (Hamilton & Adkinson, 1988). The latter is usually used for antibody determination; however, problems have been reported to arise due to poor reproducibility of results (Carpenter, 1992). Theoretically a linear relationship should exist between analyte concentration and optical density. However, since this is likely to be the exception, rather than the rule, reference standards are employed for extrapolation of unknown antigen or antibody in the test sample (Kemeny & Chantler, 1988). A reference standard is used for calibration when it is desirable to report ELISA data in absolute quantitative amounts. A dose response curve is generated using the known concentration of the reference standard plotted against its absorbance. It is important to obtain values for samples falling along the linear portion of the standard curve. Either homologous or heterologous interpolation can be performed for translating the response into a dose estimate.

Homologous interpolation utilizes a standard containing a homologous or identical analyte to that being measured in the test sample. Heterologous interpolation is used when it may be impossible or too costly to use a homologous reference serum for each analyte. The analyte is interpolated from a standard curve using a similar, but not identical test serum (Hamilton & Atkinson, 1988).
Figure 6. Indirect ELISA. Antigen is added to the microtitre plate and incubated. Unadsorbed antigen is removed by washing with PBS-T prior to addition of antibody. Specific antibody is incubated with the antigen. Unbound material is removed by washing. Enzyme labelled secondary antibody (species specific anti-IgE) is added and incubated with the bound IgE. The enzyme chromogenic substrate is added as the final step. Figure adapted from Kemeny & Chantler (1988).
The magnitude of the response provided by the unknown sample (i.e., absorbance) can be interpolated from the standard dose response curve. The test sample must be analyzed with good parallelism between the dilution curves (Hamilton & Atkinson, 1988). This is important in order to have an accurate assessment of the analyte concentration where the interpolated test sample result does not vary as a function of the serum dilution analyzed. In order to evaluate parallelism, a test sample is measured in several different dilutions, interpolated from the standard, and corrected for its respective dilution. Less than 10% interdilutional coefficient of variation of the analyte is considered to be within reasonable statistical error (Hamilton & Atkinson, 1988). In addition, variability of the method can further be assessed by testing three dilutions of the reference antigen to cover the lower, middle, and upper limits of the standard curve in triplicate on several different assays. Inter- and intra-assay variation can be expressed as the coefficient of variation ([Standard Deviation X 100]/mean; Kemeny & Chantler, 1988).

Optimization

While ELISAs offer advantages due to their apparent simplicity, careful attention must be given to development of individual assay systems and optimization of assay techniques in order to assure good quality (Kemeny & Chantler, 1988). The optimal working concentration of labeled antibodies will vary depending on the application and the assay format. Optimal conditions can be verified only by trial and error (Kemeny & Chantler, 1988).

Initially, all ELISA reagents alone should be tested for their background absorbance. The basic controls on all ELISA plates should include a PBS blank, a negative sample control, and low and high positive controls. The checkerboard titration is traditionally used to establish appropriate working dilutions of coating antibody or antigen and antibody conjugate. Four dilutions of coating antibody or antigen can be tested in
duplicate horizontal rows on a 96-well microtitre plate. Coating proteins are bound passively during incubation. Non-bound material is removed by washing with phosphate buffered saline with Tween (PBS-T). Reacted sites are typically blocked with a nonspecific protein (e.g., BSA, gelatin, or nonfat dry milk; Carpenter, 1992). Test samples of antigen, which should include a high positive control, a negative control, and PBS, are added vertically in columns and incubated with the capture antibody or antigen. Following incubation, unbound material is removed by washing with PBS-T. The detection antibody conjugate is added in vertical triplicate columns and incubated. Unbound material is again removed followed by application of the appropriate substrate (Carpenter, 1992). The optimal antibody or antigen coating concentration is provided by the least quantity yielding near maximal binding of the analyte. This is used to determine the impact of varying the concentration of the secondary antibody conjugate (Kemeny & Chantler, 1988). Typically, higher levels of antibody conjugate increase the background noise and decrease the measurable analyte range. Low levels have been demonstrated to reduce the specific absorbance measured but not the nonspecific binding (Kemeny & Chantler, 1988). The optimal conjugate dilution is one that yields an absorbance reading for the PBS blank of less than 0.05, the negative control value of less than 0.2, and a high positive of greater than 1.0 (Carpenter, 1992).

The rate at which the assay proceeds is dependent on the concentration of reactants and is directed by the Law of Mass Action. The concentration of reactants, the capacity of the solid phase, the concentration of detector, assay speed, incubation temperature, and detection system can impact the sensitivity of the assay. Attempts to increase the rate of specific signal detection usually result in an increase in the signal in controls (Kemeny & Chantler, 1988). For example, increased concentration of labelled detector usually increases nonspecific binding and, therefore, background noise. The goal of optimization is to improve the signal-to-noise ratio. Prolonged incubation times are not desirable and
have been associated with increased intra-assay variation (Kemeny & Chantler, 1988). The rate of the reaction is dependent upon temperature. Temperature variations can be minimized by incubating under controlled conditions.

When immunoassays do not work, the first step is to check primary and secondary antibodies and detection systems. However, the most common source of failure is in attaching the antigen to the solid support (Kemeny & Chantler, 1988). Other common technical problems encountered in ELISA include nonspecific protein binding, cross-reactivity and other unwanted interactions, the presence of antibodies in sample that recognize blocking proteins, antibody or antigen excess, temperature variation, and edge effects (Kemeny & Chantler, 1988). Application of too much antigen or antibody to the solid phase may result in stacking due to protein-protein interactions and result in desorption during the assay process (Carpenter, 1992). This problem can be circumvented by additional washing prior to application of the next layer. Undesirable cross reactivity with assay components must be removed. Problems with nonspecific and specific undesirable binding can often be reduced by the addition of BSA, animal sera with or without Tween 20, or casein hydrolysate to buffers (Kemeny & Chantler, 1988). Serum albumin, gelatin, casein, nonfat milk, and other proteins have been used to block unreacted sites on the solid phase following binding of the primary antibody or antigen and can reduce nonspecific binding. Controlling for nonspecific binding to the plate makes it possible to use an excess of detection antibody. There are some reports that a so-called "edge effect" occurs by which coating proteins bind to peripheral wells better than to interior wells; however, well-to-well variability is likely to be greater than edge effects (Carpenter, 1992).

The development of each individual ELISA system requires careful attention to essential steps in order to ensure accurate and reproducible results. The type of assay must be individually tailored to the particular analyte in question (Kemeny & Chantler, 1988).
Many aspects of ELISA methodology can prove to be problematic (Carpenter, 1992). However, if the details of the general ELISA procedures are strictly followed in the development of specific assays coupled with appropriate troubleshooting, reliable and useful test results can be obtained.

Immunoaffinity Chromatography

The basic concept of affinity chromatography is based on a selective interaction between the material to be isolated and a molecule, the ligand, that has been immobilized on a chromatographic support (Jack, 1992). The ligand is immobilized on the chromatography support, and the molecule of interest specifically binds to the ligand and remains bound until the complex is broken with an eluting agent (Scopes, 1992).

Immunoaffinity chromatography provides the specificity of immunologic reactions to affinity separations. Immunoaffinity chromatography uses antibodies or antigens as the affinity ligand whereby separation comes about through immunological reactions (Jack, 1992). Immunoaffinity chromatography is an effective technique for isolation of proteins. As with any immunological assay, the purity of the antibody and the affinity of the antibody for the antigen is critical.

The overall process of immunoaffinity chromatography for separation and extraction of proteins involves immobilization of a specific antibody by chemical bonding to an inert support matrix. The antibody matrix may be packed into a column, and a solution containing the protein antigen to be captured is passed over the antibody coated matrix (Harlow & Lane, 1988). The antigen binding sites located on the arm of the antibody come in contact with the antigen and capture it. The captured protein is retained, while unbound material passes through the column. The captured antigen can be recovered by a variety of elution techniques, which releases the antigen from the solid phase (Scopes,
Column techniques are designed to allow maximum contact between the material to be separated and the immobilized ligand (Harlow & Lane, 1988).

Alternatively, batch techniques can be used to isolate proteins. Batch techniques are used when large amounts of protein antigen need to be captured with minimal effort. The availability of magnetic beads has greatly enhanced batch techniques with ease of recovery of the ligand bound protein (Wang, Borysiewicz, & Weetman, 1992). The activated support is incubated with the sample media. Constant stirring is required to prevent sedimentation of the solid support and loss of ligand efficiency (Blake, 1996). Application of a magnet for holding the protein ligand complex while removing unreacted material facilitates the recovery process. The batch technique using paramagnetic beads is shown in Figure 7.

For both traditional column and batch techniques, immunoaffinity chromatography can be divided into three steps: (1) preparation of the antibody matrix, (2) binding of the antigen or protein to the antibody matrix, and (3) elution of the antigen from the matrix is desired (Harlow & Lane, 1988). Details of some of the more common techniques for preparing the antibody matrix and binding of the antigen to the antibody matrix are reviewed below. Since the research presented in the following chapter did not involve elution of the antigen, a limited discussion of elution techniques is provided.

**Preparation of the Antibody Matrix**

**Characteristics of Common Solid Supports**

The desirable characteristic of support matrices used for chromatographic separations is a large surface area that is mechanically stable with suitable sidechains that can be easily modified for ligand attachment. The support matrix must be chemically inert to avoid nonspecific binding (Harlow & Lane, 1988).
Figure 7. Immunoaffinity chromatography batch technique using magnetic beads. Magnetic beads with an activated ligand, an antibody, are added to a mixture containing a number of proteins (x) and the protein (Ag) of interest. The mixture is incubated allowing the protein of interest to bind to the antibody ligand (#1). The captured protein can be extracted by applying a magnetic field to the test tube (#2).

A number of supports are available for chromatography. For chromatographic separations, solid phase supports are usually used in beaded form. Organic supports include cellulose, dextran, and agarose. Inorganic supports include silica and solid glass beads. Silica supports can be chemically modified to produce silane groups to which sidechains (e.g., epoxy and thiol groups) are attached. These sidechains provide attachment sites for the ligand to be immobilized (Bhatia et al., 1989). Silica can also be modified with a thin coat of hydrophilic polymers, which form both a protective coat and a spacer arm for ligand attachment (Hermanson, Mallia, & Smith, 1992). Glass beads can also be silanized followed by chemical modification of the silanol group for attachment of reactive side chains (Bhatia et al., 1989). Synthetic supports include polyacrylamide, methacrylate, and acrylic, which are mechanically strong (Hermanson et al., 1992). Composite matrices include polysaccharide-polyacrylamide and magnetic beads.
Magnetic beads are gaining popularity as ligand supports for batch separation methods as the affinity ligand can easily be recovered with a magnet (Wang et al., 1992). Beads are good supports for batch purification and immunoassays using a wide variety of ligands (Harlow & Lane, 1988). Immunomagnetic beads coated with a polystyrene shell provide a smooth hydrophobic surface that adsorbs molecules (e.g., antibodies) or can bind other bioreactive molecules covalently through surface hydroxyl groups (Wang et al., 1992). Several types of beads can be activated by chemical treatment to produce binding sites for proteins with free amino groups. Ligands can be coupled by a variety of techniques. The disadvantage of the use of magnetic beads as the solid phase is that washing becomes a tedious and time-consuming process (Harlow & Lane, 1988).

**Antibody Ligands**

A number of different molecules can serve as affinity ligands for chromatographic separation and extraction. The major constraints are that the ligand binds specifically, and usually reversibly to the material being isolated and contain groups within its chemical structure that bind the solid support (Harlow & Lane, 1988). Immunoaffinity chromatography utilizes antibodies or antigens as the ligand. The discussion here will be limited to the use of antibodies as the capture ligand.

Either monoclonal antibodies or polyclonal antibodies to the antigen in question are covalently attached to the solid phase (Harlow & Lane, 1988). Monoclonal antibodies can be made to any number of specific antigenic determinants. Polyclonal antibodies react well with more common antigenic determinants. Polyclonal antibodies usually bind to several different sites on the antigen with high avidity. This binding pattern, however, makes it difficult to elute the protein (Harlow & Lane, 1988; Jack, 1992).

The strength and selectivity of the immobilized antibody to the material being isolated are critical (Pepper, 1992). Polyclonal antibodies used in affinity chromatography
require purification. Isolation of the IgG fraction can be achieved with either ion exchange chromatography or salt purification with ammonium or dextran sulfate (Hermanson et al., 1992). Further purification by affinity chromatography using the target antigen as the immobilized ligand may be desirable in some cases. Antibodies with high affinity can yield quantitative removal in less than 1 hour of incubation. Those with low affinity, even if in high concentration, will never bind all the antigen in solution (Harlow & Lane, 1988).

**Attachment of Antibodies to the Solid Phase**

A number of methods have been described to attach antibodies to the solid phase. Antibodies can be immobilized by chemical linkage to reactive sidegroups on the solid phase or via specialized protein coatings (Bhatia et al., 1989). Direct attachment of antibodies to the solid phase can be accomplished using a number of functional side chains (e.g., thiol, epoxy, carbonyl, and carbonyl diimidazole; Hermanson et al., 1992). Direct attachment of antibodies has been reported to be haphazard. In this case the antibody is attached by amine groups at any point along the heavy chain and may not be optimally oriented for antigen capture. For maximum capture efficiency, antibodies should be attached to the solid phase via the Fc or tail portion, leaving the arms containing the two antigen receptors free to bind the antigen (Harlow & Lane, 1988). For this reason, hydrazine has become popular to attach the antibody by the Fc fragment. This can be achieved following treatment of the antibody carbohydrate moiety with periodate (Hermanson et al., 1992).

To facilitate optimal binding and orientation of antibodies to the support matrix, special coatings can be applied to the support matrix. The general methods for coupling antibodies to the solid phase include use of protein A/G beads, activated beads, and activated antibodies (Harlow & Lane, 1988). Protein A binds specifically to the Fc, or tail...
portion, of the antibody. The antibody is stabilized to the matrix by cross-linking by a bifunctional coupling reagent (Harlow & Lane, 1988).

The second method involves use of chemically activated beads containing reactive groups that are coupled to the antibody (Harlow & Lane, 1988). Several commercially prepared matrices are available that contain secondary reagents that bind specifically to antibodies. Reagents used to activate beads include carbonyldiimidazole, cyanogen bromide, and tosyl chloride which bind the solid phase matrix by an -OH group; gluteraldehyde, which binds by an -NH2 group; and hydroxysuccinate, which binds by the -COOH moiety (Harlow & Lane, 1988). Antibodies are coupled to the activated beads through amine groups. Overcoupling can decrease antibody activity and can usually be avoided by letting the reaction proceed at neutral pH (at of above the pK of the amino groups; Harlow & Lane, 1988). The major advantage of using activated beads is that the antibody can be attached covalently ensuring a stable reactive phase (Harlow & Lane, 1988).

Alternately, antibodies can be chemically activated and then attached to the solid phase (Harlow & Lane, 1988). Purified antibodies may be activated by a number of bifunctional reagents where one group binds to the antibody and the other to the solid phase (Harlow & Lane, 1988). Common reagents for indirect coupling of antibodies to the solid phase include water-soluble carbodiimides (e.g., 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimides, EDAC and EDCI), condensing agents for peptide synthesis (e.g., N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, EEDQ), glutaraldehyde, and periodate (Harlow & Lane, 1988). Antibodies activated by carbodiimides and condensing agents bind to carboxylic acid. Glutaraldehyde and periodate-activated antibodies bind to amines (Harlow & Lane, 1988). The primary advantage of antibody activation is that it allows a choice of a wide range of beads and spacer arms where necessary (Harlow & Lane, 1988).
Spacer arms, which extend the immobilized ligand away from the support matrix, are utilized to reduce steric hindrance between the immobilized support and the material to be isolated. Steric hindrance can reduce or prevent specific binding. This can be overcome by binding a spacer arm to the support matrix prior to attachment of the ligand. The ligand can be attached by a gluteraldehyde or carbodiimide-mediated reaction (Hermanson et al., 1992).

Nonspecific interactions can occur between the substance being isolated and either the support matrix or the spacer arm (Bhatia et al., 1989). Regardless of the means of attaching the antibody to the support matrix, once the antibodies are attached, unreacted side chains should be blocked to avoid both specific and nonspecific binding with components of the test media (Bhatia et al., 1989).

**Attachment of Antigen to the Antibody Bead Matrix**

The two recommended procedures involve binding of the antigen in a constantly mixing slurry of antibody-activated beads and a sample containing the antigen or by passing the antigen solution down an antibody-activated bead column (Harlow & Lane, 1988). The sample is exposed to the antibody bead matrix, and the antigen becomes bound to the immobilized antibody during incubation (Harlow & Lane, 1988). The remainder of the unreacted sample is removed by washing (Harlow & Lane, 1988).

**Elution**

Elution involves desorption of the bound material from the ligand. The forces holding the antibody-antigen complex intact are the same forces contributing to the intramolecular structure and stability of the two proteins. Both antibodies and antigens can easily be denatured by elution buffer (Scopes, 1992). When an antigen is bound to several different antibodies, as occurs with polyclonal antibodies, the harsh elution conditions
required to remove the antigen from the solid support can also partially denature the antigen (Harlow & Lane, 1988). More conservative approaches can be used to elute monoclonal antibodies due to the limited number and similar characteristics of bonds formed with the antigen. Antigen-antibody complexes can be broken by treating with harsh conditions, adding a small amount of an agent that resembles the antigen-antibody binding site, or by treating with an agent that causes an allosteric change in the binding site. The antigen is released into the eluate and subsequently recovered (Harlow & Lane, 1988).

CONCLUSION

IDDM develops in a relatively small number of individuals who carry specific genetic characteristics associated with increased disease susceptibility. The interaction of genetic, immunological, and environmental factors working over the extended diabetic prodrome appears to be extensively involved in the pathogenesis of the disease. To date, the exact mechanism of autoimmunity and its interplay with genetic predisposition and environmental factors is unknown.

There appear to be a number of potential environmental factors that may trigger and sustain the autoimmune process that eventually leads to total destruction of the insulin-producing beta-cells of the islets of Langerhans in the pancreas and overt clinical disease. If the initial insult triggering the autoimmune response could be identified and subsequently eliminated, a substantial reduction in the incidence of IDDM could be achieved.

Epidemiological evidence points to an important role of diet in the etiology of IDDM. The complexity of the search for a diabetogenic agent is compounded by the likelihood of multiple dietary factors and potential interaction with other environmental determinants. Studies have failed to look at both the qualitative and quantitative aspects of infant diet against a background of genetic predisposition and the progression of autoimmunity to pancreatic beta-cells. While there is still much controversy surrounding
infant feeding practices and their relationship to the pathogenesis of IDDM, there is substantial evidence to suggest that reduced incidence and duration of breast-feeding, particularly exclusive breast-feeding, and early introduction of cow's milk protein are contributing factors.

An elaborate scheme involving genetic and immunological factors interacting with the specific BSA epitope, ABBOS, in the pathogenesis of IDDM has been proposed. Substantial evidence of an immunological response to BSA, and specifically to the ABBOS epitope, has been repeatedly and independently documented. There are, however, conflicting reports linking immunization with BSA to specific genetic and immunological markers of IDDM. The role of BSA as an antigenic trigger in the pathogenesis of IDDM is controversial and will require further investigation.

Assessment of the BSA content of cow's milk and commercial infant formula preparations will provide critical information to assist professionals and public health agencies in making sound nutritional recommendations to help reduce the incidence of IDDM in those not exclusively breast fed for the first year of life. The American Academy of Pediatrics (1994) "strongly encourages" families with a history of IDDM to adopt the practice of breast-feeding and avoid cow's milk and products containing intact cow's milk protein during the first year of life. Since routine screening to detect the "diabetic genes" is not practical at this time as a component of public health policy, it may be appropriate for all infants to avoid BSA. Development of technology to commercially produce an affordable BSA-free breast-milk substitute for use in feeding all infants not exclusively breast-fed may prophylactically help to reduce the incidence of IDDM. The results of the research reported within this dissertation provide vital information that infant formula is heterogeneous in terms of its BSA content. This information can be used to further evaluate infant feeding practices and the contribution of BSA to the immunopathogenesis of IDDM.
CHAPTER III

METHODS

The dissertation research described below was designed to determine the amount of BSA present in commercially available infant formulas and to provide a laboratory model for extraction of BSA from cow's milk and whey protein isolates used in the manufacture of infant formula. A sensitive enzyme-linked immunosorbant assay (ELISA) was developed to detect and quantitate the amount of BSA present in both polymeric and hydrolyzed infant formulas. The objective of the second component of the research was to develop a laboratory model to produce modified cow's milk and whey isolates free of BSA. An immunocapture method, using paramagnetic beads, was developed to specifically remove BSA and immunologically active epitopes of BSA from cow's milk and whey isolates. These BSA-free protein sources could be used in the manufacture of infant formulas, which may in turn decrease the expression of IDDM in genetically susceptible individuals.

HYPOTHESES

1. An ELISA can be developed to detect and quantitate the BSA composition of cow's milk and whole formula samples.

2. The BSA content of cow's milk and polymeric cow's-milk-based infant formulas varies directly with the whey protein fraction.

3. Hydrolyzed infant formulas contain antigenic epitopes of BSA that can be detected by ELISA.

4. BSA can be captured and extracted from cow's milk and whey protein isolates utilizing a specific anti-BSA immunoaffinity chromatography batch technique.
MEASUREMENT OF THE BOVINE SERUM ALBUMIN CONTENT OF INFANT FORMULAS

Direct and indirect ELISAs were evaluated as methods for detecting and quantitating BSA in cow's milk and cow's-milk-based infant formulas. Indirect ELISA is currently used by the formula industry to detect residual antigenicity of hydrolyzed infant formulas (Cordle, 1994). The two-site, direct ELISA is generally considered to be more sensitive than indirect methods (Kemeny & Chantler, 1988). A sensitive two-site, direct ELISA was developed and adopted to quantitate BSA in cow's milk and formula following preliminary evaluation of direct and indirect methods. Preliminary studies are briefly described below.

Preliminary Studies

Indirect ELISA Methodology

The indirect ELISA was evaluated as a means of detecting and quantitating BSA antigen (Figure 8). With indirect ELISA, the antigen, in this case BSA, was bound passively to the microtitre plate during incubation of the formula sample. Excess sample was removed by washing. The second layer of the assay consisted of anti-BSA antibody, added in excess, that was incubated with the bound antigen. Unbound antibody was removed by washing. Bound antibody, and therefore antigen, was detected using a horse-radish peroxidase (HRP) labelled anti-immunoglobulin (IgG). The most sensitive substrate for detection of low levels of HRP enzyme is 3, 3', 5, 5'-tetramethylbenzidine hydrochloride (TMB). The color formed is proportional to the amount of labelled antibody and, therefore, antigen (Makarananda & Neal, 1992). Details of the specific ELISA protocol established to detect and quantitate BSA are outlined in Table 2.
Figure 8: Indirect ELISA for quantitation of BSA. Antigen is added to the microtitre plate and incubated. Unadsorbed antigen is removed by washing with PBS-T prior to addition of antibody. Specific antibody is incubated with the antigen. Unbound material is removed by washing. Enzyme-labelled secondary antibody (species specific anti-IgE) is added and incubated with the bound IgE. The enzyme chromagenic substrate is added as the final step.
Table 2

Indirect ELISA Protocol

1. BSA standards and formula samples diluted in carbonate-bicarbonate coating buffer (pH 9.6).
   (PBS-Tween 20 used as blank).
2. 100 µl diluted standard or sample added, in triplicate each microtitre plate well.
3. Plates incubated for 1 hour at 37 degrees Celsius in a humid chamber.
4. Wells washed 3 times with PBS-0.2% Tween 20 (pH 7.4).
5. Anti-Bovine Serum Albumin (BSA), developed in rabbit, fractionated antiserum (Sigma B-7276, lot: 024H8960) diluted 1/2000 in PBS-Tween 20.
6. 100 µl added to microtitre plate wells.
7. Plates incubated for 1 hour at 37 degrees Celsius in a humid chamber.
8. Wells washed 3 times with PBS-Tween 20.
10. 100 µl added to microtitre plate wells.
11. Plates incubated for 1 hour at 37 degrees Celsius in a humid chamber.
12. 100 µl TMB substrate added to microtitre plate wells.
13. 100 µl 2 M H₂SO₄ added to each microtitre plate well to stop reaction at 30 minutes.
14. Absorbance read at 450 nm using the Bio-Rad EIA Mac Reader 2.0.

(Adapted from Bono, Fisher, Abuodeh, Legendre, & Scalarone, 1995).
The initial indirect ELISAs were performed using an enzyme-labelled anti-BSA as the detector antibody. The availability of the enzyme-labelled anti-BSA eliminated the need for the secondary antibody conjugate. Unfortunately, the anti-BSA HRP-labelled antibody used in the first set of experiments gave no response to substrate. It was concluded that the HRP label was not bound to the antibody.

In the second set of experiments, anti-BSA antibody was used in conjunction with a secondary HRP-labeled anti-immunoglobulin conjugate, as described in Table 2. This ELISA system responded well in the presence of substrate with anti-BSA antibody demonstrating avidity for the BSA antigen. Several trial runs were performed using various concentrations of primary and secondary antibody with serial dilutions of BSA and positive control formula samples on several independent occasions. Problems were encountered with high background interference. It was concluded that the low signal-to-noise ratio could be attributed to inability of the BSA antigen in the test sample to bind to the microtitre plate, a known limitation of indirect ELISA (Kemeny & Chanter, 1988). In addition, specific and nonspecific binding could result from interaction of either the primary or secondary antibody and the unreacted sites on the microtitre plate. The target antigen, in this case BSA, is the most common agent used to block unreacted sites and prevent nonspecific binding in ELISA. For these reasons, the indirect ELISA was aborted, at this point, in exchange for the more sensitive, direct, two-site ELISA.

Direct ELISA Methodology

The second approach utilized the direct, two-site ELISA (Figure 9). Anti-BSA antibody was passively bound to the microtitre plate and served as the capture antibody. Samples containing BSA antigen were incubated with the capture antibody. Bound BSA was subsequently detected and quantitated using a second HRP-labelled anti-BSA antibody that binds to a different region of BSA than the capture antibody. With the direct approach,
Figure 9: Direct (two-site) ELISA for quantitation of BSA. Anti-BSA antibody added to the microtitre plate and incubated. Unadsorbed antibody removed by washing with PBS-T prior to addition of the sample containing the BSA antigen. Test sample incubated with the capture antibody. Unbound sample removed by washing. A secondary enzyme-labelled anti-BSA antibody added and incubated with the captured BSA. TMB substrate is added as the final step.
BSA is "sandwiched" between two layers of antibodies. Table 3 provides a detailed description of the final direct ELISA protocol for detection and quantification of BSA in cow's milk and infant formula. Several optimization strategies were employed to arrive at this final direct ELISA protocol and are briefly reviewed below.

**Optimization of the Direct ELISA**

**Determining Working Antibody Concentrations**

The first step was to measure the background absorbance of each ELISA reagent. The ELISA reagents included the primary anti-BSA antibody in coating buffer, the secondary anti-BSA HRP-labelled detector antibody in PBS-Tween, BSA standards in PBS, a negative sample (soy formula), a positive sample, TMB substrate, and a PBS blank. Baseline absorbance of individual ELISA components was unremarkable.

Optimal working conditions for each antibody were determined using checkerboard titration. Figure 10 shows the scheme for optimization of the primary capture and secondary detector antibodies. A working concentration of 1/1,500 for the capture antibody and 1/6,000 for the detector antibody was selected. Together, these concentrations provided an ELISA system with the maximum signal for the BSA standards to background absorbance of the PBS-Tween and soy blanks. Consistent with optimization guidelines of Kemeny and Chantler (1988) and Carpenter (1992), these dilutions yielded a high positive control of approximately 1 absorbance unit (0.978) and a negative sample absorbance of less than 0.2 (0.15).

The preliminary titration studies confirmed the efficacy of the direct ELISA as a means of quantitating BSA in cow's milk and infant formula. A graded absorbance reading was obtained for serial dilutions of BSA. BSA could be recovered from the spiked soy formula. At this point, the direct ELISA was adopted for further experiments.
Table 3

Direct (Two-Site) ELISA Protocol

1. Anti-Bovine Serum Albumin (BSA), developed in rabbit, fractionated antiserum (Sigma B-7276, lot: 024H8960) diluted 1/1500 in carbonate bicarbonate coating buffer (pH 9.6).

2. 100 µl diluted antibody preparation added to each well of 96-well microtitre plate (Corning, disposable non-sterile polystyrene assay plates, 25880-96).

3. Plates incubated in a humid chamber at room temperature for 2 hours or overnight at 5 degrees Celsius.

4. Wells washed 3 times with PBS-Tween 20 (0.2%).

5. BSA standards and samples diluted in PBS-Tween 20 (0.2%). (PBS-Tween used alone in selected wells to blank absorbance on EIA Reader.)

6. 100 µl standard or samples added to each microtitre plate well, in triplicate.

7. Microtitre plates incubated at room temperature for 1 hour in a humid chamber.

8. Wells washed 3 times with PBS-Tween 20 (0.2%).


10. 100 µl diluted antibody conjugate added to each microtitre plate well.

11. Microtitre plates incubated for 1 hour at room temperature.

12. Wells washed 3 times with PBS-Tween 20, last wash no Tween.

13. 100 µl TMB substrate added to each microtitre plate well

14. 100 µl 2 M H₂SO₄ added to each microtitre plate well to stop reaction at 30 minutes.

15. Absorbance read at 450 nm using the Bio-Rad EIA Mac Reader 2.0.
Figure 10. Checkerboard titration for antibody dilution optimization. The primary antibody was tested in duplicate rows in concentrations ranging from 1/1,500 to 1/12,000. The secondary antibody conjugate was tested in columns in dilutions of 1/3,000 and 1/6000 over each dilution of the primary antibody. Dilutions of 1/9000 and 1/12,000 of secondary antibody conjugate were tested on a second plate. Three BSA dilutions of 1, 10, and 100 nanograms of BSA per 100 µl; a PBS-Tween and soy blank; and soy formula spiked with 10 nanograms of BSA were used as samples and controls.

Assessment of Blocking Agents

It is common in ELISA to block unreacted sites on the microtitre plate following binding of the capture antibody. Since BSA itself is the most common blocking agent used in ELISA, and likely present in other common blocking agents (e.g., powdered milk and gelatin derived from beef), alternative blocking agents were evaluated.
To optimize the signal-to-noise ratio, 3% gelatin extracted from pork, was incubated 1 hour at 38 degrees Celsius with the previously bound antibody on the solid phase. Unbound material was removed using three washes of PBS-Tween. The direct ELISA protocol was subsequently followed from step 5 (Table 3). The background was found to be much higher when gelatin was used to block unreacted sites on the solid phase. The gelatin was found to be an unsuitable blocking agent due to nonspecific binding of the detector antibody. This could possibly be explained by the stickiness of the gelatin attributed to high bloom.

Casein and salmine at concentrations of 1, 3, and 5% and Tween 20 at 0.2 and 0.4% diluted in PBS were evaluated as alternative blocking agents. The blocking effect of salmine was greater than that of casein. The Tween exhibited the greatest blocking effect, which was similar at both concentrations.

It was postulated that the formula sample could be diluted in PBS-Tween, avoiding the blocking step all together. To test this hypothesis, identical plates were run with (a) 100 µl PBS-Tween 20 (0.2%) added to the antibody matrix and incubated for 1 hour at 37 degrees Celsius prior to addition of a positive formula sample diluted in PBS and (b) direct addition of the positive formula sample diluted in PBS-Tween 20 (0.2%). The latter proved to be as effective and eliminated the time and effort of a separate blocking step.

Comparison of Effectiveness of the Direct ELISA in Whole Samples Versus the Soluble Fraction

Preliminary studies were performed using the soluble fraction of cow's milk and select formula samples. Results were compared to whole formula samples to determine if the ELISA could be performed using whole formula samples. Since BSA is a whey protein, it should be isolated to the soluble fraction. Formula samples were centrifuged at 5,000 RPMs for 90 minutes at 4 degrees Celsius and the soluble fraction extracted for assay. Initial assays performed using the soluble fraction did not yield superior results.
relative to whole formula samples. The latter method was preferred due to relative ease of assay and quantitation of the actual BSA load provided to the infant from the formula being evaluated.

**Estimation of Recovery of BSA**

In order to determine if BSA could be effectively captured from whole formula samples by the refined ELISA system, whole formula samples, two high-whey, two high-casein, and a negative soy control were spiked with 10 nanograms of BSA. Recovery of BSA was estimated by subtracting the amount of BSA in the nonspiked sample from the amount of BSA detected in the spiked sample. The capture efficiency confirmed the direct ELISA protocol for quantitating the BSA antigen load in cow's milk and infant formula.

**Samples**

Preliminary evaluation of direct and indirect ELISA methodology was conducted using BSA samples. BSA samples were diluted in the appropriate ELISA buffer. BSA samples were diluted in carbonate-bicarbonate coating buffer for assessment of indirect ELISA methodology. All other BSA standards and samples were diluted in phosphate-buffered-saline (PBS) with Tween 20 (Polyoxy-ethylenesorbitan Monolaurate, Sigma P-7949, Lot 15H09291).

The BSA content of cow's milk (2%), 12 polymeric infant formulas (Table 4), and 4 hydrolyzed infant formulas was measured (Table 5). Ready to serve, concentrated liquid, and powdered infant formulas were selected where available. Formula samples were prepared according to manufacturers' directions. Powdered formula samples were weighed and reconstituted with the appropriate amount of deionized water. Whole formula samples were chosen for analysis, which is consistent with that currently used in the
industrial setting in quality control evaluations for residual antigenicity of hypoallergenic formula preparations.

Cow's milk and infant formulas were tested in serial dilutions from each of three batches. Samples were diluted in phosphate buffered saline (PBS) with Tween 20 (Butler, 1988). Sample dilutions were as follows: full strength, 1/100, 1/500, 1/1,000, 1/5,000, 1/10,000, and 1/50,000. Individual sample dilutions were tested in triplicate.

Controls

BSA standards were weighed in triplicate and diluted in PBS-Tween 20. BSA standards ranged from 0.08 ng/100 µl to 50 ng/100 µl. Each of the three sets of BSA standards was run in triplicate on three microtitre plates by direct ELISA. The standard curve was determined by plotting the actual absorbance measured by the EIA Mac Reader 2.0 against the known BSA concentration. Construction of the BSA standard curve provided an absolute precalibrated standard as a reference for homologous interpolation and a quantitative evaluation of the BSA content of samples (Kemeny & Chantler, 1988). The BSA concentration of unknowns was interpolated from the BSA standard curve based on the absorbance of the formula sample. In addition, BSA standards for high, middle, and low positive controls were performed on each plate in order to assess the interassay coefficient of variation.

A follow-up study was performed to determine whether our direct ELISA system detected the presence of the ABBOS epitope. Synthetic ABBOS peptide was obtained from Hans-Michael Dosch, M.D., Hospital for Sick Kids, Toronto, Canada. The direct and indirect ELISA procedure was performed using the ABBOS peptide as the antigen in place of BSA. Using both direct and indirect ELISA provided a means of individually assessing the avidity of the capture and detector antibody for the ABBOS epitope.
Table 4

Polymeric Infant Formulas

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Table 5

Hydrolyzed Infant Formulas

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Electrophoresis

Formulas testing negative for BSA by ELISA were evaluated by electrophoresis to confirm the absence of BSA from the sample. Defatted formula samples, skim milk, and BSA standards were prepared for analysis by mixing 100 µl sample, 100 µl 10 mM Tris Buffer, 350 µl 10% SDS, 350 µl deionized water, and 50 µl 2-mercaptoethanol. Prepared samples and controls were brought to a boil for 5 minutes and vortexed to separate proteins in order to facilitate their migration on the polyacrylamide gel. Proteins from the powdered hydrolyzed formulas migrated well in the polyacrylamide gel; however, the initial runs performed on the polymeric ready-to-serve and liquid concentrate preparations did not migrate into the polyacrylamide gel. This observation was hypothesized to be due to agglutination of the proteins during heat sterilization of the formula. To enhance separation
of the agglutinated proteins, the Tris buffer was increased to a 100-mM concentration; 400 µl of 10% SDS was added, and the samples were boiled for 10 minutes. Following preparation, formula samples and controls were analyzed by the PhastSystem, PhastGel Electrophoresis System (Pharmacia LKB Biotechnology). BSA has a molecular weight of 66 kilodaltons, which can be easily detected by electrophoresis.

**IMMUNOCAPTURE AND EXTRACTION OF BOVINE SERUM ALBUMIN FROM COW'S MILK AND WHEY ISOLATES**

Affinity chromatography, using magnetic beads with an immobilized antibody against BSA, was utilized to selectively extract BSA from cow's milk and whey isolates. Immobilization of antibodies to hydroxyl groups on silica surfaces with heterobifunctional crosslinkers has previously been reported (Bhatia et al., 1989). Bhatia's methodology has been modified in Dr. Weimer's laboratory at Utah State University by Michael Blake for immunocapture, detection, and quantification of bacterial spores in food products and environmental samples, patent pending (Blake, 1996). Blake's methodology was applied and adapted for immunocapture and extraction of BSA from cow's milk and whey protein isolates. The experiments outlined below provide proof of concept of a laboratory model demonstrating that BSA, and antigenic epitopes of BSA, can be captured and extracted from cow's milk and whey protein isolates.

**Purification of Polyclonal Antibodies**

Two sets of polyclonal antibodies (1) Polyclonal Rabbit Anti-Serum to BSA (Chappel 55269; lot # 39879) containing 61.8 mg/ml total protein and 6 mg/ml antibody and (2) Polyclonal Rabbit Anti-Bovine Serum Albumin, Delipidized Whole Antiserum (Sigma Bio Sciences B-1520; lot # 055H4811) containing 60.4 mg/ml total protein and 3.0 mg/ml specific antibody were purified using Pierce ImmunoPure (A/G) IgG
Purification Kit (No. 44902) according to the standard protocol (Table 6). The IgG fraction of purified antibodies was subsequently determined by measuring the absorbance at 280 nm. Purified antibody samples were blanked against ImmunoPure IgG Elution Buffer. At 280 nanometers an absorbance of 1.35 corresponds to an IgG protein concentration of 1 mg/ml (Harlow & Lane, 1988). The protein concentration of selected aliquots of purified antibody measured 1.66 mg IgG/200 μl and 2.9 mg IgG/200 μl sample sets 1 and 2, respectively.

Washing and Desalting of Antibodies

Purified polyclonal and monoclonal antibodies were washed to remove excess salts and stored in appropriate buffer solutions depending on the intended use of the antibodies. General procedures for washing and desalting antibodies are outlined in Table 7. Antibodies to be used for standard ELISA were washed in phosphate buffered saline (PBS) (pH 7.2) and stored at -20 degrees Celsius. Antibodies to be attached to paramagnetic beads and used in immunocapture and BSA extraction experiments were washed in 0.1 M sodium phosphate (pH 7.0) and stored at 5 degrees Celsius for use within 48 hours.

Monoclonal antibodies to BSA, Mouse, IgG 1K, Ig Fraction (American Qualex MMO 250 P, lot # 60658G) containing an antibody concentration 4.11 mg/ml (0.5 mg) in PBS were also prepared for ELISA and immunocapture BSA extraction experiments. Five microliters of the monoclonal antibody preparation was aliquotted for running standard ELISA and stored at -20 degrees Celsius. The remaining 116.65 μl of monoclonal antibody was washed in 0.1 M sodium phosphate (pH 7.0) according to the general procedures for washing and desalting antibodies outlined in Table 7. In variation to the above procedure, each wash consisted of a 20-minute centrifuge cycle at 2500 RPMs (Blake, 1996). Washed and desalted monoclonal antibodies to be attached to paramagnetic beads were stored at 5 degrees Celsius for use within 48 hours.
Table 6

Standard Protocol for the Isolation and Purification of IgG

1. Protein A/G column opened. Top stopper removed first and storage solution poured off column.

2. Protein A/G column equilibrated with 10 ml of the ImmunoPure IgG Binding Buffer.

3. Diluted sample applied to the column and allowed to flow completely into the gel. (Affinity PAK Columns stop flowing automatically when the liquid reaches the top disc.)

4. Protein A/G column washed with 20 ml of the ImmunoPure IgG Binding Buffer.

5. Bound IgG eluted with 10 ml of the ImmunoPure IgG Elution Buffer. To obtain the most concentrated sample, ten 1.0 ml fractions were collected. Elution of bound proteins was monitored by absorbance at 280 nm.

6. Column regenerated by flushing and storing with 10 ml 0.02% sodium azide.

(Instructions ImmunoPure [A/G] IgG Purification Kit; Pierce, 1995)

Attachment of Antibodies to Paramagnetic Beads

Tosylactivated, super paramagnetic, 2.8 µm, polystyrene beads (Dynabeads M-280; Dynal 142.03) were used as the solid phase for immunocapture and biomagnetic extraction of BSA from cow's milk and whey protein isolates. A polythreonine cross-linker (60 nm, MW [vis] 12,100) was covalently coupled to the tosylactivated groups of the bead via the terminal amine (Hermanson et al., 1992). This configuration, as shown in Figure 11, provided an extended cross-linker to minimize steric hindrance between the BSA molecule
Table 7

General Procedures for Washing and Desalting Antibodies

1. Antibodies washed with PBS (pH 7.2) or NaPO₄ (pH 7.0).

2. Filtered concentrator used for centrifuging and washing antibodies. (Centricon 30 concentrator 4208, MDB093 or Filtron 30K Macrosep 0D030C36).

3. Antibodies placed in top of tube (above filter).

4. To wash, top of tube filled with PBS or NaPO₄. Sample centrifuged 30 to 60 minutes at 5000 RPMs (centrifuged with filter perpendicular to centrifuge pull). Wash in bottom of centrifuge filter tube discarded, antibody (above filter) saved. Washing repeated 5 times.

5. After final wash, wash discarded, centrifuge concentrator tube recapped, placed upside-down in centrifuge, and spun at 1000-2000 rpms for 1 minute to collect antibody. Antibody aliquotted and stored for future use.

(Adapted from Blake, 1996)

to be captured and the bead. In addition, the long flexible arm allowed the antibody to establish the correct binding orientation with the BSA antigen (Blake, 1996). Tris buffer, in particular the amine group, served to block the remaining tosylactivated sites. Adenine dihydrazide (ADH) was linked to the carboxyl terminal of the bound polythreonine using an ethylene diamine carbodiimide mediated reaction (Hermanson et al., 1992).

The ADH functions to convert the hydroxyl end of the polythreonine to an activated amine. Either monoclonal or polyclonal anti-BSA antibodies were attached to the carboxyl, ADH-activated end of the polythreonine spacer via the carbohydrate moiety of the Fc or tail of
Figure 11. Immunomagnetic capture of BSA antigen using a polythreonine spacer. (Adapted from Blake, 1996)

the antibody during incubation. This methodology provided an immunomagnetic capture medium that allowed for extraction of BSA from cow’s milk and whey protein isolates.

Paramagnetic beads were prepared in two batches according to the bead washing and preparation procedures (Table 8). Batch one, 1 ml of beads, was washed and prepared for coating with purified Polyclonal Rabbit Anti-serum to BSA (Cappel). Batch 2, 1250 µl of beads, was prepared accordingly; 1 ml of beads was prepared for coating with purified Polyclonal Rabbit Anti-Bovine Serum Albumin (Sigma) and 250 µl of beads was prepared for coating with monoclonal antibodies to BSA (American Qualex). The two sets of purified polyclonal antibodies and one set of monoclonal antibodies were prepared for coating of the prepared beads as outlined in Table 9. Sodium meta-periodate was used to
oxidize the carbohydrate moiety of the antibody and later removed by washing (Hermanson et al., 1992).

Each set of oxidized antibody preparation was combined with an equal volume of activated beads in a 1.5-ml microcentrifuge tube and incubated overnight on a parastaltic pump at room temperature. Coated beads were washed three times with sodium phosphate (pH 7.0) by gentle hand motion, approximately 3 minutes per wash (Blake, 1996). Subsequent assays revealed that four additional washes were required to remove unbound antibody from the bead suspension. Beads were resuspended in the original volume of PBS-Tween 20 to block unactivated sites on the bead surface. One set of control beads was prepared with polystyrene (Steps 1-9, Table 8), without the addition of antibody, and stored in PBS-Tween 20. Sodium azide was added to each set of beads at a final concentration of 0.02% to prevent bacterial growth. Beads were stored at 5 degrees Celsius.

Enzyme-Linked Immunosorbant Assay (ELISA)

The two-site, direct ELISA described in the preceding section was utilized to quantitate BSA for establishing BSA standards; baseline BSA concentrations of skim milk, acid whey, and sweet whey; and for analysis of residual BSA in samples treated with polyclonal and monoclonal anti-BSA activated antibody-coated paramagnetic beads. An overview of the direct ELISA procedures has previously been provided, Table 3.

BSA standards were weighed in triplicate and diluted in PBS-Tween 20. BSA standards ranged from 0.08 ng/100 µl to 50 ng/100 µl. Each of the three sets of BSA standards was run in triplicate on three microtitre plates by direct ELISA. The standard curve was determined by plotting the actual absorbance measured by the EIA Mac Reader 2.0 against the known BSA concentration. Construction of the BSA standard curve provided an absolute precalibrated standard as a reference for homologous
Table 8

Washing and Preparation of Beads

1. Beads resuspended by pipetting and vortexing for 1 minute (avoid foaming).
2. Desired volume of paramagnetic beads pipetted into 1.5 ml microcentrifuge tube.
3. Beads sequestered in magnetic chamber, 3 minutes.
   (MPG 3-in-1 MPS, Magnetic Particle Separator, MPSO301, lot 112895, Cpg Inc.)
4. Sodium phosphate pipetted off.
5. Beads resuspended in half volume 0.05 M borate buffer (pH 9.5).
6. Equal volume of polythreonine added to bead suspension:
   (polythreonine protein concentration 400 µg/ml 0.05 M borate buffer)
   polythreonine weighed,
   polythreonine dissolved in appropriate volume deionized water,
   borate buffer (0.5 M stock solution) added for final concentration
   of 0.05 M.
7. Beads incubated 24 hours at 37 degrees Celsius on parastaltic pump.
8. Beads sequestered in magnetic chamber. Borate supernatant pipetted off and
discarded.
9. Beads washed in 0.2 M Tris Buffer (pH 8.5) (three times):
   -Tris Buffer added to beads in microcentrifuge tube to bring to original volume,
   -beads placed on parastaltic pump at room temperature for 10 minutes
     (washed by gentle rolling motion),
   -beads sequestered in magnetic chamber, supernatant removed and discarded,
   -fourth wash done running on parastaltic pump for 30 minutes.
   (table continues)
10. Beads washed twice with 0.1 M 2-[N-Morpholino]ethanesulfonic acid (MES) (pH 4.75):
   - MES added to beads in microcentrifuge tube to bring to original volume,
   - beads washed by gentle hand washing, no parastaltic pump,
   - beads sequestered, supernatant removed and discarded.

11. Adipic Acid Dihydrizide (ADH), 0.5 M (pH 4.75) in 0.1 M MES added to beads to bring to original volume:
   - ADH weighed into 1.5 ml microcentrifuge tube,
   - appropriate volume 0.1 M MES added to yield 0.5 M ADH,
   - added to beads.

12. ADH and beads added to 1-ethyl-3 (3-dimethylaminopropyl) carbomide (EDAC):
   - EDAC weighed to 1.5 ml microcentrifuge tube,
   - goal 0.03 gms/ml,
   - ADH and bead suspension added to EDAC.

13. Beads incubated 3 hours at room temperature on parastaltic pump.

14. Beads washed by gentle hand motion as follows:
   - two washes sterile water at original volume,
   - two washes in 1 M NaCl at original volume,
   - three washes sterile water at original volume,
   - two washes 0.1 M sodium phosphate (pH 7.0) at original volume.

15. Beads sequestered in magnetic chamber, supernatant pipetted off and discarded.

16. Beads resuspended in half volume sodium phosphate (pH 7.0).

(Adapted from Blake, 1996)
Table 9

Preparation of Antibodies

1. Volume of purified antibody measured using pipette.
2. Sodium phosphate (pH 7.0) added to bring to 1 ml.
3. Antibody concentration measured by absorbance at 280 nm (1.35 = 1 mg/ml IgG).
4. Sodium phosphate (pH 7.0) added to bring antibody concentration to 1 mg/ml.
5. Antibody preparation added to sodium-m-periodate:
   (5 mg sodium-m-periodate/ml antibody)
   -weight sodium-m-periodate to 1.5 ml microcentrifuge tube,
   -add antibody.
7. Antibodies washed six times with 0.1 M sodium phosphate (pH 7.0) in order to
   remove sodium-m-periodate:
   -antibody transferred by pipette to filtered concentrator centrifuge tube
     (Centricon 30 concentrator 4208, MDB093);
   -antibodies placed in top of tube (above filter);
   -to wash, top of tube filled with sodium phosphate, sample centrifuged 30
     minutes at 5000 RPMs, wash in bottom of centrifuge filter tube discarded,
     antibody (above filter) saved; washing repeated 6 times;
   -after final wash, waste discarded, centrifuge tube recapped, placed upside-
     down in centrifuge, and spun until centrifuge comes up to 2000 RPMs.
8. Antibodies brought to half original volume with sodium phosphate (pH 7.0).

(Blake, 1996)
interpolation and a quantitative evaluation of the BSA content of samples (Kemeny &
Chantler, 1988). Baseline BSA concentration of Skim Milk (Meadow Gold Nonfat Skim
Milk; Vitamin A and D, 49-7, August 18; Meadow Gold Dairies Inc., Columbus, Ohio)
was determined by the direct ELISA. Acid Whey (Utah State University, Dairies;
delactosed whey) and Sweet Whey (Utah State University, Dairies; from cheddar cheese)
were reconstituted in deionized water to a 10% solution. Whey isolates were adjusted to
a pH of 7.0 using 1 M and 0.1 M sodium hydroxide. Baseline BSA concentration of the
whey isolates was determined by direct ELISA.

Preliminary Immunocapture Experiments

Preliminary antibody capture BSA extraction experiments are summarized in
Appendix B. Several studies were performed on independent occasions using 5, 10, and
20 µl of polyclonal anti-BSA antibody activated paramagnetic beads added to milk samples
ranging from 100 to 500 microliters. Samples and beads were incubated at room
temperature on a rotating parastaltic pump for 1 hour. Beads were sequestered in a
magnetic chamber. Samples were removed and saved for evaluation of residual BSA
content by the direct ELISA.

ELISA evaluation of the residual BSA content of the treated milk samples did not
show a measurable reduction in the BSA content of the samples following treatment with
polyclonal anti-BSA antibody activated paramagnetic beads. It was hypothesized that
these observations could be explained by a large excess of BSA in the milk sample relative
to the capacity of the immunocapture system to bind BSA. However, these observations
raised several additional questions. Was antibody present on activated beads? Was
unbound antibody present in the bead suspension that could bind BSA and remain in the
treated sample after the beads were sequestered? Could BSA be captured from the milk
sample? What was the capture efficiency of BSA in skim milk, acid whey, and sweet
whey using polyclonal and monoclonal antibody-activated beads? In order to answer these questions, additional assays were developed to quantitate the amount of antibody attached to the activated paramagnetic beads and the amount of BSA captured by the antibody activated beads. This methodology is described below.

Initial follow-up studies revealed that antibody was attached to the activated beads. Some antibody was detected in the bead suspension that was not bound to paramagnetic beads, but the amount present was not felt to have a significant impact relative to the total amount of BSA present in the treated sample. The unbound antibody could be removed with additional washes. BSA could be captured by the antibody-activated beads; however, a large excess of BSA remained in the treated sample. Subsequent studies focused on determining the capture efficiency of polyclonal and monoclonal antibody-activated beads in skim milk, acid whey, and sweet whey. This information could be utilized to determine the efficiency of the system and evaluate the feasibility of its application to the industrial setting.

Quantitation of Antibody Attached to Paramagnetic Beads

In order to provide a means to compare antibody attached to beads and efficiency of BSA capture between polyclonal, monoclonal, and control polythreoneine-activated paramagnetic beads, bead concentrations were measured spectrophometrically. Refraction was measured at 590 nanometers for each set of beads. Bead concentrations were equilibrated using PBS-Tween 20.

The antibody attached to the activated paramagnetic beads was quantitated using an antibody excess ELISA (Figure 12). The detailed modified ELISA methodology, using the bead as the solid support, is described in Table 10. Three sets each of polyclonal anti-BSA activated paramagnetic beads and monoclonal anti-BSA activated paramagnetic beads were assayed in serial triplicate dilutions. Polyclonal antibodies were quantitated using
Horseradish Conjugated Goat Anti-Rabbit IgG (Sigma Bio Sciences, A-6154, lot 026H8896) as the capture antibody. Monoclonal antibodies were quantitated using Anti-Mouse IgG Peroxidase Conjugate (Sigma Bio Sciences, A-4416, lot 082H8892) for capture. Polythreonine beads devoid of antibody served as the control.

A standard curve for each set of species specific capture antibodies was developed in triplicate using serial dilutions of the HRP-labeled antibody. Diluted anti-IgG-HRP standards were placed in triplicate (10 µl) in microtitre plate wells. Ninety microliters of TMB substrate were added to each well. Standards were incubated for 30 minutes at room temperature. The reaction was stopped with 2 M sulfuric acid. Microtitre plates were read at 450 nanometers on Bio-Rad EIA Mac Reader 2.0. Each of the three sets of anti-IgG standards was run in triplicate on three microtitre plates. Construction of a standard curve for each anti-BSA HRP-labelled antibody was developed based on the signal elicited when the TMB substrate was added to use as a reference standard for quantitating antibody content of beads. Absorbance of the sample, taken from the bead supernatant, was plotted against the species-specific IgG standard. The amount of antibodies present on beads is reported in nanograms of IgG. Values reported assume a one-to-one molar capture efficiency between the antibody on the bead and the anti-rabbit or anti-mouse IgG and represent relative, semiquantitative findings.

Antibody Capture BSA Extraction

Antibody capture BSA extraction experiments were repeated three times for each set of polyclonal and the monoclonal anti-BSA antibody activated paramagnetic beads and controls in order to provide a means to calculate and optimize the ratio of beads to skim milk and whey isolates necessary for BSA extraction. Capture efficiency of each set of monoclonal and polyclonal anti-BSA activated paramagnetic beads was compared. In
Figure 12. Antibody excess ELISA for quantitation of antibody attached to activated paramagnetic beads.

addition, capture efficiency was compared between the media of skim milk, acid whey, and sweet whey for the monoclonal and polyclonal antibody-activated beads.

Samples of skim milk, acid whey, and sweet whey were aliquotted to 1.5-ml microcentrifuge tubes. Activated beads were resuspended by rotating on a parastaltic pump for 5 to 10 minutes and then mixed with a pipette. Ten microliters of polyclonal or monoclonal anti-BSA antibody activated paramagnetic beads was added to a 500-µl sample for treatment. Control samples were treated with matching amounts of polythreonine treated beads devoid of antibody. Samples and beads were incubated at room temperature
Table 10

**ELISA Procedures for Quantitation of Antibody on Activated Paramagnetic Beads**

1. 10 µl active and control beads suspended in 500 µl of PBS-Tween.
2. Serial dilutions made in 1.5-ml microcentrifuge tubes (full-strength, 1/10, 1/100, 1/1000).
4. Beads incubated on parastaltic pump for 1 hour at room temperature.
5. Beads sequestered in magnetic chamber and washed three times in PBS-Tween (gentle hand washing).
7. Beads incubated at room temperature for 30 minutes in TMB on parastaltic pump.
8. Beads sequestered, 100 µl supernatant (reacted TMB) added to microtitre plate wells in triplicate.
9. 100 µl 2 M H₂SO₄ added to each well to stop reaction.
10. Absorbance read at 450 nm using the Bio-Rad EIA Mac Reader 2.0.

on a rotating parastaltic pump for 1 hour. Beads were sequestered in a magnetic chamber. Treatment and control samples were removed and saved for evaluation of residual BSA content by the direct ELISA. The beads were washed once with equal volume to sample of PBS-Tween. Beads were sequestered. Wash was pipetted off and saved for analysis of residual BSA. Beads were resuspended in equal volume to samples of PBS-Tween 20 and saved for quantitation of captured BSA.
A trial run of molecular sieving was performed to determine whether the bulk of the BSA could be removed from the sample, followed by use of the immunocapture to remove residual BSA. Skim milk was filtered in two Centricon tubes using a 50-kilodalton and 100-kilodalton separation device. Samples were centrifuged at 3000 RPMs for 3.5 hours. The filtrate less than 50 and greater than 100 kilodaltons was assayed by direct ELISA to determine residual BSA content. Since the bulk of the BSA, but not all BSA, could be removed by molecular sieving, it was proposed that coupling the immunocapture to molecular sieving to remove the relatively small amount of BSA remaining in the sample may be advantageous in order to overcome the limitations of the immunocapture system and strengthen its potential application to the industrial setting.

In order to determine if BSA could be removed totally from a sample and assess capture efficiency of activated beads at substantially lower baseline BSA concentrations, known samples of BSA (25 ng/500 µl in PBS-Tween) were treated with 10 µl of the Polyclonal Rabbit Anti-Bovine Serum Albumin (Sigma) activated paramagnetic beads and polythreonine control. The experiment was performed in triplicate according to the above procedures.

Quantitation of Captured BSA on Beads

Quantitation of BSA captured by polyclonal and monoclonal anti-BSA antibody-activated paramagnetic beads was achieved using a direct ELISA, with the immunomagnetic bead serving as the solid support, Figure 13. This procedure was developed and selected due to its relative ease and minimal potential loss of BSA, which might otherwise occur during elution procedures. The antibody attached to the activated bead served as the capture and antibody. Anti-Bovine Albumin-Peroxidase (horseradish-peroxidase labelled; developed in sheep; The Binding Site, PP204, batch 5939) diluted 1/5,000 in PBS-Tween 20 was used as the detector antibody. The specific steps in the direct ELISA are the same
Figure 13. Direct ELISA for quantitation of captured BSA on bead. The immunomagnetic beads function as the solid support in the ELISA. The antibody attached to the activated bead serves as the primary capture antibody. Following incubation of the antibody activated beads with the treatment sample, the beads are sequestered and washed with PBS-Tween. Captured BSA is detected by the addition of the secondary anti-BSA HRP-conjugate. Following incubation, the beads are sequestered and washed with PBS-Tween. Substrate is added to the microcentrifuge tube. The supernatant, carrying the enzyme/substrate reaction product is removed and added to a microtitre plate for analysis on the EIA MAC reader 2.0.

as those previously described in Table 10 for quantitation of the amount of antibody attached to the paramagnetic beads. Once BSA is bound to the bead, the secondary antibody conjugate can be added "sandwiching" the BSA between the two antibodies. A standard curve was developed for the anti-BSA-HRP as previously described for the species specific anti-IgG standards. The amount of BSA present on beads is reported in
nanograms and assumes a one-to-one molar capture efficiency between the antibody on the bead and anti-BSA-HRP conjugate and represents relative, semiquantitative findings.

The capture efficiency of each set of polyclonal and monoclonal antibody activated beads was determined after treatment of sample media (i.e., skim milk, acid whey, and sweet whey). The molar to molar capture efficiency was calculated using the following equation.

\[
\text{Capture Efficiency} = \frac{\text{ng BSA captured by 10 } \mu\text{l beads / 66,000 kD}}{\text{ng IgG bound to 10 } \mu\text{l beads / 160,000 kD}}
\]

This calculation assumes a one-to-one molar capture efficiency between the anti-BSA antibody attached to the activated beads and the species-specific detector antibody conjugate used to quantitate the amount of antibody attached to the beads. In addition, a one-to-one capture efficiency is assumed between the antibody present on the activated beads, BSA, and the secondary anti-BSA HRP-labelled antibody used to quantitate the captured BSA. Given this limitation, results represent relative semiquantitative data.

Data Analysis and Report

The standard curves for BSA, anti-mouse IgG-HRP, anti-rabbit IgG-HRP, and anti-BSA IgG-HRP were developed using known concentrations of the standard plotted against absorbance of the standard. The Kruskal-Wallis nonparametric test, which is designed to compare two or more groups of cases on one variable, was used to determine variability between plates and sets of known standards. The plate and set variation of each standard is reported as the chi-square value, $Q$ value, and degrees of freedom. Linear regression analysis was performed to determine the line of best fit for all standards based on a 95% confidence interval. The $R$-Square, standard error, $F$ value, and significance of $F$ are reported with each standard curve. The equation for the regression line was used to interpret the respective sample concentration as indicated above.
The unknown sample concentrations of BSA, IgG, and captured BSA were interpolated from their respective standard curve based on the absorbance of the sample. Sample concentrations are reported along with the standard deviation. The coefficient of variation for the ELISA was determined for the BSA quantitated in formula samples.

Three high, medium, and low standards were included on each plate used to quantitate the BSA concentration of infant formulas in order to assess the reproducibility of the assay. The interassay coefficient of variation and standard deviation is reported along with the mean of the BSA standards. The BSA content of infant formulas is also reported with both the standard deviation and coefficient of variation. The coefficient of variation, in this case, reflects both intra-assay and interdilutional variation.

The Kruskal-Wallis nonparametric test was used to determine the variance in amount of antibody bound to activated paramagnetic beads and in capture efficiency between monoclonal and polyclonal antibodies within and between sample media (milk, acid whey, sweet whey, and the known 25-ng BSA sample). In addition, the Kruskal-Wallis test was used to assess the significance of BSA extraction from the 25-ng BSA standard. Standards and sample unknowns are reported along with the chi-square value, p value, and degrees of freedom. All statistical analysis was performed using SPSS Advanced PowerMac Version 6.1.1.

SUMMARY OF METHODS

Measurement of the BSA content of infant formulas by ELISA provides a quantitative assessment of BSA and antigenic epitopes of BSA present in infant formulas. A summary of individual experiments performed in the preliminary stages to evaluate ELISA techniques and quantitation of BSA in cow's milk and formula is provided in Appendix A. Appendix B summarizes experiments for antibody capture and BSA extraction from cow's milk and whey protein isolates. This includes prelimi-
nary experiments as well as experiments leading to proof of concept that BSA can be extracted from cow's milk and whey protein isolates. A list of reagents and solutions used in the experiments outlined above is provided in Appendix C.
CHAPTER IV
RESULTS

MEASUREMENT OF BOVINE SERUM ALBUMIN
CONTENT OF INFANT FORMULAS

Evaluation of Indirect and Direct ELISA Methodology

Preliminary evaluation of the indirect ELISA for quantitating BSA content of known samples of BSA resulted in poor coating of the plate with BSA and high background interference due to difficulty finding a suitable blocking agent. As a result, efforts were directed toward development of the two-site, direct ELISA. The development of the direct ELISA is described in detail in the preceding chapter. The direct ELISA was utilized for quantitation of the BSA content of infant formulas.

Several blocking agents were evaluated for use in the direct ELISA since BSA the traditional blocking agent could not be used. Blocking with gelatin at 1% and 3% resulted in an extremely high background on the control blank. The blank was visibly in excess of the positive BSA sample on the indirect ELISA. The mean background absorbance for casein was 0.210 (1%), 0.249 (3%), and 0.290 (5%). Salmine exhibited a mean absorbance of 0.235 (1%), 0.229 (3%), and 0.197 (5%). PBS-Tween 20 at 0.2% and 0.4% exhibited a mean absorbance of 0.170 and 0.176, respectively. PBS-Tween 20 (0.2%) exhibited a lower background than casein, salmine, or gelatin, but the blocking effect did not reach the level of statistical significance (p > 0.05). The blocking effect of PBS-Tween 20 (0.4%) was essentially equivocal to the 0.4% PBS-Tween 20. PBS-Tween 20 (0.2%) was chosen as the blocking agent for the direct ELISA.

The recovery of BSA for the direct ELISA system was determined by spiking two high-whey, two high-casein, and a negative soy control with 10 nanograms of BSA. The
percent recovery of BSA was 100 and 96% for the two high-whey formulas, 99 and 63% for the two high-casein preparations, and 82% for the spiked soy control. The capture efficiency did not differ significantly between sample formula media (p > 0.5). The overall average percent recovery was 88% (± 15.7).

BSA Content of Infant Formula

The BSA standard curve for the quantitative, two-site direct ELISA for quantitation of the BSA content of infant formulas is shown in Figure 14. Data reflect BSA standards from three independent sample sets of BSA across dilutions; each was performed in triplicate on three separate microtitre plates. There were no significant differences collectively or within dilutions between the three independent sample sets or between plates (p > 0.05). The interassay coefficient of variation (CV) was determined for BSA standards of 50, 10, and 5 nanograms per 100 µl performed on each assay plate. The mean absorbance values were 0.660 (standard deviation = 0.13; CV = 19), 0.550 (Standard Deviation = 0.10; CV = 18), and 0.410 (standard deviation = 0.07; CV = 17). All baseline and residual sample BSA content was interpolated from the BSA standard curve.

The mean BSA content of 20% milk and infant formulas is reported in Table 11. BSA was detected and quantitated in all polymeric powdered infant formulas and 2% milk. BSA was not detected by the ELISA in any of the ready-to-serve or concentrated liquid formulas, including those that are counterparts to the powdered formulas containing BSA. All elemental, partially hydrolyzed formulas tested negative for BSA regardless of form. There were no statistically significant differences between powdered high-whey (60%) formulas from different manufacturers (p > 0.05). No statistically significant differences were observed between brands of high-casein formulas (p > 0.05). In order to demonstrate reproducibility of the test, the test was repeated in one high-whey formula,
Figure 14. BSA standard for infant formula. The equation for the regression line is:
\[ Y = 55.971886 \text{ (absorbance)} + (-0.605137); \text{ standard error} = 1.69; \text{ } F = 929; \]
significance of \( F = 0.0000 \). The mean absorbance of each BSA standard coordinate is:
2.5 ng/100 \( \mu l \) = 0.042 (±0.011), 5 ng/100 \( \mu l \) = 0.13 (±0.022), 10 ng/100 \( \mu l \) = 0.218 (±0.022), and 25 ng/100 \( \mu l \) = 0.439 (±0.035).

SMA powder. The mean BSA content of SMA run 1 was 37 mg/100 ml compared to
SMA run 2 of 45 mg/100 ml, which was not significantly different (\( p > 0.05 \)). Since
polyclonal antibodies were used for capture and detection of BSA in these preparations,
both BSA and antigenic epitopes of BSA should be able to be detected, if present. Anti-
BSA antibodies used for capture and detection of BSA failed to recognize the ABBOS
epitope in levels that might have been present in the test samples. The bulk of the antibody
response from the polyclonal antibodies did not appear to be directed toward the ABBOS
peptide.
Table 11

BSA Content of Polymeric and Hydrolyzed Infant Formulas

<table>
<thead>
<tr>
<th>Product type</th>
<th>Manufacturer</th>
<th>BSA mg/100ml</th>
<th>Std. deviation</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% milk</td>
<td>Meadow Gold</td>
<td>52</td>
<td>(± 4)</td>
<td>7.7</td>
</tr>
<tr>
<td>High-casein, powder</td>
<td>Ross Laboratories</td>
<td>13</td>
<td>(± 2)</td>
<td>7.5</td>
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<tr>
<td><em>Similac Fe Powder</em></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>High-casein, ready-to-serve</td>
<td>Ross Laboratories</td>
<td>none detected</td>
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<td></td>
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<tr>
<td><em>Similac Fe Ready-to-Serve</em></td>
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<td></td>
</tr>
<tr>
<td>Preemie, ready-to-serve</td>
<td>Ross Laboratories</td>
<td>none detected</td>
<td></td>
<td></td>
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<tr>
<td><em>Similac Special Care</em></td>
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<tr>
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<td>Ross Laboratories</td>
<td>0.6</td>
<td>(± 0.075)</td>
<td>12.5</td>
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<tr>
<td><em>Similac Neocare</em></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Preemie, ready-to-serve</td>
<td>Ross Laboratories</td>
<td>none detected</td>
<td></td>
<td></td>
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<tr>
<td><em>Similac Neocare</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-whey, powder</td>
<td>Mead Johnson Brystol-Meyers</td>
<td>41</td>
<td>(± 6)</td>
<td>14.6</td>
</tr>
<tr>
<td><em>Enfamil Fe Powder</em></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Preemie, ready-to-serve</td>
<td>Mead Johnson Brystol-Meyers</td>
<td>none detected</td>
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</tr>
<tr>
<td><em>Enfamil Premature 24</em></td>
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<tr>
<td>High-whey, powder</td>
<td>Wyeth-Ayerst Laboratories</td>
<td>37</td>
<td>(± 5)</td>
<td>13.5</td>
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<td>45</td>
<td>(± 10)</td>
<td>22.2</td>
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<tr>
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<td>Wyeth-Ayerst Laboratories</td>
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<td></td>
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<tr>
<td><em>SMA Preemie</em></td>
<td></td>
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<td></td>
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<tr>
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<td>Brystol-Meyers</td>
<td>none detected</td>
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<td></td>
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<tr>
<td>concentrate</td>
<td><em>Gerber Liquid Concentrate</em></td>
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<td></td>
</tr>
<tr>
<td>High-casein, powder</td>
<td>Brystol-Meyers</td>
<td>13</td>
<td>(± 2)</td>
<td>15.4</td>
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<tr>
<td><em>Gerber Powder</em></td>
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<td></td>
</tr>
<tr>
<td>High-casein, powder</td>
<td>Wyeth-Ayerst Laboratories</td>
<td>13</td>
<td>(± 2)</td>
<td>15.4</td>
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<tr>
<td><em>Bonamil</em></td>
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(table continues)
<table>
<thead>
<tr>
<th>Product type</th>
<th>Manufacturer</th>
<th>BSA mg/100ml</th>
<th>Std. deviation</th>
<th>CV</th>
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</thead>
<tbody>
<tr>
<td>Hydrolyzed whey, powder</td>
<td>Carnation, Nestle Co.</td>
<td>none detected</td>
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<tr>
<td>Carnation Good Start</td>
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<td></td>
</tr>
<tr>
<td>Casein hydrolysate, powder</td>
<td>Ross Laboratories</td>
<td>none detected</td>
<td></td>
<td></td>
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<tr>
<td>Pregestimil</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein hydrolysate, ready-to-serve</td>
<td>Ross Laboratories</td>
<td>none detected</td>
<td></td>
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<tr>
<td>Alimentum</td>
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<td></td>
</tr>
<tr>
<td>Casein hydrolysate, powder</td>
<td>Mead Johnson</td>
<td>none detected</td>
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<td></td>
</tr>
<tr>
<td>Nutramagen</td>
<td>Nutritional Brystol-Meyers</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CV = coefficient of variation

PhastGel electrophoresis was used to evaluate formulas testing negative for BSA by the two-site, direct ELISA. All hydrolyzed formulas, regardless of form, ran no detectable protein bands and were negative for BSA. This further confirmed that the hydrolyzed formulas were devoid of BSA. No BSA band was detected for the ready-to-serve and concentrated liquid formulas on SDS-PAGE.

A correlation was run to determine the relationship between the whey protein fraction of the formula and the BSA concentration. Results are summarized in Figure 15. The BSA content was highly correlated with the whey protein concentration in powdered polymeric infant formulas and 2% milk (correlation coefficient = 0.8445; p = 0.008).
Figure 15. Comparison of BSA content to whey protein fraction. Formula: (1) high-casein powder, *Bonamil*; (2) high-casein powder, *Similac*; (3) high-whey powder, *Enfamil*; (4) high-whey powder, *SMA*; (5) high-casein powder, *Gerber*; and (6) 2% milk (correlation coefficient = 0.85; \( P = 0.008 \)).

### IMMUNOCAPTURE AND EXTRACTION OF BOVINE SERUM ALBUMIN FROM COW'S MILK AND WHEY ISOLATES

**Baseline and Residual BSA Content of Cow's Milk and Whey Isolates**

The BSA standard curve for the quantitative, two-site direct ELISA for baseline BSA content of cow's milk, whey isolates, and residual BSA content of samples treated by immunomagnetic capture is shown in Figure 16. Data reflect BSA standards from three independent sample sets of BSA across dilutions; each performed in triplicate on three separate microtitre plates. There were no significant differences collectively or within
Figure 16. BSA standard curve for quantitation of BSA in milk, whey, and treated samples. The equation for the regression line is:
\[ Y = 73.571532 \text{ (absorbance)} + (-0.153229); \text{ standard error} = 2.25; F = 508; \text{ significance of } F = 0.0000. \]
The mean absorbance of each BSA standard coordinate is:
- 2.5 ng/100 µl = 0.031 (±0.019),
- 5 ng/100 µl = 0.074 (±0.028),
- 10 ng/100 µl = 0.158 (±0.029),
- and 25 ng/100 µl = 0.323 (±0.033).

dilutions between the three independent sample sets or between plates (p > 0.05). All baseline and residual sample BSA content was interpolated from the BSA standard curve.

The mean baseline BSA concentration of skim milk measured 28 mg/100 ml (± 0.8). Solutions of 10% delactosed acid whey and 10% sweet whey measured 17 mg/100 ml (± 0.2) and 12 mg/100 ml (± 0.1), respectively.

Preliminary immunocapture BSA extraction experiments using 5, 10, and 20 µl of polyclonal antibody activated paramagnetic beads incubated with 100 to 500 µl of skim milk demonstrated that the antibody-activated beads were able to capture BSA from the skim milk. However, no significant reduction in the amount of BSA in the milk sample
was observed. BSA remaining in the treated sample was well in excess (10,000-fold) of that captured by the beads.

**Anti-BSA Antibody Present on Activated Paramagnetic Beads**

In order to quantitate the amount of antibody present on each set of antibody-activated beads by ELISA, a standard curve for each species-specific labelled capture antibody was developed. Anti-mouse IgG-HRP standards were used to quantitate the amount of antibody present on the monoclonal antibody activated paramagnetic beads, Figure 17. Data reflect anti-mouse IgG standards from three independent sample sets of antibody across dilutions; each was performed in triplicate on three separate microtitre plates. There were no significant differences collectively within dilutions or between plates ($p > 0.05$). The difference between set standards reached the level of significance for 0.03813 ng IgG/100 µl sample (chi-square = 7.2; $p = 0.0273$; 2 d.f.), the 0.07625 ng IgG/100 µl sample (chi-square = 7.2; $p = 0.0273$; 2 d.f.), the 0.1525 ng IgG/100 µl (chi-square = 7.2; $p = .0273$; 2 d.f.), and the 0.305 ng/100 µl sample (chi-square = 6.49; $p = 0.039$; 2 d.f.). The amount of monoclonal antibody attached to the beads was interpolated from the anti-mouse IgG-HRP standard. Polythreonine-treated beads devoid of antibody served as the control.

Anti-rabbit IgG-HRP standards were used to quantitate the amount of antibody present on each set of polyclonal antibody activated paramagnetic beads, Figure 18. Data reflect anti-rabbit IgG standards from three independent sample sets of antibody across dilutions; each was performed in triplicate on three separate microtitre plates. There were no significant differences collectively within dilutions between the three independent sample sets or between plates ($p > 0.05$). The amount of polyclonal antibody on activated beads was interpolated from the anti-rabbit IgG-HRP standard. Polythreonine-treated beads devoid of antibody served as the control.
Figure 17. Anti-mouse IgG-HRP standard for quantitation of monoclonal antibody on beads. The equation for the regression line is: \( Y = 0.674769 \text{ (absorbance)} + 0.00001; \) standard error = 0.02968; \( F = 2167; \) significance of \( F = 0.0000. \) The mean absorbance at each IgG coordinate is: 0.03813 ng IgG/100 \( \mu \)l = 0.059 (\( \pm 0.009 \)), 0.07625 ng IgG/100 \( \mu \)l = 0.117 (\( \pm 0.016 \)), 0.1525 ng IgG/100 \( \mu \)l = 0.232 (0.023), 0.305 ng IgG/100 \( \mu \)l = 0.453 (\( \pm 0.047 \)), and 0.610 ng IgG/100 \( \mu \)l = 0.891 (\( \pm 0.084 \)).

The amount of antibody bound to each set of paramagnetic beads is reported in Table 12. Values are expressed in nanograms of IgG present on 10 \( \mu \)l of activated beads. Reported values assume a one to one capture efficiency between the anti-mouse or anti-rabbit IgG and the antibody present on the bead and provide relative, semiquantitative data. The amount of antibody attached to the activated beads varied significantly between monoclonal antibody activated beads and both sets of polyclonal antibody-activated beads (chi-square = 7.2; \( p = 0.0273 \); 2 d.f.). Polyclonal (Sigma) antibody-activated beads contained 17 ng IgG/10 \( \mu \)l beads, which was significantly more bound antibody than either the monoclonal antibody-activated beads with 3.9 ng IgG (chi-square = 3.86;
Figure 18: Anti-rabbit IgG-HRP standard for quantitation of polyclonal antibody on beads. The equation for the regression line is: $Y = 0.55376 \times \text{(absorbance)} + (-0.006459)$; standard error = 0.01782; $E = 4584$; significance of $E = 0.0000$. The mean absorbance at each IgG coordinate is: 0.0331 ng IgG/100 µl = 0.059 (±0.015), 0.0662 ng IgG/100 µl = 0.122 (±0.005), 0.1325 ng IgG/100 µl = 0.270 (0.026), 0.265 ng IgG/100 µl = 0.511 (±0.035), and 0.530 ng IgG/100 µl = 0.950 (±0.043).

$p = 0.0495; 1 \text{ d.f.}$) or polyclonal (Cappel) antibody activated beads with 8.1 ng IgG (chi-square = 3.86; $p = 0.0495; 1 \text{ d.f.}$). The polyclonal (Cappel) antibody-activated beads contained significantly more bound antibody than the monoclonal antibody-activated beads (chi-square = 3.86; $p = 0.0495; 1 \text{ d.f.}$). Control polythreonine beads tested negative for antibodies.

**BSA Captured by Activated Paramagnetic Beads**

In order to quantitate the amount of BSA captured by the antibody-activated beads by the modified ELISA procedures, a standard curve of the capture anti-BSA-HRP antibody was developed. Anti-BSA-HRP standards are shown in Figure 19.
The equation for the regression line is: $Y = 0.223718 \times \text{absorbance} + (-0.005117)$; standard error: $0.009$; $F = 1446$; significance of $F = 0.0000$. The mean absorbance at each IgG coordinate is: 0.0375 ng IgG/100 µl = 0.116 (±0.012), 0.047 ng IgG/100 µl = 0.240 (±0.016), 0.095 ng IgG/100 µl = 0.478 (0.044), and 0.19 ng IgG/100 µl = 0.848 (± 0.06 4).

Data reflect anti-BSA-HRP standards from three independent sample sets of antibody across dilutions; each was performed in triplicate on three separate microtitre plates. There were no significant differences collectively within dilutions between the three independent sample sets or between plates ($p > 0.05$). BSA captured by antibody-activated and control polythreonine paramagnetic beads was interpolated from the anti-BSA-HRP standard curve.

BSA captured by each set of paramagnetic beads is reported in Table 12. Values are expressed in nanograms of BSA captured by 10 µl of activated beads. Reported values assume a one-to-one capture efficiency between the anti-BSA-HRP and the BSA captured.
# Table 12

## Immunocapture and Extraction of BSA Data Summary

<table>
<thead>
<tr>
<th>Beads</th>
<th>ng IgG/10 ul beads</th>
<th>Milk</th>
<th>Acid whey</th>
<th>Sweet whey</th>
<th>Molar-to-molar capture efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Milk</td>
</tr>
<tr>
<td>Monoclonal</td>
<td>3.9 (±1.62)</td>
<td>0.94 (±0.19)</td>
<td>1.46 (±0.38)</td>
<td>1.47 (±0.39)</td>
<td>59% (±12)</td>
</tr>
<tr>
<td>Polyclonal (Sigma)</td>
<td>17.0 (±0.749)</td>
<td>1.34 (±0.51)</td>
<td>2.20 (±1.1)</td>
<td>1.66 (±0.27)</td>
<td>19% (±7)</td>
</tr>
<tr>
<td>Polyclonal (Chappel)</td>
<td>8.1 (±0.899)</td>
<td>0.04 (±0.015)</td>
<td>-</td>
<td>-</td>
<td>11% (±4)</td>
</tr>
<tr>
<td>Control 0.00 polythreonine</td>
<td>negligible</td>
<td>negligible</td>
<td>negligible</td>
<td>negligible</td>
<td>negligible</td>
</tr>
</tbody>
</table>

Molar to Molar Capture Efficiency = \( \frac{\text{ng BSA captured by 10 µl beads}}{66,000 \text{ kD}} \)

\[ \frac{\text{ng IgG bound to 10 µl beads}}{160,000 \text{ kD}} \]
by the bead and provide relative, semiquantitative data. BSA captured by the control polythreonine beads devoid of antibody was negligible.

Capture efficiency is reported in a one-to-one molar ratio based on a molecular weight of IgG of 160,000 kilodaltons and a molecular weight of BSA of 66,000 kilodaltons (Table 12). The capture efficiency of the monoclonal and polyclonal antibody activated beads did not differ significantly between sample media of milk, acid whey, or sweet whey ($p > 0.05$).

Capture efficiency in milk varied significantly between monoclonal and both sets of polyclonal antibody-activated paramagnetic beads (chi-square = 5.96; significance = 0.05; 2 d.f.). The monoclonal antibody activated beads exhibited the highest capture efficiency of 59% compared to 19% (chi-square = 3.86; $p = 0.0495$; 1 d.f.) and 11% (chi-square = 3.86; $p = 0.0495$; 1 d.f.) for the polyclonal (Sigma) and polyclonal (Cappel) antibody-activated beads, respectively. Capture efficiency between the two sets of polyclonal antibody-activated beads did not differ significantly ($p = 0.05$). Capture efficiency of the monoclonal antibody-activated beads (91%) was significantly greater in both acid whey and sweet whey compared to the polyclonal (Sigma) antibody-activated beads. Capture efficiency of polyclonal antibody-activated beads was 31% (chi-square = 3.86; $p = 0.0495$; 2 d.f.) and 24% (chi-square = 3.86; $p = 0.0495$; 2 d.f.) for acid and sweet whey, respectively.

Molecular Sieving

The initial molecular sieving experiments performed on the milk sample resulted in incomplete removal of BSA. BSA was not detected in the less than 50,000 molecular weight effluent, but was detected in excess of 1 mg/100 ml from the greater than 100,000 molecular weight effluent.
BSA Extraction

Treatment of a known sample of 25 ng of BSA in 500 µl of PBS-Tween 20 with 10 µl of polyclonal (Sigma) antibody-activated beads resulted in a high capture efficiency. Compared to skim milk containing 138,640 ng BSA/500 µl treated with 10 µl beads where the capture efficiency averaged 19%, treatment of only 25 ng BSA/500 µl sample resulted in an average capture efficiency of 35% (±3), which was significantly greater (chi-square = 3.86; p = 0.0495; 1 d.f.). The residual BSA content of the treated sample measured 7.7 ng/500 µl (± 1.7) and represents a 70% reduction in the BSA content of the treated sample (chi-square = 4.35; significance = 0.0369; 1 d.f.). No BSA was detected in treated samples that were diluted beyond full strength.
CHAPTER V
DISCUSSION

MEASUREMENT OF BOVINE SERUM ALBUMIN CONTENT OF INFANT FORMULAS

Enzyme-Linked Immunosorbant Assay (ELISA)

Enzyme-linked immunosorbant assays (ELISAs) have been utilized to measure antigenicity of milk proteins found in infant formulas and residual antigenicity following extensive acid and enzymatic hydrolysis, as well as heat treatment. The ELISA has been reported to be an effective means of quantitating immunologically active antigen at concentrations as low as 10 ng/ml or 30 PPM (Cordle et al., 1994). The critical element used to determine the interaction between the preformed antibody and the whole protein or antigenic epitope of the protein antigen resides in the quality of the antiserum. Immunoassay results are affected by the proportion of epitopes (residual epitopes) and intact protein recognized by the antiserum and functional avidity of the specific antibodies for the antigenic regions (Leary, 1992). Since the ELISA measures antigenicity, it should detect fragmented antigen, antigenic epitopes, as well as intact protein (Cordle et al., 1994; Lahl & Braun, 1994; Mahmoud et al., 1992). Differences in residual antigenicity may not be strictly quantitative. However, ELISA is the system of choice for determining residual antigenicity because of its inherent sensitivity (Leary, 1992) and was used in this study to detect and quantitate the BSA content of cow's milk and infant formulas.

Preliminary evaluation of the indirect ELISA for quantitating the BSA content of known samples of BSA resulted in poor coating of the plate with BSA and high background interference due to difficulty finding a suitable blocking agent. As a result, a sensitive two-site, direct ELISA was developed and utilized for assessment of the BSA
content of infant formulas. PBS-Tween 20 (0.2%) was a more effective blocking agent than casein, salmine, and gelatin. The sensitivity was high with detection of antigen at concentrations as low as 1 ng/100 µl, which is consistent with that reported by Cordle and colleagues (1994). Polyclonal antibodies to BSA were used to capture and quantitate BSA in cow’s milk and infant formulas. Polyclonal antibodies, directed towards various antigen binding sites, should recognize antigenic epitopes of BSA as well as the native protein. The antibody response of our polyclonal capture and detector antibodies to the ABBOS peptide was negligible. It would appear that the bulk of the polyclonal antibodies were not directed against the ABBOS epitope.

BSA Standards

The BSA standard was developed by diluting BSA in phosphate-buffered saline. Use of isolated BSA in buffer avoided potential interference due to the presence of large quantities of carbohydrates, insoluble lipid, large molecular weight vitamins, minerals, and emulsifiers (Knights, 1985). However, these factors remained an issue in whole formula samples. In order to determine the impact of these background factors, cow’s milk and formula samples were spiked with a known concentration (5 and 10 ng) of BSA. Preliminary experiments showed a mean recovery of 88% of the total amount of BSA present in the sample. Therefore, the values reported in Table 11 of the BSA content of cow’s milk and polymeric infant formulas may underestimate the absolute amount of BSA by approximately 10%.

BSA Content of Cow’s Milk

The BSA content of 2% cow’s milk measured by the direct ELISA was 52 mg/100 ml (0.5 g/L). There are multiple other reports of the BSA content of cow’s milk in the literature with values ranging from 10 to 40 mg/100 ml (0.1 to 0.4 grams of
BSA per liter of milk; Robin et al., 1993; Swaisgood, 1982; Whitney, 1988). The variability in the BSA content of milk likely reflects assay techniques and the known heterogeneity of the BSA molecule. BSA exhibits several properties that contribute to the heterogeneity, including polymer formation, related sulfur linkages, and microheterogeneity (Whitney, 1988). This may impact detection of BSA in a sample by different methodologies. It is unlikely that this amount of variability in the BSA content between pooled samples of cow's milk could be explained by differences in breed, feed, or season.

**BSA Content of Powdered Polymeric Infant Formula**

BSA was detected and quantitated in all polymeric powdered infant formulas (Table 11). The high-whey formulas, 40% casein and 60% whey, contained 37 to 44 milligrams of BSA per 100-ml sample. The high-casein preparations, 80% casein and 20% whey, contained 12 to 13 milligrams of BSA per 100-ml sample. The BSA content of the powdered polymeric infant formulas and 2% milk was highly correlated with the whey protein concentration (correlation coefficient = 0.84; p = 0.008). Given that BSA is present in the whey fraction of dairy products, this finding was expected and lends support to the ELISA methodology as a means of quantitating BSA in whole formula samples and milk.

**BSA Content of Hydrolyzed Infant Formulas**

All elemental and partially hydrolyzed formulas tested negative for BSA using ELISA. The extensively hydrolyzed preparations Nutramagen, Alimentum, and Pregestimil, derived from intact casein, would be unlikely to test positive for any intact protein based on manufacturers reported molecular weight limits and degree of extensive enzymatic hydrolysis. These formulas contain approximately 70% free amino acids with
remaining peptides of less than eight amino acids. In contrast, the second generation whey hydrolysates (e.g., Good Start HA) contain from 40% to 60% free amino acids with remaining peptides up to 12 amino acid residues (Lahl & Braum, 1994). Based on these characteristics, none of these formulas would be expected to contain BSA or the 17 amino acid ABBOS epitope.

Polyacrylamide gel electrophoresis is an indication of intact parent protein (Cordle, 1994) and was utilized to confirm the results of the ELISA. No detectable protein bands were observed for any of the three extensively hydrolyzed products that we evaluated. This is consistent with earlier reports of SDS-PAGE analysis employing the more sensitive silver stain. Consistent with our findings, a high degree of degraded cow's milk protein with no visible protein bands was observed from the casein-based hydrolysates Nutramagen and Alimentum (Cordle, 1994; Oldaeus et al., 1991; Sampson et al., 1991). The presence of immunologically active whey following immunization of animals with these highly hydrolyzed infant formulas (Alimentum, Nutramagen, and Pregestimil) has previously been reported to be almost negligible (Cordle et al., 1994) although specific reactivity to BSA was not addressed. Follow-up ultrafiltration of tryptic hydrolysates has been proven to be effective in removing the BSA (Lahl & Braum, 1994). Lack of oral sensitization with extensively hydrolyzed whey suggests successful elimination of existing epitopes of the native protein (Pahud et al., 1985). The combination of enzymatic hydrolysis, thermal-processing, and particle separation has been reported to lead to marked reduction in the allergenicity of these proteins (Siemensma et al., 1993). Our results, taken together with these reports focusing exclusively on the allergenicity of infant formulas, suggest that the extensively hydrolyzed infant formulas are devoid of BSA and, most likely, antigenic epitopes of BSA.

While enzymatic hydrolysis of whey protein antigens has been reported to significantly reduce antigenicity, serum albumin appears to be resistant to enzymatic
hydrolysis (Figure 20; Jost et al., 1987). Polyvalent peptide fragments of BSA have been isolated and identified following hydrolysis with trypsin, chymotrypsin, and pepsin (Alting, Meijer, & von Berstein, 1997; Habeeb & Atassi, 1976; Wahn et al., 1981). BSA has been detected in trypsin-hydrolyzed whey and whey protein concentrates on SDS-PAGE (66 kilodaltons; Jost et al., 1987; Pahud et al., 1985). Resistance of BSA to enzymatic hydrolysis by trypsin and other proteases has been attributed to shielding of its sensitive sites by a strongly disulfide-bonded primary structure (disulfide loops; Jost et al., 1987). In addition, those fragments comprising the last third of BSA are reported to account for all the antigenic reactivity of the native protein (Habeeb & Atassi, 1976). This is consistent with the position of the ABBOS epitope.

Despite our observation that BSA was not detected by ELISA in any of the elemental or partially hydrolyzed products, some concern exists that BSA and/or antigenic epitopes of BSA may remain intact in the hydrolyzed formulas, particularly the partially hydrolyzed product Good Start HA. We found no detectable BSA in Good Start HA powdered formula on ELISA or with the PhastGel electrophoresis using Coomassi-blue stain. However, reports in the literature are varied regarding the presence of BSA and other milk proteins in this partially hydrolyzed product, as well as, residual antigenicity.

Good Start HA has previously been reported to show a number of unresolved proteins and non-degraded and partially degraded whey proteins in the range of 5 to 20 kilodaltons (Oldaeus et al., 1991). Identifiable fractions of peptides of greater than 4000 daltons have been detected by silver nitrate stained SDS-polyacrylamide gel electrophoresis (Sampson et al., 1991). A weak protein-stained band corresponding to BSA (69,000) has also been reported, but was not determined specifically to be BSA (Oldaeus et al., 1991). However, most recently Cordle and colleagues (1994) reported the more sensitive silver-stained polyacrylamide gel electrophoresis of Good Start to be
highly positive for BSA. The conflicting reports regarding the presence of the BSA antigen may potentially be explained by revisions in thermoprocessing and enzymatic hydrolysis of the product.

Good Start has been shown to cause allergic reactions in 24% of cow's-milk-sensitive infants (Oldaeus et al., 1991; Oldaeus, Bradley, Bjorksten, & Kjellman, 1992). Good Start has been reported to exhibit whey protein levels by ELISA at 700 times the amount detectable in the extensively hydrolyzed products Alimentum and Nutramagen (Sampson et al., 1991). In addition, the polymeric products Enfamil and Similac, in comparison to Good Start, have been shown to contain immunologically active whey proportional to their respective whey protein concentration independent of hydrolysis.
Despite reduction in antigenicity of the whey protein fraction of Good Start compared to these polymeric formulas, immunogenicity, as measured by indirect (inhibition) ELISA, has been reported to be very high (Cordle et al., 1994). While we did not detect intact BSA in Good Start, these findings suggest that antigenic epitopes of BSA, in particular the ABBOS epitope, may still be present. Development of an ELISA system using antibody specific to the ABBOS epitope would provide additional insight into this question.

BSA Content of Liquid Concentrate and Ready-to-Serve Formula Preparations

BSA was not detected by the ELISA in any of the ready-to-serve or concentrated liquid formulas, including those that are counterparts to the powdered formulas containing BSA. This is consistent with the findings by Monte and colleagues (1994), who also failed to detect BSA in any of six liquid formulas tested, using radial immunodiffusion. These polymeric concentrated liquid and ready-to-serve formulas are sterilized under heat-treatment, which may denature proteins reducing or eliminating immunogenicity. Serum antibody production to BSA in guinea-pigs has previously been shown to be greatly reduced in SMA Gold Cap liquid concentrate compared to the corresponding spray-dried powder (Heppel, 1984). In addition, extensive heating and shelf storage may result in agglutination, coagulation, and precipitation of BSA and other proteins, masking binding sites and preventing interaction of the BSA antigen with the antibodies. Similarly, disruption of antigenic determinants may make the BSA undetectable by radial immunodiffusion (Monte et al., 1994). While BSA was not detected, the proposed antigenic trigger of autoimmunity in IDDM, the ABBOS epitope, may still be intact.

No detectable band was observed in the region of BSA on follow-up polyacrylamide gel electrophoresis (PAGE) using Coomassi stain for polymeric liquid
concentrate and ready-to-serve infant formulas tested. This is consistent with findings reported by Monte and colleagues (1994), who demonstrated that liquid formulas testing negative for BSA via radial immunodiffusion demonstrated protein migration on an antibody-free agarose gel and appeared to be BSA free. Fox (1982) has previously reported considerably altered protein migration with failure to resolve the proteins of milk or sodium caseinate following heat treatment at 140 degrees Celsius for 10 minutes on PAGE (Fox, 1982). However, these temperatures are in excess of thermal processing of sterilized milk. Our initial runs of formula samples on SDS-PAGE revealed heavy protein aggregation with failure of the sample to migrate into the gel. An increase in the concentration of SDS and an increase in boiling time were required to allow migration of proteins from the heat-treated formula samples into the gel.

While BSA is markedly resistant to hydrolysis, it appears to be sensitive to heat-induced denaturation. BSA is heavily disulfide bonded (cysteine, cystine) and is easily denatured by heat. Heating whey proteins has been demonstrated to result in disruption of hydrogen and ionic bonds. The destabilized structure generally unfolds. BSA precipitates after heat treatment at 40 to 50 degrees Celsius due to hydrophobicity-directed unfolding (Brown, 1988). Serum albumin can be reduced below detectable limits of immunodiffusion analysis by heating at 80 to 90 degrees Celsius for up to 30 minutes or 125 degrees Celsius for 3 minutes (Jost et al., 1987). However, the whey had previously been subjected to enzymatic hydrolysis, and it is possible that this situation would not parallel that seen in sterilized canned formula preparations. Table 13 provides a summary of various degrees and duration of heat treatment and the degree of residual antigenicity of BSA. Extensive heat treatment of whey proteins, including BSA, has been reported to result in almost complete loss of their unique three-dimensional structure, reduces solubility, and decreases antigenicity as measured by passive cutaneous anaphalaxis (PCA) testing (McLaughlan et al., 1981).
Table 13

Heat-Induced Destruction of BSA Antigens in Trypsin-Hydrolyzed Demineralized Whey

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BSA (1/d.f.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demineralized whey</td>
<td>32</td>
</tr>
<tr>
<td>Trypsin hydrolyzed whey (240 min)</td>
<td>32</td>
</tr>
<tr>
<td>Hydrolysate:</td>
<td></td>
</tr>
<tr>
<td>Heated at 80 degrees Celsius</td>
<td></td>
</tr>
<tr>
<td>3 min</td>
<td>32</td>
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<tr>
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Note. Units of BSA 1/df is the reciprocal of the dilution factor for the double immunodiffusion. No residual antigen activity is observed after 10 minutes at 90 degrees Celsius or 180 seconds at 125 degrees Celsius (Jost et al., 1987).

Aside from denaturing whey proteins, heating milk may result in coagulation, a decrease in the pH, Maillard browning, and interaction with caseins. Protein-protein interactions are enhanced by heating. Aggregation and precipitation due to intermolecular disulfide binding can occur (Robin et al., 1993). Precipitation with K-casein by sulphydryl-disulfide interaction has been observed following heat treatment at 90 degrees Celsius for 5 minutes (Fox, 1982). A drop in pH to as low as 5.5 to 6.0 due to produc-
tion of organic acids and formic acid from lactose may further promote changes in the BSA molecule. Hydrogen ion and ionized calcium work together to promote heat-induced coagulation (Fox, 1982). These interactions have been observed in sterilized milk. In addition, there is some evidence to suggest that proteolysis occurs in ultra-high-temperature (UHT)-treated milk and to a lesser extent in products sterilized by the indirect method of autoclaving. Proteases of microbial origin survive UHT treatment (Harwalkar, 1982).

Heat-induced changes in caseins treated at high temperatures for long periods of time (120 degrees Celsius for 5 hours) include proteolysis of 10 to 20% of caseins.

Protein denaturation secondary to heat treatment has been demonstrated to result in loss of conformational epitopes of intact protein (Siemensma et al., 1993). Bottled sterilized milks that are highly heat treated have been reported to be less sensitizing than the spray-dried powders (McLaughlan et al., 1981). Evaporated milk, commonly used as the breast-milk substitute prior to the upsurge of the infant formula industry, is extensively heat treated at high temperature (110-115 degrees Celsius for 15-20 minutes) where virtually all whey proteins are denatured. Evaporated milk shows significantly decreased oral sensitization in guinea-pigs compared with cow's milk and SMA gold cap liquid concentrate. Oral sensitization has been shown to be inversely related to the degree of heat used in the preparation of the final milk-based product (McLaughlan et al., 1981).

Strand (1994) has suggested that primary prevention of insulin-dependent diabetes could be achieved by simply increasing the temperature used to process milk from 60 to 72 degrees Celsius (pasteurization) to at least 85 degrees Celsius. Serum albumin is heat labile and the rate of denaturation increases exponentially with increasing temperature. Based on milk processing temperatures, BSA-free commercially available milk products include those canned at greater than 100 degrees Celsius and include infant formula, those ultrapasteurized at 138 degrees Celsius, and UHT-processed at 149
The absence of BSA in these preparations has been confirmed by radial immunodiffusion (Strand, 1994). In addition, BSA could be destroyed and, therefore, eliminated in any milk product simply by bringing the product to a boil (> 85 degrees Celsius). However, radial-immuno-diffusion is sensitive down to 40 mg BSA/L which is about 10% of that found in cow's milk. Although Strand (1994) reports that heat treatment has been shown to eliminate allergenicity and antigenicity associated with BSA according to the work of Ratner, Dworetzky, Oguri, and Ascheim (1958), currently available comparatively highly sensitive techniques for assessing residual antigenicity give conflicting reports.

It would appear that heat treatment of milk denatures whey proteins and would, therefore, destroy conformational epitopes. Enzymatic hydrolysis is required to destroy sequential epitopes (Siemensma et al., 1993). It has not been established that heat treatment destroys the ABBOS epitope which appears to be the culprit in the pathogenesis of IDDM. Alting and colleagues (1997) have recently shown that heat treatment of BSA to denature the protein followed by simulated gastrointestinal conditions in infants results in reduced proteolysis. Increased aggregation and insolubilization due to heat treatment may decrease potential enzymatic cleavage sites and have been demonstrated to leave significant amounts of the ABBOS epitope intact (Alting et al., 1997). While heating has been shown to significantly reduce antigenicity, theoretically heating to denature the protein will destroy only the conformational antigenic epitopes, leaving sequential epitopes intact. More sensitive indicators may be warranted to further confirm whether heat modification destroys the primary sequence of amino acids in BSA, including the ABBOS epitope and is protective against BSA triggered disease.
Conclusion BSA Content of Infant Formulas

Taken together these findings point to the heterogeneous nature of cow's-milk-based infant formula, specifically in terms of its BSA content. Powdered polymeric cow's-milk-based infant formula contains BSA in amounts proportional to the whey protein concentration. The first generation, extensively hydrolyzed casein-based formulas are devoid of BSA and almost certainly, antigenic epitopes of BSA. The partially hydrolyzed product also appeared to be free of intact BSA; however, we cannot say with certainty that the ABBOS epitope was not present. The commercially sterile liquid concentrate and ready-to-serve infant formulas also appear to be free of immunologically active BSA. Again the question as to whether the ABBOS epitope is present and masked due to protein aggregation or absent due to hydrolysis is unresolved. Use of a monoclonal antibody specific for the ABBOS epitope could resolve these questions with certainty.

Epidemiological evidence suggests that milk consumption follows a "dose response" pattern in the pathogenesis of IDDM. A significant positive correlation has been reported between milk consumption and incidence of IDDM (Cerrato, 1993; Dahl-Jorgensen et al., 1991; Scott, 1990). Case-control studies, to date, examining the contribution of BSA in the pathogenesis of IDDM have failed to decipher individual formula characteristics. The wide variety of commercially available cow's-milk-based infant formula preparations on the market, as well as homemade preparations derived from whole cow's milk or commercially sterilized canned milk products, further complicates the issue. In addition, the possibility exists for BSA to cross into the mammary gland and, therefore, be present even in human milk (Paul & Peterson, 1993). However, Monte and colleagues (1994) did not detect BSA in pooled human milk. The potential presence of a small amount of BSA even in breast milk, while unlikely, could pose a confounding problem that should not be ignored (Rennie, 1992).
While several studies have specifically looked at the issue of formula feeding in comparison to breast-feeding and the incidence of IDDM, none have attempted to address the issue of the heterogeneous nature of breast milk substitutes revealed in the present study. For instance, an infant receiving 75% breast milk and 25% of its nutrition from a high-whey formula would receive more BSA than an infant receiving 25% breast milk and as much as 75% supplementation with a high-casein preparation. An infant receiving 100% of its nutrition from a hydrolyzed product, even the partially hydrolyzed standard infant formula, Good Start HA, would have a negligible BSA intake similar to the breast-fed infant. Given the observed heterogeneous nature of breast milk substitutes, it is not surprising that case-control studies attempting to establish a link between breast-feeding practices and early introduction of cow's milk proteins have been able only to demonstrate a weak, though consistent, relationship between infant feeding practices and the incidence of IDDM (Gerstein, 1994; Norris & Scott, 1996). If BSA is indeed a trigger of IDDM, the trend toward a decrease in the casein and an increase in the whey content of infant formula may be a contributing factor in the incidence of IDDM independent of breast-feeding.

Infant feeding practices, in particular introduction of cow's-milk-based infant formulas as breast-milk substitutes, need to be reevaluated for their potential contribution to the pathogenesis of IDDM in genetically predisposed individuals. A large multicenter, international prospective cohort study of 3,000 families, to be followed over a 10-year period, where cow's milk proteins are withheld for the first 9 months of life, has been initiated (Alberti, 1993). While exclusive breast-feeding certainly remains the preferred method of feeding infants the first 6 months of life, many new mothers have difficulty initiating and maintaining breast-feeding. It is estimated that approximately 50% of newborns and 80% of infants at 6 months of age are currently receiving cow's-milk-based infant formula containing intact protein (Ross Laboratories, 1996). While numerous
groups have been actively working to promote breast-feeding worldwide, formula feeding remains a commonly practiced alternative. The American Academy of Pediatrics (1991) "strongly encourages" that families with a strong history of IDDM, particularly if a sibling has diabetes, adopt the practice of breast-feeding and avoid commercially available cow's milk and products containing intact cow's milk protein during the first year of life. Since routine screening to detect the "diabetic gene" is not practical at this time and is not a component of public health policy, it may be appropriate for all infants not exclusively breast-fed to receive BSA-free formulas. Prophylactic use of BSA-free infant feeding may reduce the incidence of IDDM, improve life-expectancy and quality of life for those at risk of developing IDDM, and reduce overall health care cost related to the long-term medical management of IDDM and the advanced complications of its chronic disease process. If standard polymeric formulas are used, it may be prudent to choose a formula with a higher proportion of casein to whey.

IMMUNOCAPTURE AND EXTRACTION OF BOVINE SERUM ALBUMIN FROM COW'S MILK AND WHEY ISOLATES

Preliminary Studies

The mean baseline BSA concentration of skim milk measured 28 mg/100 ml (± 0.8). Solutions of 10% delactosed acid whey and 10% sweet whey measured 17 mg/100 ml (± 0.2) and 12 mg/100 ml (± 0.1), respectively. Preliminary immunocapture BSA extraction experiments using 5, 10, and 20 µl of polyclonal antibody-activated paramagnetic beads incubated with 100 to 500 µl of skim milk demonstrated that the antibody-activated beads were able to capture BSA from the skim milk. However, no significant reduction in the amount of BSA in the milk sample was observed. BSA
remaining in the treated sample was well in excess (10,000-fold) of that captured by the beads.

The immunocapture methodology employed for extraction of BSA from cow's milk has previously been reported by Blake (1996). The strengths of this method relative to use for extraction of BSA from cow's milk include: (a) use of small 2.8 µm paramagnetic beads, which are easily dispersed in the sample and permit greater bead-area to be used; (b) use of a long flexible polythreonine spacer that was attached to the tosyl-activated groups on the bead surface, allowing correct orientation of the antibody to the BSA antigen; (c) avoidance of steric hindrance between the BSA antigen and the bead; and (d) proven effectiveness of antigen (spore) capture in cow's milk, formula, and a variety of media, indicating that the sample media did not influence antigen binding. However, since this methodology has previously been used to detect and capture small amounts of spores down to 8 x 10³ cfu/ml (Blake, 1996), it would seem most likely, in our application, that the system did not provide sufficient antibody to fully extract BSA from the milk sample. While Blake confirmed the presence of antibody on beads using ELISA, no quantification of the amount of antibody attached to the beads was reported.

Anti-BSA Antibody Present on Activated Paramagnetic Beads

The amount of antibody bound to each set of paramagnetic beads is reported in Table 12. Values are expressed in nanograms of IgG present on 10 µl of activated beads. Reported values assume a one-to-one capture efficiency between the anti-mouse or anti-rabbit IgG and the antibody present on the bead and provide relative, semiquantitative data. Polyclonal (Sigma) antibody activated beads contained 17 ng IgG/10 µl beads, polyclonal (Cappel) antibody activated beads 8.1 ng IgG, and the monoclonal antibody-activated beads 3.9 ng IgG. Control polythreonine beads tested negative for antibody.
The differences in the amount of each set of antibody attached to the beads were significant in all cases at the $p < 0.5$ level. While these findings were statistically significant, the practical significance points to the need to know how much antibody is present on the bead. Since each set of antibody might be expected to act differently in binding to the beads via the polythreonine cross linker, it is important to quantify the amount of antibody on beads in order to be able to calculate capture efficiency of the antibody-activated beads when presented with antigen.

**Capture Efficiency of Activated Paramagnetic Beads**

**Against BSA Antigen**

BSA captured by each set of antibody-activated paramagnetic beads is reported in Table 12. Values are expressed in nanograms of BSA captured by 10 µl of activated beads. Reported values assume a one-to-one capture efficiency between the anti-BSA-HRP and the BSA captured by the bead and provide relative, semiquantitative data. BSA captured by the control polythreonine beads devoid of antibody was negligible and confirms that nonspecific binding between the bead and the antigen did not occur.

Capture efficiency is reported in a one-to-one molar ratio based on a molecular weight of IgG of 160,000 kilodaltons and a molecular weight of BSA of 66,000 kilodaltons (Table 12). The capture efficiency for BSA by the monoclonal and polyclonal antibody-activated beads did not differ significantly between treatment of samples of milk, acid whey, or sweet whey ($p > 0.05$). These findings are consistent with those reported by Blake (1996) demonstrating that antibody-activated paramagnetic beads can effectively capture spores in a variety of media and that the media do not impact antigen binding.

While the sample media did not affect antigen binding, significant differences were observed between the capture efficiency of monoclonal and polyclonal antibody-
activated paramagnetic beads in milk, acid whey, and sweet whey (p < 0.05). The monoclonal antibody-activated beads exhibited the highest capture efficiency in all three media. It is likely that each set of antibodies would vary in their avidity for the antigen, in this case BSA. The specificity and sensitivity of any ELISA is highly dependent upon the efficiency with which the solid phase antibody can bind the sample antigen (Kemeny & Chantler, 1988). In a commercial application of this immunocapture technology, monoclonal antibodies could be produced with consistency and in quantity to BSA, or specifically, to the ABBOS epitope. Monoclonal antibodies provide the advantage of ensuring continuity of reagents, which normally poses a considerable source of error in ELISA (Kemeny & Chantler, 1988).

**BSA Extraction**

The primary focus at the outset of this study was to provide proof of concept that BSA could be extracted from cow's milk and whey protein isolates and, therefore, significantly be reduced or eliminated from the test samples. Despite a respectable capture efficiency of BSA by antibody-activated paramagnetic beads, it is apparent, given the amount of antibody present on the beads, that the BSA in the sample media greatly overrides the limitations of the system. It would be impractical from a cost standpoint if not physically impossible to add enough beads to the full-strength cow's milk or whey isolates to totally remove BSA from the sample. While BSA is considered a minor whey protein, there are still substantial quantities of the BSA antigen present in cow's milk and whey isolates. In order for the immunocapture methodology to be commercially practical, it would need to be coupled to another method of removing individual proteins from the sample media. Molecular sieving could be used to remove gross amounts of BSA from cow's milk or whey isolates followed by the immunocapture in order to remove residual BSA.
In order to determine whether antibodies were effective in capturing BSA when BSA was present in small amounts, such as that which might remain after completion of molecular sieving, a known sample of 25 ng of BSA in 500 µl of PBS-Tween 20 was treated with 10 µl of polyclonal (Sigma) antibody-activated beads. The capture efficiency exceeded that in skim milk containing 138,640 ng in 500 µl. The residual BSA content of the treated sample measured 7.7 ng/500 µl and represents a significant 70% reduction in the BSA content of the treated sample (chi-square = 4.36; d.f. = 1; p = 0.0369). Given that the immunocapture was effective in removing small residual amounts of BSA, the immunocapture together with molecular sieving could be effective in reducing the BSA content of cow's milk from 138,640 ng/500 µl to 7.7 ng/500 µl. This represents a 99.99% decrease in the BSA content of cow's milk. Addition of a greater quantity of beads to the sample and an increase in the incubation time allowed for binding would likely increase the efficacy of the BSA extraction. As the absolute zero point is approached, the standard direct (two-site) would require amplification to discriminate diminutive nanogram differences in BSA content.

Conclusion Immunocapture and Extraction of Bovine Serum Albumin from Cow's Milk and Whey Isolates

The research presented here provides proof of concept that BSA can be extracted from cow's milk and whey protein isolates using immunocapture methodology. Both monoclonal and polyclonal antibody-activated paramagnetic beads were effective in capturing BSA from the sample media. Given the significant amount of BSA present in cow's milk and whey protein isolates and the large quantities of antibody-activated paramagnetic beads that would be required to totally remove these quantities of BSA, it would appear that coupling of the immunocapture methodology with another means of extracting individual proteins from sample media may be warranted. A trial run of
molecular sieving proved less than 100% effective in extracting BSA. We were able to demonstrate that the immunocapture methodology was effective in capturing and extracting BSA when only small amounts (25 ng) were present in the sample. Use of glass or ceramic beads with greater potential for recycling would increase the practicality of the immunocapture technology outside the laboratory setting. Immunocapture methodology would appear to be most effective in capturing and removing proteins when only small amounts are present in the sample or to clean up a sample when other means of extracting individual proteins are less than optimal (100%).
CHAPTER VI
CONCLUSION

IDDM is one of the most prevalent and costly severe diseases of childhood in North America and Europe. Despite advances in medical management, disease morbidity and mortality remain high. IDDM develops in a relatively small number of individuals who carry specific genetic characteristics associated with increased disease susceptibility. A chronic progressive autoimmune process leads to destruction of the insulin-producing pancreatic beta-cells and ultimately to clinical IDDM. The interaction of genetic, immunological, and environmental factors working over the extended diabetic prodrome appears to be intricately involved in the pathogenesis of the disease.

There appear to be a number of potential environmental factors that could trigger and sustain the autoimmune process that eventually leads to IDDM, most notably viruses. There is also agreement that an epidemiological role of diet in the etiology of IDDM exists. If the initial insult triggering the autoimmune response could be identified and subsequently eliminated, a substantial reduction in the incidence of IDDM could be achieved.

The interrelationship between breast-feeding and the timing of introduction of breast-milk substitutes, cow's milk, and solid foods into the infant's diet has been extensively studied. To date, studies have failed to look at both the qualitative and quantitative aspects of infant diet against a background of genetic predisposition and the progression of autoimmunity to pancreatic beta-cells. While there is still much controversy surrounding infant feeding practices and their relationship to the pathogenesis of IDDM, there is substantial evidence to suggest that reduced incidence and duration of breast-feeding, particularly exclusive breast-feeding, and early introduction of cow's milk protein are contributing factors.
An elaborate scheme involving genetic and immunological factors interacting with the specific BSA epitope, ABBOS, in the pathogenesis of IDDM has been proposed. Immunization with BSA and the ABBOS epitope prior to establishment of normal self-tolerance in the infant precipitates a cross-reactive immunological response with the beta-cell protein p69 via molecular mimicry. The chronic autoimmune process proceeds until beta-cell destruction is complete and manifestation of clinical IDDM becomes apparent.

Substantial evidence of an immunological response to BSA, and specifically the ABBOS epitope, has been repeatedly and independently documented. However, there are conflicting reports linking immunization with BSA to specific genetic and immunological markers of IDDM. The role of BSA as an antigenic trigger in the pathogenesis of IDDM is controversial and requires further investigation.

The research presented here points to the heterogeneous nature of cow's-milk-based infant formula, specifically in terms of its BSA content. Powdered polymeric cow's-milk-based infant formulas contain BSA in amounts proportional to the whey protein concentration. The first generation, extensively hydrolyzed casein-based formulas are devoid of BSA and, almost certainly, antigenic epitopes of BSA. The partially hydrolyzed whey product also appeared to be free of intact BSA; however, we cannot say with certainty that the ABBOS epitope was not present. The commercially sterile liquid concentrate and ready-to-serve infant formulas also appear to be free of immunologically active BSA. Additional research will be required to answer the question as to whether the ABBOS epitope is present and masked due to protein aggregation or absent due to hydrolysis. Development of an ELISA system using a monoclonal antibody specific for the ABBOS epitope could be one alternative approach used to resolve these questions with certainty.

Studies to date attempting to establish a link between early exposure to cow's milk proteins, in particular the BSA antigen, have failed to take into consideration
whether or not BSA is indeed present in infant formulas and to what degree. Given the results obtained from this study, which clearly demonstrate that the BSA composition of breast-milk substitutes varies widely, it is not surprising that case-control studies attempting to establish a link between breast-feeding practices and early introduction of cow's milk proteins have been able only to demonstrate a weak, though consistent, relationship between infant feeding practices and the incidence of IDDM. If BSA is indeed a trigger of IDDM, the trend toward a decrease in the casein and an increase in the whey content of infant formula may be a contributing factor in disease pathogenesis independent of breast-feeding. Given the knowledge of formula composition gained in this study, it may now be feasible to look at diabetes incidence rates from Center for Disease Control (CDC) or the Supplemental Program for Women, Infants, and Children (WIC) databases and determine method of infant feeding, including the specific formula used. Information gained from such a study could provide relative quantitative information on the BSA load received by the infant. Comparative studies of infant feeding practices and disease incidence could help to resolve the cow's milk/BSA controversy.

Breast milk, formula, or cow's milk is the primary source of an infant's nutritional intake the first year of life. It is estimated that approximately 50% of newborns and 80% of infants at 6 months of age are currently receiving cow's-milk-based infant formula containing intact protein (Ross Laboratories, 1996). While numerous groups have been actively working to promote breast-feeding worldwide, formula-feeding remains a commonly practiced alternative. While exclusive breast-feeding certainly remains the preferred method of feeding infants the first 6 months, approximately 50% of newborns are initiated on infant formula at birth. It can be assumed that most infants who are not breast-fed or who are breast-fed for only a short duration will receive cow's-milk-based infant formula.
Infant feeding practices, in particular introduction of cow's-milk-based infant formulas as breast-milk substitutes, need to be reevaluated for their potential contribution to the pathogenesis of IDDM in genetically predisposed individuals. Evidence of an immune defect that can be triggered by cow's milk or BSA has raised concerns regarding the safety and advisability of feeding intact cow's milk protein to infants (Gerstein, 1994). Since gut closure is variable over the first year of life when many new foods are introduced, caution may need to be exercised during this vulnerable period (Paul & Peterson, 1993). Introduction of artificial cow's-milk-based breast-milk substitutes and introduction of solid foods should be avoided for a minimum of the first 3 months of life. The American Academy of Pediatrics recommends breast-feeding for all infants the first 6 to 12 months. In addition, several recommendations have specifically been put forward to address dietary risk factors associated with IDDM (American Academy of Pediatrics, Work Group on Cow's Milk Protein and Diabetes, 1994). In families with a strong history of IDDM, particularly if the sibling has diabetes, breast-feeding and avoidance of commercially available cow's milk and products containing intact cow's milk protein during the first year of life are strongly encouraged. The substitution of soy-based formulas for milk-based formulas is not advised for either general or high-risk infant feeding practices because of animal studies linking the ingestion of soy protein intake to the development of diabetes (American Academy of Pediatrics, Work Group on Cow's Milk Protein and Diabetes, 1994). While the substitution of elemental formulas may eliminate potentially harmful large proteins and has been recommended for use in those with identified genetic predisposition, the American Academy of Pediatrics, Work Group on Cow's Milk Protein and Diabetes (1994) does not endorse this feeding option as prophylaxis for IDDM given the lack of scientific studies in humans confirming the benefits. Appropriate solid foods should be introduced between 4 to 6 months of age (American Academy of Pediatrics, 1992). Prophylactic use of BSA-free infant feeding may
reduce the incidence of IDDM, improve life-expectancy and quality of life for those at risk of developing IDDM, and reduce overall health care cost related to the long-term medical management of IDDM and the advanced complications of its chronic disease process. If standard polymeric formulas are used, it may be prudent to choose a formula with a higher proportion of casein to whey in order to minimize the BSA load to the infant.

Manufacturers of infant formulas should be encouraged to develop processing procedures to destroy BSA and its ABBOS subunit. Based on our findings, several commercial infant formulas appear to be devoid of BSA. With the exception of the extensively hydrolyzed preparations, which can cost up to three times the amount of standard formulas, we cannot say with certainly that the ABBOS epitope is not present. The research presented here provides an alternative means by which BSA can be selectively extracted from cow's milk and whey protein isolates using immunocapture methodology. We were able to demonstrate that the immunocapture methodology was effective in capturing and extracting BSA when only small amounts were present in the sample. Immunocapture methodology would appear to be most effective in capturing and removing proteins when only small amounts are present in the sample or a follow-up process when other means of removing BSA (e.g., molecular sieving, enzymatic hydrolysis, and thermal processing) are less than optimal. Commercial availability of an affordable BSA-free infant feeding may prophylactically help to reduce the incidence of IDDM and would be relatively harmless.
REFERENCES


Bodington, M., & Burden, A. (1993). Early exposure to cow's milk or cow's milk formula does not appear to increase the risk of childhood onset of IDDM in Leicestershire (Abstract). Diabetic Medicine, 10 (Supplement 1), S41.


Kostraba, J. (1994). What can epidemiology tell us about the role of infant diet in the etiology of IDDM. *Diabetes Care*, 17(1), 87-91.


APPENDICES
APPENDIX A
MEASUREMENT OF THE BOVINE SERUM ALBUMIN CONTENT OF INFANT FORMULA

Assessment of Indirect ELISA Methodology.
Evaluation of BSA standards.
Evaluation of anti-BSA antibodies.
Reevaluation of anti-BSA antibodies, first HRP-labeled detection antibody inactive.
Assessment of blocking agents.
Optimization of antibody concentration using checkerboard titration.
Optimize blocking and washing procedures.
Evaluate differences between whole formula samples or soluble fraction for quantitation of BSA.
Rule out indirect ELISA for quantitation of BSA in samples.

Assessment of direct (two-Site) ELISA Methodology.
Evaluation of BSA standards.
Evaluation of anti-BSA antibodies.
Obtain primary and secondary anti-BSA antibody, HRP-labeled secondary antibody.
Assessment of blocking agents.
Optimization of antibody concentration using checkerboard titration.
Optimize blocking and washing procedures.
Evaluate differences between whole formula samples or soluble fraction for quantitation of BSA.
Adopt direct ELISA for quantitation of BSA in cow's milk and formula samples.

Develop BSA Standards.
Weigh BSA independently three times and dilute in appropriate buffer.
Evaluate linearity.
Redo standards with a greater number of dilutions in linear response range.
Complete BSA standard for each plate.
Complete collective set of BSA standards, 3 plates, 3 replicates, from each of 3 sets of BSA standards.
Preliminary statistical analysis.

Quantitation of BSA in cow's milk and infant formula using direct ELISA.
Assay repeated for each of 15 formulas and cow's milk.

Statistical Analysis.
Interpolation of BSA content of formulas from BSA standard curve.

SDS-PAGE PhastGel of those formulas testing negative for BSA.
Centrifuge samples to remove fat and obtain soluble fraction of protein.
Prepare reagents.
Optimize heating temperature and times, reagent concentration, and protein.
Run 10 formulas testing negative for BSA by ELISA.
APPENDIX B
IMMUNOCAPTURE AND EXTRACTION OF BOVINE SERUM ALBUMIN
FROM COW’S MILK AND WHEY PROTEIN ISOLATES
SUMMARY OF EXPERIMENTS

Preparation and Antibodies and Beads.
- Purification of polyclonal rabbit anti-serum to BSA (Chappel).
- Activation of paramagnetic beads.
- Attachment of polyclonal antibodies to beads.
- Direct ELISA performed to test activity of set 1 polyclonal rabbit anti-serum to BSA.
- Direct ELISA repeated on set 1 polyclonal rabbit anti-serum to BSA.
- Purification of polyclonal rabbit anti-bovine serum albumin, delipidized whole antiserum (Sigma).
- Direct ELISA performed to test activity of set 2 Polyclonal Rabbit Anti-Bovine Serum Albumin (Sigma).
- Preparation of monoclonal antibodies to BSA, Mouse, IgG 1K, Ig Fraction for ELISA and attachment to beads (American Qualex).
- Direct ELISA performed to test activity of Monoclonal antibodies to BSA.
- Activation of second batch of paramagnetic beads to beads (Sigma).
- Attachment of Monoclonal antibodies against BSA to beads (American Qualex).
- ELISA performed to test blocking agents, Tween 20, salmine, and casein.
- Preparation of BSA standards.
- Preparation of polythreonine beads devoid of antibody to use as true control.

Preliminary Capture Experiments.
- 500 ul of skim milk treated with 5, 10, and 20 ul polyclonal anti-BSA active beads.
  - Direct ELISA performed on milk, wash, and beads.
- 500 ul of skim milk treated with 5, 10, and 20 ul polyclonal anti-BSA activated beads.
  - Direct ELISA performed on milk, wash, and beads.
  - Experiment performed three times.
- 150 ul of skim milk treated with 5, 10, and 20 ul polyclonal anti-BSA activated beads, 10 ul "inactive" anti-BSA activated beads as control.
  - Direct ELISA performed on milk, wash, and beads.
- 100 ul of skim milk treated with 5, 10, and 20 ul polyclonal anti-BSA activated beads, 10 ul "inactive" anti-BSA activated beads as control.
  - Direct ELISA performed on milk, wash, and beads.
- 500 ul of skim milk diluted 1/10, 1/100, 1/1,000, and 1/10,000 treated with 10 ul active polyclonal and "inactive" control beads.
  - Direct ELISA performed on beads, milk, and wash.
  - Direct ELISA performed on supernatant of beads to determine if free antibody existed in bead preparation.
  - ELISA for detection of antibody on polyclonal anti-BSA activated beads, "inactive" beads, and polythreonine treated control beads.
  - 150 ul skim milk treated with 5, 10, and 20 ul polyclonal anti-BSA activated beads, and 5, 10, and 20 ul "inactive" anti-BSA activated beads as control.
  - Direct ELISA performed on milk, wash, and beads.
Proof of Concept Antibody Capture BSA Extraction.

Direct ELISA to quantitate baseline BSA concentration of skim milk and whey isolates.

Quantitation of polyclonal anti-BSA antibody on set 1 and 2 of activated beads and control polythreonine beads.
Experiments performed three times.

Development of anti-rabbit-IgG-HRP standard curves.
Experiments performed three times.

Antibody capture BSA extraction in skim milk using set 2 polyclonal antibody activated and polythreonine control beads.
Quantitation of BSA captured by beads.
Experiments performed three times.

Development of anti-BSA-HRP standard curves.
Experiments performed three times.

Antibody capture BSA extraction in skim milk using set 1 polyclonal antibody activated and polythreonine control beads.
Quantitation of BSA captured by beads.
Experiments performed three times.

Quantitation of monoclonal anti-BSA antibody on activated beads and control polythreonine beads.
Experiments performed three times.

Development of anti-mouse-IgG-HRP standard curves.
Experiments performed three times.

Antibody capture BSA extraction in acid whey using set 2 polyclonal antibody activated and polythreonine control beads.
Quantitation of BSA captured by beads.
Experiments performed three times.

Antibody capture BSA extraction in sweet whey using set 2 polyclonal antibody activated and polythreonine control beads.
Quantitation of BSA captured by beads.
Experiments performed three times.

Antibody capture BSA extraction in acid whey using monoclonal antibody activated and polythreonine control beads.
Quantitation of BSA captured by beads.
Experiments performed three times.

Antibody capture BSA extraction in sweet whey using monoclonal antibody activated and polythreonine control beads.
Quantitation of BSA captured by beads.
Experiments performed three times.

Molecular sieving of whey protein isolates.
Direct ELISA to assess residual BSA.

Antibody capture BSA extraction in 25 ng BSA/500 ul PBS-T using polyclonal antibody activated and polythreonine control beads.
Quantitation of BSA captured by beads.
Experiments performed three times.

Statistical analysis.
APPENDIX C
REAGENTS AND SOLUTIONS

Carbinate-bicarbonate coating buffer (pH 9.6)
0.159 g Na₂CO₃
0.293 g NaHCO₃
100 ml distilled water

Phosphate Buffered Saline (PBS) (pH 7.4)
16.0 g NaCl
0.4 g KH₂PO₄
5.8 g Na₂HPO₄-7H₂O
0.4 g KCl
2000 ml distilled water

PBS-Tween 20 (0.2% Tween)
1500 ml PBS
3.0 ml Tween 20
(Tween 20 Sigma, Polyoxyethylene-sorbitan monoluarate,
P-7949: Lot: 15HO9291)

5 M Stock Solution Sulfuric Acid
50 ml H₂SO₄ (98%)
50 ml distilled water

Stop Solution, 1 M Sulfuric Acid
10 ml stock solution
40 ml distilled water

Sodium Phosphate (0.1 M NaPO₄) (pH 7.0):
Sodium Phosphate, Dibasic (MW 268.07) (pH 6.0)
0.1 M 26.8 g
1000 ml distilled water
Titrated with: Sodium Phosphate, Monobasic (MW 120.0) (pH 11.0)
0.1 M 12.0 g
1000 ml distilled water
Borate Buffer (0.05 M Borate Buffer) (pH 9.5):
3.09 g H2BO3 (MW 61.83)
1000 ml distilled water
Mallinckrodt 2549-500: B1320

Tris Buffer (0.2 M Tris Buffer) (pH 8.5):
2.42 g Trisma Base C4H11NO3 (Tris[hydroxymethyl]amino methane) FW 121.1
100 ml distilled water
(adjust pH)
Sigma, T-6791: Lot: 95H5731

MES (0.1 M) (pH 4.75):
2.132 g MES C6H13NO4S-H2O
(2-[N-Morpholino]ethane-sulfonic acid, monohydrate)
100 ml distilled water
Sigma, M-5287: Lot: 114H57281

Adipic Acid Dihydrizide (ADH):
C6H14N4O2 FW 174.2
Sigma, A-0638: Lot: 60H5008

EDAC:
C8H17N3-HCl FW 191.7
(1-ethyl-3(3-dimethylaminopropyl) carbomide, hydrochloride)
Sigma, E-1769: Lot: 85HO1521

Sodium m-Periodate:
NaIO4 FW 213.9
Sigma, S-1878: Lot: 95HO766

Poly-L-Threonine:
MW (vis) 12,100
Sigma, P-8077: Lot: 24H5536

2-mercaptoethanol:
C2H6O5 FW 78.13
Sigma, M-3148: Lot: 53HO74115
10% SDS (Sodiumdodecylsulfate):
\[ \text{C}_{12}\text{H}_{25}\text{O}_{4}\text{SN}_{9} \quad \text{FW 288.4} \]
2.884 g Laurel Sulfate
100 ml water
Sigma, L-3771: Lot: 55H06061

Tris Buffer:
10 mM Trisma + 1 mM EDTA
100 ml distilled water
0.1211 g Trisma Base
0.029225 g EDTA (Ethylenediamine-tetra acetic acid)
\[ \text{C}_{10}\text{H}_{14}\text{N}_{2}\text{O}_{8}\text{Na}_{2}\cdot2\text{H}_{2}\text{O} \quad \text{FW 372.2} \]
Sigma, ED2SS: Lot: 55H1360
100 mM Tris + 10 mM EDTA
Trisma 1.211 g
EDTA 0.29225
100 ml distilled water

Destaining Solution:
(30% methanol + 10% acetic acid)
2 Liters: 600 ml methanol
200 ml acetic acid
1200 ml distilled water

Preserving Solution:
(5% glycerol + 10% acetic acid)
500 ml: 25 ml glycerol
50 ml acetic acid
425 ml distilled water

20% Acetic Acid:
1000 ml: 200 ml acetic acid
800 ml distilled water

Fast Commassie Staining:
(0.1% PhastGel R Blue in 30% methanol and 10% acetic acid)
100 ml: 50 ml stain stock
50 ml 20% acetic acid
CURRICULUM VITAE

Patricia Zelinski Marincic

Education:

Bachelor of Science in Dietetics, Purdue University, 1982, with Honors (GPA 5.64/6.0).
Completed Coordinated Undergraduate Program in Dietetics as route to registration.
Purdue University Residence Halls, August 1981-December 1981.

Master of Science in Applied Nutrition, Colorado State University, 1984 (GPA 3.72/4.0).
Research emphasis in enteral nutrition support.

Ph.D. in Nutrition and Food Science, Utah State University, Dec. 1997 (GPA 4.0/4.0).
Research emphasis in infant nutrition and Type 1 diabetes mellitus.

Professional Experience:

Assistant Professor and Program Director of Dietetics, Idaho State University, Pocatello, Idaho. January 1988 - present.

Administration of Didactic and Internship Programs in Dietetics
- Evaluate curriculum/implement revisions.
- Coordinate course offerings in nutrition and dietetics.
- Serve as liaison with other College and University departments regarding coordination of course offerings, laboratory facilities, and classroom availability.
- File annual reports with the American Dietetic Association.
- Maintain records and liaison with the American Dietetic Association and the Commission on Dietetic Registration.
- Advise all dietetics majors (80).
- Review and select applicants for admission to January and August classes.
- Develop recruitment strategies and market nutrition programs.
- Maintain on-going relationships with clinical facilities and affiliate faculty.

Development of Dietetic Internship Program
(Formerly the Approved Preprofessional Practice Program in Dietetics)
- Complete needs assessment.
- Survey prospective students and professionals in practice in Idaho.
- Establish cooperative relationships with approximately 18 clinical facilities and 25 preceptors.
- Develop curriculum objectives, planned clinical experiences, and evaluation procedures for 1440 hours of supervised practice.
Development of Dietetic Internship Program (con't):

- Develop New Program Proposal Idaho State Board of Education.
- Change program status to accredited Dietetic Internship.

### Teaching Courses developed* and taught:

<table>
<thead>
<tr>
<th>Course Code</th>
<th>Course Title</th>
<th>Credits</th>
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<tbody>
<tr>
<td>HEC g481</td>
<td>Current Issues in Nutrition</td>
<td>2</td>
</tr>
<tr>
<td>HEC g482</td>
<td>Special Problems: BCHM Assess</td>
<td>1</td>
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<tr>
<td>NTD 300</td>
<td>Clinical Nutrition I</td>
<td>3</td>
</tr>
<tr>
<td>NTD 300L</td>
<td>Clinical Nutrition I Lab</td>
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<tr>
<td>NTD 301</td>
<td>Clinical Nutrition II</td>
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<td>NTD 301L</td>
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<tr>
<td>NTD 340</td>
<td>Therapeutic Nutrition</td>
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<tr>
<td>NTD 405</td>
<td>Community Nutrition</td>
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<tr>
<td>NTD 405L</td>
<td>Community Nutrition Lab</td>
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<tr>
<td>NTD g439</td>
<td>Sports Nutrition</td>
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<tr>
<td>NTD g461</td>
<td>Advanced Nutrition</td>
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<tr>
<td>NTD 488</td>
<td>Adv. Dietetics Practicum I</td>
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<tr>
<td>NTD 489</td>
<td>Adv. Dietetics Practicum II</td>
<td>30</td>
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<tr>
<td>NTD 622</td>
<td>Maternal, Infant, &amp; Child Nutrition</td>
<td>2</td>
</tr>
<tr>
<td>BIOSg485</td>
<td>Nutritional Biochem</td>
<td>3</td>
</tr>
<tr>
<td>PAS 301</td>
<td>Clinical Nutrition</td>
<td>1</td>
</tr>
</tbody>
</table>

* HEC g481 Current Issues in Nutrition 2 credits (Home Economists)
* HEC g482 Special Problems: BCHM Assess 1 credit
* NTD 300 Clinical Nutrition I 3 credits
* NTD 300L Clinical Nutrition I Lab 6 hour practicum in hospitals
* NTD 301 Clinical Nutrition II 3 credits
* NTD 301L Clinical Nutrition II Lab 6 hour practicum in hospitals
* NTD 340 Therapeutic Nutrition 3 credits (Nurses)
* NTD 405 Community Nutrition 3 credits
* NTD 405L Community Nutrition Lab 2 credits
* NTD g439 Sports Nutrition 3 credits
* NTD g461 Advanced Nutrition 3 credits
* NTD 488 Adv. Dietetics Practicum I 30 credits
* NTD 489 Adv. Dietetics Practicum II 30 credits
* NTD 622 Maternal, Infant, & Child Nutrition 2 credits (MPH)
* BIOSg485 Nutritional Biochem 3 credits
* PAS 301 Clinical Nutrition 1 credit (Physician Assistants)

### Additional Teaching and Curriculum Development:

- Develop curriculum for the MPH Nutrition Track.
- Develop nutrition curriculum for biochemistry series for proposed Idaho Medical School.
- Develop nutrition and food science curriculum for Home Economics option.
- Develop nutrition curriculum for nutrition minor.
- Develop Preschool through Grade Three Nutrition Education Curriculum for Idaho. Fourteen workshops presented throughout Idaho to disseminate program to teachers in the field.
- Rural Outreach Nursing Program, teach Therapeutic Nutrition.
- Teach nutrition support/medical nutrition therapy courses for Family Practice Residency Program.

### Other:

- Clinical nutrition consultant:
  - ISU Student Health Center Nutrition Clinic
  - **Bannock Regional Medical Center**
  - **Pocatello Regional Medical Center**
  - **Columbia Eastern Idaho Regional Medical Center**
  - **Pocatello Veteran's Home**
  - Super Save Drug, Home Nutrition Support and Diabetes Programs

** Serve as technical consultant for clinical nutrition, nutrition management, staffing for both foodservice and clinical areas, and in the development of clinical nutrition protocols for specialized practice areas. Provided as service as University faculty member.

Clinical Responsibilities:
Primary responsibilities included patient care in the Trauma Life-Support and Burn Center, Medical ICU, Surgical ICU, Rehabilitative Medicine, Neurology, Gastroenterology and General Surgery with relief in Pediatric ICU. Development and monitoring of nutrition support regimens. Provision of medical nutrition therapy to all patients on assigned services. Participation in daily medical rounds. Staff dietitian Surgical Clinics, Neurology Clinic, and ENT Clinic. Nutrition Home Tube Feeding and TPN outreach programs.

Administrative Responsibilities:

Academic Responsibilities:
Education and supervision of dietetic interns assigned to service. Education and training of dietetic interns, medical students, medical and surgical residents, critical care nursing staff, and pharmacy in critical care nutrition, tube feeding, and TPN. Coordinated Undergraduate Program in Dietetics Advisory Committee. Planning committee, nutrition curriculum for medical students, Department of Medicine. Implementation of nutrition clerkship, Department of Medicine. Speaker miscellaneous university and hospital sponsored conferences.

Clinical Dietitian, Waynesboro Community Hospital, 501 Oak Avenue, Waynesboro, Virginia. June 1982 to August 1983.

Responsibilities Included:

Responsibilities Included:
Nutritional care of residents consistent with Federal guidelines, in-service training, and menu writing.
Preceptor of Dietetic Assistant Course.

Publications:

Books:

Refereed Journals:
Marincic, P., & Francfort, E. Post-graduate dietetic education and training in the rural setting. Manuscript submitted for publication.

Journals/Newsletters:

Unpublished Manuscripts:
Unpublished Manuscripts, con't:


Grants:

Kasiska Foundation grant to update equipment Quantity Foods Laboratory
Funded $6,000.
State Department of Education Grant, Child Nutrition Program. Preschool through Grade Three Nutrition Education Curriculum and Workshops.
Funded $12,850.
Kasiska Foundation grant for computer to conduct nutrient assessment analysis and develop educational materials for clients. Funded $4,000.
Dissertation research component entitled "Immunocapture and Extraction of Bovine Serum Albumin from Cow's Milk and Whey Isolates" funded with small business grant from USDA through WendTech Industries International. Funded $50,000.

Professional Presentations:

International:

National:
State and Regional:
(First Place Award, Contribution to the Field)

Awards and Recognition:
First place recognition for "Contribution to the Field", Idaho Dietetic Association, 1991. (For development of the Dietetic Internship Program)
Recognized Young Dietitian of the Year (RYDY), Idaho, 1992.
American Dietetic Associated selected RYDY (1/165 RYDYs) to present at national meeting. (Award Presentation: Biochemical Assessment of Nutritional Anemias), 1992.
Tenured faculty, Idaho State University, 1993.
Phi Upsilon Omicron, honorary member, 1994.
Life Rich Scholarship, 1996.
Nominated, "Outstanding Dietetic Educator" Area I, 1996.
National Dean's List, 1996.
Professional Organizations and Participation:

American Dietetic Association (ADA):
- Representative, 1993 State Professional Recruitment Chair.

American Dietetic Association (ADA):

Dietetic Educators of Practitioners (DEP):
- Attend regional meetings and participate in strategies meetings to improve dietetic education and training.

Idaho Dietetic Association (IDA):
- Planning Committee Tri-state Meeting (Idaho, Wyoming, and Montana).
- Moderator, Critical Care Sessions.
- Plan and implement annual state conference, Idaho Falls.
- Implement ADA strategic initiatives in Idaho.

Healthy Mothers, Health Babies Coalition:

Pocatello Dietetic Association (1988-present):
- Planning and implementation of 1988 annual meeting.
- IDA (Fun Run).
- Legislative/licensure chair.

American Society of Parenteral & Enteral Nutrition (ASPEN).