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MINIREVIEW

Casein: A Milk Protein with Diverse Biologic Consequences(43129)

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There is little debate that maternal milk is the best food for suckling infants, with the possible exception of the severely premature infant. For the non-breast fed infant, cow milk-based formulas are often regarded as a suitable substitute. Human milk is markedly different from that of other mammalian species in terms of protein content and composition; human milk has the lowest protein content (approximately 0.9% protein/100 g of whole milk) and the lowest ratio of casein/whey (0.2). Furthermore, the subclasses of casein, amino acid sequence, and profiles of human and bovine milk differ markedly.

The purpose of this review is to address the remarkable and diverse biologic effects of bovine casein or its digestive fragments and, where possible, draw comparisons to other milk or non-milk proteins. While historically our consumption of bovine casein parallels the domestication of the cow, clinical and experimental evidence suggests that the myriad of effects associated with the consumption of this convenient source of protein necessitates a reevaluation of its effects on human health.

Milk Protein Biochemistry

Milks are complex oil-in-water emulsions containing protein, fat, lactose, vitamins and minerals, as well as biologic products such as enzymes, cells, hormones, and immunoglobulins. There are tremendous differences in milk composition among mammalian species, particularly in protein quantity, quality, and profile of amino acids (1). Of greatest relevance to this review are cow's and human milk, two mammalian species whose milk is markedly different in protein quantity and profile (Table I). Cow's milk contains an average of 3.5 g/dl of total protein (compared with 0.9 g/dl for human milk) divided into two major protein classes—the casein phosphoproteins (α, β, κ, γ) and the whey proteins (β-lactoglobulin, α-lactalbumin, proteose peptones, serum albumins, and immunoglobulins) (2). Food scientists consider bovine casein as the fraction of milk protein that precipitates from raw milk at pH 4.6 and 20°C. However, as noted by Kunz and Lonnerdal (3), certain whey proteins may coprecipitate with casein at 4.6. Thus, up to 5% of the measured casein content may be contaminated with α-lactalbumin and lactoferrin (4). Hence, pure casein extraction is possible using ultracentrifugation after pH adjustment to pH 4.3 and the addition of calcium. The casein fraction of bovine milk dominates milk as evidenced by the casein to whey ratio of 82:18 (5). Prior to these refined separation techniques the concentration of casein in bovine milk was underestimated, and a bovine casein to whey ratio of 60:40 is often misreported.

Bovine casein is a mixture of subclasses (Table I): α-casein (50–55%) containing several hydrophobic phosophoseryl residues, β-casein (30–35%), κ-casein (15%) which contains the calcium-rich portion, and γ-casein (5%) which may arise from proteolysis of β-casein (6). The linear casein molecule possesses excellent emulsification properties due to bipolar lipophilic and hydrophilic amino acid sequences. In solution, however, bovine casein associates into complex micelles that bind calcium, magnesium, and phosphate. It is the interaction between casein, calcium, and phosphate in these micelles that gives milk its white appearance (7).

Human milk caseins differ from bovine in quantity, amino acid composition, and physiochemical properties. In contrast to bovine casein, only two classes of human casein exist—the highly phosphorylated, 24,000 dalton β-casein and the carbohydrate-rich κ-

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casein (Table I). Furthermore, there is relatively little homology between the bovine and human forms, unlike the whey proteins. While α-casein is the predominant subclass of bovine milk, it is absent in human milk (3). Although quite difficult to quantify, β- like and κ-like caseins account for the remaining 30%, respectively, of human casein. In general, human casein micelles are smaller, contain less calcium and phosphorus, and form much softer and easier to digest curds in an acid environment (6, 8). Although George and DeFrancesca (6) reported that bovine and human β-casein were homologous, Jost (9) reported only a 47% sequence homology and there is a 10-residue shifted alignment (10). Differences in amino acid homology are often predictive of the potential allergenicity of a protein (9). Human whey differs from bovine by the total absence of β-lactoglobulin and a preponderance of α-lactalbumin (29%), lactoferrin (20%), and secretory IgA (11%) (11).

Gastric Emptying

Although there is variability in the gastric emptying times of adults, the individual variation is more striking in newborns. Gastric emptying is delayed in premature infants compared with term infants (12). Gastric emptying in the newborn may be delayed as much as 24 hr (13). Good peristaltic waves may not be developed until the baby is approximately 3 months old.

Furthermore, gastric emptying time differs in the same individual under different circumstances, particularly with respect to diet. In general, isosmotic feedings pass more rapidly than those that are more concentrated and require dilution. The two primary food substances thought to delay gastric emptying are fats and milk proteins. Although complex fats delay gastric emptying, Pascale *et al.* (14) demonstrated that medium chain triglycerides do not affect gastric emptying in the newborn. Bovine casein and cow milk-based formula both delay gastric emptying in humans (Table II). Newport and Herschel (15) reported increased dry matter in the stomach contents, an index of delayed gastric emptying, of 16-day-old pigs fed milk with a high casein to whey ratio (80:20) compared with formulations with low or no casein.

Table I. Major Milk Proteins in Human and Bovine Milk

	Bovine	Human	% Homology[a]
Total protein (g/dl)	3.5[b]	0.9[c]	
Total casein (g/dl)	2.8[d]	0.18[d]	
α-casein (%)	50–55[b]	Absent	—
β-casein (%)	30–35[b]	50[b]	47
κ-casein (%)	15[b]	20[b]	Low
Casein nitrogen/whey nitrogen[d,e]	80/20 0.7[b]	20/80 0.72[b]	
Total whey (g/dl)	19[c]	30.2[c]	72
α-lactalbumin (%)	51[c]	Absent	—
β-lactoglobulin (%)	6.4[c]	6.4[c]	80
Serum albumin (%)	3–5	20–25	
Nonprotein nitrogen[f] (%)			

[a] Ref. 9.
[b] Ref. 6.
[c] Ref. 8.
[d] Ref. 53
[e] Kunz and Lonnerdal (185) recently demonstrated that the casein content of human milk increases from 20% in early to 40% in late lactation.
[f] Ref. 186.

Lactobezoars

Lactobezoars were reported with increased frequency from the late 1970s until the mid-1980s (16–18). This proteinaceous gastric coagulum has not been reported in babies fed breast milk or a whey-predominant proprietary formula (19). Schreiner *et al.* (19) reported that as high as 6.6% of low birth weight babies (less than 2000 g) developed a lactobezoar when fed a bovine casein-predominant formula.

The cause of lactobezoar formation is thought to be multifactorial relating primarily to aggressive feeding of high caloric density formulas to preterm and growth-retarded neonates (16, 19). In contrast to full-term infant formulas, these formulas have higher concentrations of fats and casein which may delay gastric emptying. The role of bovine casein in lactobezoar formation may be attributed to its insolubility under acidic conditions, with the precipitate forming the nucleus of the lactobezoar. In addition, calcium concentrations are raised in an attempt to prevent osteopenia of prematurity. This combination of calcium, fats, and greater than 80% bovine casein may result in the formation of a firm undigestible mass.

A lactobezoar presents itself in an infant as abdominal distension, feeding intolerance, and, occasionally, gastric perforation (20). Currently, low birth weight babies are fed more conservatively and formulas with greater than 40% bovine casein are generally no longer used. Consequently, the incidence of lactobezoars is declining.

Digestion of Bovine Casein and Metabolic Response

Caseins are a series of related milk proteins that will curd at an acid pH, and for bovine milk, in the presence of the gastric enzyme rennin (21, 22). They are distinct from the whey proteins in that caseins are found only in milk whereas the whey proteins are analogous or identical to proteins found in blood or other vital secretions. The molecular weight for casein is approximately 25,000 (23, 24). However, the caseins in milk aggregate to form a micelle containing as many as 10,000 casein molecules, in addition to the fat, calcium, and other abundant cations (21, 23, 24).

Protein digestion is initiated in the stomach. There

Table II. Gastrointestinal Effects of Casein or Casein-Predominant Formulas

Subject	Effect	References
Gastric emptying and lactobezoars	Delayed, casein-component critical for lactobezoar formation	(15, 19)
Digestion	Attenuated release of pancreatic carboxypeptidases A and B, binds to bile salts limiting fatty acid solubility and promoting cholesterol absorption	(31, 32, 126, 132, 134, 140)
Divalent cations	Binding of calcium, manganese, magnesium, zinc, iron, copper, thereby reducing bioavailability	(53–58)
Intestinal allergy and inflammation	Major component of cow's allergy	(97, 98, 101–104)
	Implicated in necrotizing enterocolitis	(68, 89, 90)
	Increased antibody titer in inflammatory bowel diseases	(95, 96)
	Iron-deficiency anemia and occult gastrointestinal bleeding	(108–110)
Bacteria	Prevents tooth enamel demineralization	(61)
	Promotes bacterial growth in milk which would otherwise be attenuated by cocoa powder or acidification	(62, 63)
	Impairs phagocytosis by milk leukocytes	(64)
	Fragments induce β-lactamase in *Enterobacter cloacae* and β-galactosidase activity and carbohydrate fermentation rates to organic acid in stool flora	(66) (67, 69)
	Promotes the cholera toxin-induced fluid shifts in the ileum. β-Casomorphins attenuate this response	(71, 72)
	Binds to heat-stable enterotoxin of *E. coli* at acidic pH	(73)

is a reciprocal relationship between solubility and digestion, i.e., proteolysis (25). At an alkaline pH, the proteolysis of casein by the pancreatic enzymes (pancreatin, α-chymotrypsin, trypsin) was more rapid than the degradation of soy protein. However, under conditions mimicking gastric digestion (acidic pH), casein resisted digestion by pepsin whereas soy protein, which is more soluble, was degraded at a faster rate. This is likely due to the limited access of proteases to specific bonds when the proteins are complexed or precipitated as in a curd. This explains why whole milk, in which casein is incorporated into micelles or potentially a curd, has a reduced digestibility compared with pure protein preparations (26).

The digestion of bovine casein in cattle is intimately linked to the action of gastric rennet (rennin) on the α and κ fractions. Rennet, at a low pH, promotes the formation of an irreversible curd. In contrast, while acidification of casein causes precipitation, this process is reversible upon alkalinization. Thus, in the absence

of rennet a true curd is not formed (21). The purpose of curd formation is to slowly release casein into the small bowel where it is rapidly digested. Human milk does not contain α-casein and the human stomach is not known to secrete rennet. Thus, following consumption of bovine milk or casein by humans, curd formation is absent although a precipitate is formed. It is therefore possible that casein may then be delivered to the small bowel in quantities that may exceed the digestive process that is designed for human milk.

In animals the time course of gastric casein digestion is slower than the digestion of whey proteins (26, 27). The curd in the stomach is relatively resistant to the enzymatic disaggregation. Therefore, casein is only slowly released into the duodenum for digestion. In contrast, whey and soy proteins are degraded both in the stomach and in the intestinal tract (25). Heat treatment or sonication may eliminate some of the differences in digestibility of soy and casein, an effect which may be due to improved solubility. Thus, the rate of *in vivo* digestion of casein must account for both gastric and intestinal proteolysis, gastric emptying, and various pretreatments of the proteins.

Casein not digested in the stomach or the first portion of the small intestine may pass more distally and has been found in the ileum and colon of babies with necrotizing enterocolitis requiring surgical excision of the intestine (28). In the presence of an adequate source of carbohydrate, casein may be partly degraded and utilized by the colonic flora and yield ammonia, free peptides, and volatile fatty acids (29). The primary organic acids produced are acetic, propionic, butyric, and isovaleric acids (30). During intestinal transit, casein is less effective than either soy protein or egg albumin in stimulating the release of pancreatic carboxypeptidases A and B (31). It also binds to bile acids, limiting their capacity to solubilize fatty acids (32). Divalent cations preferentially complex to the casein micelle or bind to its phosphoseryl residues as phosphopeptides (33, 34). Although calcium, a constituent of the casein micelle, and iron have been most studied, zinc, magnesium, and manganese have also been found to bind to casein, thus potentially limiting their bioavailability (33–37).

Donovan and Lonnerdal (38) have recently demonstrated that the casein content of human milk contributes less than 25% of the total available nitrogen. The remainder is whey protein and nonprotein nitrogen. On the other hand, cow milk has approximately three times as much total protein (3–4 g %) as human milk, is casein predominant (greater than 80%), and its residual whey fraction is primarily the lactoglobulins which are not found in human milk (6, 7). The net protein utilization of bovine or human whey protein is superior to that of casein, 95% vs 80%, respectively (6).

Numerous metabolic effects have been associated with casein-predominant diets. Babies fed a casein-predominant formula have higher levels of blood urea nitrogen, phenylalanine, methionine tyrosine, and ammonia (39–41). They also have a lower serum pH (late metabolic acidosis) and lower levels of taurine and cystine (39–41). The aminograms of preterm newborn babies fed a whey-predominant formula more closely resemble the aminograms of breast-fed infants (41).

One measure of the protein quality of milk proteins involves determination of the protein efficiency ratio (PER) or the amount of weight gain divided by the amount of protein eaten. Although simple to perform, PER are not sensitive to small differences in protein quality and the number generated has no intrinsic meaning beyond its use for comparative purposes. In general, the whey fraction of cow's milk generates higher PER than the casein fraction due to the higher percentages of essential amino acids and, in particular, cystine and threonine (6). Compared with the nonadjusted PER of cow milk (3.6), the casein (3.3) fraction is consistently lower than the average for whey (3.8), which summates the individual contributions of β-lactoglobulin (3.5) and α-lactalbumin (4.0) (6). These values agree with the nonadjusted PER values of 2.80 for casein and 3.4 for whey protein provided by the National Dairy Council.

Another presumed measure of the dietary adequacy of milk proteins is comparison of plasma amino acid concentrations to these human milk-fed infants after a feeding. Other selected measures of protein nutriture (e.g., urea, albumin) may be evaluated concurrently. With the marked differences in the amino acids present in casein and whey protein, it is not surprising that infants fed whey-dominant and casein-dominant formulas exhibit different plasma amino acid profiles. Jarvenpaa *et al.* (39) fed infants whey-predominant or casein-predominant (whey to casein, 60:40 and 18:82, respectively) formulas and noted higher concentrations of tyrosine and phenylalanine in the casein-predominant group and higher threonine in the whey-predominant group. The authors expressed concern over the significance of increased plasma concentrations of tyrosine and phenylalanine in terms of potential neurotoxicity and adverse effects on nervous system development. Kashyap *et al.* (42) also studied growth, nitrogen retention, and chemical indices of protein metabolic tolerance of whey-predominant versus casein-predominant formulas in low birthweight infants. Standard anthropometric measures of height and weight, nitrogen retention, blood urea nitrogen, and acid-base balance did not differ between the two groups. Plasma tyrosine was significantly elevated in the casein-predominant group, however, and threonine in the whey group. The authors suggested that the "transient tyrosinemia of the newborn" raises questions over the use of casein-predominant formulas for the low birth-

weight infant. Indeed, infants fed casein-predominant evaporated milk containing a naturally high protein concentration developed transient tyrosinemia and specific learning disabilities as tested by the Illinois Test of Psycholinguistic Ability (43). Although the elevated threonine in the whey group was not associated with adverse developmental outcome, neither the casein- nor the whey-predominant formulas achieved amino acid patterns similar to those of breast-fed infants. Janas *et al.* (44) also noted that altering the whey to casein ratio failed to achieve the desired blood indices common to breast-fed infants. They suggested that lowering the total nitrogen (protein) content of infant formulas may be a more effective strategy to achieve a more physiologic post-prandial amino acid pattern.

Low birthweight infants appear to be uniquely vulnerable to distortions of the amino acid profile associated with casein-predominant feedings. Raiha *et al.* (41) fed 106 low birthweight infants whey-predominant versus casein-predominant (cow milk) formulas varying in protein content of 1.5 or 3.0 g/100 ml of formula. Blood ammonia concentration was relatively elevated in the casein groups along with a more frequent, severe, and prolonged state of metabolic acidosis. Metabolic acidosis and failure to thrive have also been reported in formulas containing 100% casein hydrolysate. Healy (45) observed that infants receiving Nutramigen (a casein hydrolysate) developed metabolic acidosis and weight loss that was corrected by the addition of a base solution or the substitution of another formula. Shenai *et al.* (46) also observed that casein-predominant formulas increased the risk of metabolic acidosis in low birthweight infants, although casein predominant and whey predominant achieved similar nitrogen retention.

Imbalances in plasma amino acid levels may influence the transport of neurotransmitter precursors into the brain (47). Hence, the elevated levels of tyrosine and phenylalanine seen in the casein-dominant formulas may affect or modify catecholamine levels in the brain resulting from increased substrate availability. The elevated levels of threonine commonly seen in whey-predominant formulas (47, 48) do not have known neurotoxicity and/or act as neurotransmitter precursors. Tyrosine and phenylalanine have been shown to be highly neurotoxic when fed to rats in excess of daily requirements, whereas threonine was the least neurotoxic amino acid. Similarly, threonine is only moderately toxic in terms of growth suppression if fed to rats; tyrosine and phenylalanine caused more severe growth depression (49).

Although much effort is directed toward humanizing infant formula by manipulating casein to whey ratios, the disparity between the amino acid contents of bovine versus human milk makes plasma amino acid duplication rather difficult (50). In a recent report, Rigo *et al.* (48) reported that 1-week-old neonates fed a 100% whey formula demonstrated a pattern of plasma amino acids close to breast milk (except for elevated threonine) and similar blood urea concentrations. Lonnerdal and Chen (51) purport that a whey to casein ratio of 55:45 and a protein concentration of 13 g/liter, supplemented with iron and zinc at 7 mg/liter, resulted in growth and metabolic indices similar to those of breast-fed infants.

Interactions with Divalent Cations

Whey and casein proteins differ in binding affinities and release of certain cations, e.g. calcium, manganese, magnesium, zinc, and iron, during digestion. Bovine β-casein (25–35% of total casein) exists in a fully phosphorylated form containing four to five phosphate groups per molecule versus the much lower phosphorylation (zero to five) of human casein (52). This probably accounts for the great binding of magnesium, iron, copper, and zinc in the bovine versus human casein micelle (8). These authors note that the casein in cow's milk may not be completely digested due to the low digestion capacity of the newborn. The casein in human milk, found in much lower quantities, is structurally different, forms a relatively soft or nonexistent curd, and binds mineral to a lesser extent, all of which may help account for the high solubility and bioavailability of mineral in human milk.

Using the suckling rat pup model, Lonnerdal (53) demonstrated a depressant effect of casein on zinc absorption. Casey *et al.* (54) also noted a significantly depressed plasma zinc response in adult females consuming a casein-predominant formula. An even lower plasma response was obtained with a 100% casein hydrolysate formula. A similar effect of casein is also evident in primates; in infant rhesus monkeys, zinc absorption is lower when they are fed casein-predominant compared with whey-predominant formulas (55). Singh *et al.* (56) were concerned that the relatively high zinc-binding capacity and poorer digestibility of casein could lead to complexing and loss of this essential nutrient. In their experiments, the enzymatic dephosphorylation of casein with acid phosphatases markedly reduced, but did not eliminate, zinc binding. Although whey may also bind zinc, a substantial portion of zinc is trapped with colloidal calcium phosphate.

Cow's milk casein binds a large proportion of iron, zinc, and copper due to phosphorylated amino acid residues on α- and β-casein and the large intrinsic potential for exogenous mineral affinities (53). Hegenauer *et al.* (57) observed the ability of the casein micelle to bind iron when added to cow's milk. Lonnerdal *et al.* (58) extrinsically labeled ^{54}Mn and found that 71% of manganese is in breast milk whey (lactoferrin) versus 67% in cow milk casein.

Approximately 65% of total milk calcium is associated in colloidal bovine casein micelles in contrast to 6% or less in human casein (3). This may greatly

influence calcium absorption depending on the relative digestive capabilities of the calcium-protein complex or the total amount of casein phosphopeptides generated. Since calcium binding to bovine β-caseins is tight (59), some authors have speculated that the phosphopeptides generated during digestion may enhance calcium and mineral absorption by increasing mineral solubility (8). In general, mineral binding by casein usually lowers the availability of bound minerals compared with an alternate protein source such as whey (3). Rudloff and Lonnerdal (60) studied calcium absorption from whey-predominant formula, a 100% whey hydrolysate formula and human milk in weanling rhesus monkeys. Using extrinsic labeling with ^{47} Ca, the percentage of absorption of calcium ions was similar in all three infant formulas and inferior to human milk. However, the higher calcium concentrations in the infant formulas compensated for slightly lower absorption and delivered equivalent amounts of calcium to the infant.

Casein Interaction with Bacteria

The caseins have numerous interactions with bacteria from the mouth to anus. Bovine casein (milk phosphoprotein) can be incorporated into dental plaque and prevents enamel subsurface demineralization (61). It does not produce a significant change in the quantity or composition of plaque bacteria but rather increases the plaque calcium phosphate content. Furthermore, its buffering capacity attenuates calcium leaching associated with organic acid production by plaque bacteria (61).

Casein may indirectly affect bacterial growth. The attenuation of salmonellae growth that is associated with cow's milk acidification can be reversed by the addition of casein in a dose-dependent manner (63). Five percent cocoa powder in a nutrient broth is bactericidal to the salmonellae but the addition of 5% bovine casein to this broth neutralizes this bactericidal effect (62). A 1:1 molar ratio of casein to cocoa powder allows for uninhibited salmonellae growth.

Casein may also interfere with neutrophil function. The phagocytosis of *Staphylococcus aureus* by milk leukocytes was impaired in a manner that can be directly attributed to the casein content of milk (64). Immunofluorescence studies reveal that both milk neutrophils and blood neutrophils, when incubated in milk, absorb casein on the cell surface and that casein is also ingested by the neutrophil. In addition, the combination of fat and casein inhibits both the phagocytosis and intracellular killing of *S. aureus* by neutrophils (65).

The production of β-lactamase by *Enterobacter cloacae* is enhanced by casein derivatives in the presence of glycine (66). Casein digests and hydrolysates induce accelerated carbohydrate fermentation in enteric gram-negative rods by inducing the lactose operon and increasing β-galactosidase activity (67). This results in more rapid fermentation of carbohydrate to organic acids, which may then lower intraluminal pH. This has been proposed as an important component in the initiation of neonatal necrotizing enterocolitis (neonatal inflammatory bowel disease) (68). Lifschitz *et al.* (69) in a recent report provided evidence that cow's milk formulas may accelerate carbohydrate fermentation by intestinal flora in healthy term infants. Breast-fed infants have a significantly slower rate of carbohydrate fermentation to organic acids than formula-fed infants. The profile of organic acid production differed between the two groups; formula-fed infants produce considerably more propionate whereas breast-fed infants produce more homolactate. These diet-related differences in the way infants' bowels respond to colonic substrate (carbohydrate) loading may explain why acute gastroenteritis in breast-fed infants tends to be less severe than in formula-fed infants. As neonatal necrotizing enterocolitis is more evident in formula-fed infants, these alterations in carbohydrate fermentation by intestinal flora may then set the stage for the devastating form of intestinal inflammation-neonatal necrotizing enterocolitis.

Bovine casein enhances the shift of fluid into the bowel in response to enterotoxins produced by colonic flora (70, 71). In rabbit intestine, bovine α-casein promoted a 90% increase in intestinal loop fluid accumulation to cholera enterotoxin, whereas whey proteins attenuate this response (71). In contrast to the synergism seen with bovine casein, human casein decreased the intestinal fluid response to cholera enterotoxin by more than 25%. An intact bovine casein structure is necessary for this potentiation of the cholera toxin effect. β-Casomorphins, potent opioid peptides formed during casein digestion, partially block the secretory effect of cholera toxin (72). Murine and bovine casein are able to bind the heat-stable enterotoxin of enterotoxigenic *Escherichia coli*. The binding is pH dependent with 75% of the toxin bound to casein at pH 2.3 and 7.4% bound at pH 10.1. The binding of the toxin does not destroy its biologic activity. Consequently upon casein digestion, the active toxin, bound by casein in the stomach, may be released in the lower gastrointestinal tract (73).

Inflammation and Allergy

Leukocytes. Casein has considerable proinflammatory characteristics. Unlike the whey proteins (albumin, immunoglobulins, and lactoferrin) which do not cause adverse reactions when administered systemically, parenteral administration of casein can cause extensive cellular injury. Caseins, primarily α- and β-casein, are chemotactic for leukocytes (74, 75). This can be demonstrated in Boyden chambers or *in vivo* where casein administration into the peritoneal cavity

is a standard technique for harvesting peripheral neutrophils of experimental animals (76, 77). Casein not only promotes leukocyte migration but it induces the activity and release of phospholipase A_2 (77, 78), thereby promoting arachidonic acid metabolism by both cyclo-oxygenase and lipoxygenase pathways in neutrophils and neighboring cells. Casein also stimulates the activity of 5-lipoxygenase, the enzyme responsible for the formation of leukotrienes (79). These lipoxygenase products (e.g., LTB_4, LTC_4, and LTD_4) may exacerbate the inflammatory response through additional cellular activation and recruitment, as well as modulating local vascular tone and permeability (80, 81).

Casein-induced activation of neutrophils and monocytes also leads to increased formation of inflammatory cytokines. Recently, Goto *et al.* (82) described increased release and formation (mRNA expression) of interleukin 1β by peritoneal neutrophils, lymphocytes, and macrophages harvested after casein administration.

The mechanism by which casein elicits a chemotactic response is still unclear but it requires an intact structure as casein hydrolysates are not chemotactic (83). There is potential for some biologically active fragments of casein to modify leukocyte function. μ and κ opioid receptor agonists inhibit human granulocyte chemotaxis to agents like casein, raising the possibility that β-casomorphins, potent μ-receptor agonists derived from casein (84, 85), oppose the actions of intact casein. Fragments of human casein have been reported to stimulate the phagocytic activity of human monocytes (86, 87).

The binding characteristics of casein include cell specificity. Casein binds primarily to neutrophils, although some binding to monocytes and lymphocytes is demonstrable; binding to erythrocytes is negligible (74). The binding of casein to neutrophils can be distinguished from phagocytosis, as it is demonstrable at 4°C and cannot be blocked by iodoacetate, a phagocytosis inhibitor (75). Nevertheless, it is not clear if a specific binding site/receptor is involved. The substantial binding of casein to neutrophils at 4°C and the inability of unlabeled casein to fully prevent binding of fluorescein isothiocyanate-labeled or ^{125}I-labeled casein suggests that a component of neutrophil binding is nonspecific (74, 88). However, binding is not blocked by unrelated proteins, e.g., ovalbumin, and can be attenuated by treatment of the neutrophil surface with trypsin, pronase, and neuroaminidase. Under these conditions, comparable reductions in binding and chemotaxis are evident. Casein binding to neutrophils may be mediated by the large number of nonpolar side groups, the large number of acidic groups, or the random coil which exists at a neutral pH (74, 83). The heterogeneous nature of casein binding may reflect contributions from each of these potential binding sites. It is worth noting that the limited chemotactic activity of other proteins, e.g., serum albumin, increases upon denaturing, when their tightly folded structure unravels and assumes a structure which is more comparable to monomeric casein (83). In milk, casein forms micelles and this structure is considerably less chemotactic, indicating that tertiary structure is linked to binding.

Inflammatory Bowel Disease. Local inflammation of the intestine in the form of mucosal damage, edema, lymphatic dilation, exaggerated release of histamine, LTB_4, PGE_2, 6-keto $PGF1\alpha$, and TXB_2 is characteristic of a model of necrotizing enterocolitis (NEC) initiated by a luminal solution of acidified casein (89, 90). This model reproduces the intraluminal biochemistry of infants with NEC. While damage may be elicited by combination of any protein with organic acids, the greatest damage is induced by casein (68). Low intraluminal pH, as a result of carbohydrate fermentation by enteric flora, can increase epithelial permeability (91). The resultant access of luminal casein to mucosal defenses initiates a local inflammatory response and cellular necrosis. Anaerobic isolates taken from the bowel of infants with NEC greatly increase their rate of carbohydrate fermentation when exposed to tryptone, a pancreatic digest of casein (67, 92). The active moiety appears to be a di- or tripeptide.

Casein alone or in combination with bacterial toxins has been implicated in the neutropenia associated with NEC, which is the result of neutrophil activation and not a depletion of bone marrow stores (76). Under these circumstances, activated neutrophils "stick" to the vascular wall and/or migrate into tissue sites, thereby decreasing circulating number. The link between bovine casein and NEC is also supported by data demonstrating that NEC is associated with enteral feeding, particularly aggressive feeding patterns (93) and especially with cow's milk formula versus human milk (94). As premature infants have an epithelial barrier that may be easily compromised, NEC may represent a devastating form of feeding-induced inflammation of the bowel.

Adult inflammatory bowel disease (ulcerative colitis, Crohn's disease) has an unknown etiology. Increased levels of serum antibodies to cow's milk proteins, including casein, bovine serum albumin, β-lactoglobulin, and α-lactalbumin, are associated with adult inflammatory bowel disease (95). The class of immunoglobulins and patient age appear to be important in discerning this response. In pediatric patients, serum levels of IgA to casein, but not to whey proteins, were elevated in ulcerative colitis and Crohn's disease. On the other hand, IgG antibodies to β-lactoglobulin A and B and bovine serum albumin, but not to casein or α-lactalbumin, were elevated in Crohn's disease (96). IgM antibodies were not elevated. The same authors reported in a similar study that adults with ulcerative

colitis and Crohn's disease also have elevated serum antibodies to milk proteins, but not necessarily in the same subclasses as the pediatric population (95). The IgA response to casein was absent in adults, although evident with the IgG and IgM classes. Increased IgG and IgM antibodies to whey proteins were also found. As intestinal permeability is enhanced with inflammatory bowel disease, these antibody responses are suggestive of an increased uptake of macromolecules secondary to bowel damage. Thus, it is possible that when the epithelial barrier is compromised, undigested or partly digested milk proteins have access to mucosal defenses which may promote further mucosal damage or retard the healing process.

Immune Response in Infants. Antibodies to cow's milk proteins can be detected at birth and levels generally increase until weaning (97, 98). The presence of antibodies to whole milk in cord blood probably represents transplacental transfer and is more evident in mothers consuming cow's milk. Clinically, infants who display symptoms of intolerance or allergy to cow's milk proteins are often switched to a soy protein-based diet. However, it is now recognized that soy proteins are as antigenic as bovine milk proteins (99–101). A more consistently efficacious therapy would be the use of protein hydrolysate formulas; these are considerably less antigenic, as the peptide constituents generally have a molecular mass under 1600 daltons. Until recently, only casein hydrolysates have been used in the United States. Experience is now growing with whey hydrolysate formulas, particularly in Europe. The impact of the numerous and diverse effects of biologically active fragments of casein has been poorly evaluated in infants. A recent study by Walker-Smith *et al.* (102) noted that children with cow milk-sensitive enteropathy improved when switched to formulas based on hydrolysates of either casein or whey. However, infants on a whey hydrolysate formula tended to gain more weight. After whey hydrolysate feeding, the small intestinal mucosa showed a significant improvement in mucosal thickness and villus height, whereas after casein hydrolysate feeding intestinal architecture did not improve.

The question of relative immunogenicity of bovine milk proteins has also been studied without clear resolution. Antibody titers vary with time, possibly related to maturation of the intestinal epithelial barrier and hence the extent of transepithelial movement of macromolecules like milk proteins. Casein has a lower molecular weight than the majority of whey proteins, which may facilitate its passage across the epithelial barrier. This is supported by the observation that antibody titers tend to be higher to casein than individual whey proteins. Of the whey proteins, β-lactoglobulin and α-lactalbumin possess molecular weights comparable to casein and are also implicated in milk protein allergy (103, 104). Since casein can directly activate inflammatory and immune cells (i.e., without antibody recognition), circulatory titers of antibody classes to casein may not be predictive of its deleterious effects, particularly in the gastrointestinal tract.

The sensitivity of some infants to bovine casein is extraordinary. There are reports of anaphylactic reaction to casein from such unexpected sources as diaper rash ointment (105) and breast milk (106). In the latter report, the infants' adverse reactions to breast milk ceased when the mother stopped consuming cow's milk, indicating that intact casein or another cow's milk protein or at least their antigenic fragments were absorbed in the mother's gut, transported to, and secreted or released into breast milk in sufficient quantities to elicit anaphylaxis in the infant.

The immaturity of the gut's epithelial barrier in infants (100) and the strong association of bovine milk consumption with iron-deficiency anemia (107), occult gastrointestinal bleeding (108–110), cataracts (111), and the various manifestations of food allergy (112, 113) have raised sufficient concern among pediatricians that some advocate that intact bovine milk should be totally avoided in infants (114).

Animal Models of Diseases

Diet appears to play a major role in the development of spontaneous autoimmune diseases in animals. For example, rabbits fed cow's milk develop joint lesions akin to early rheumatoid arthritis (115). This response is characterized by increased numbers of nucleated cells and increased percentages of T lymphocytes in synovial fluid in a manner consistent with the severity of the joint lesions. Rabbits drinking cow's milk develop high titers of serum and synovial fluid C1q-binding activity (116). However, these values did not correlate with the degree of damage and there was no evidence of glomerulonephritis, suggesting that deposition of immune complexes is not a key event. The precise mechanism underlying this effect of cow's milk, and presumably casein, remains unresolved. Unlike other models of rheumatoid arthritis, however, this model does not require direct manipulation of immune responses with Freund's complete adjuvant.

Casein appears to augment murine systemic lupus erythematosus, as mice fed a casein-free diet display increased longevity, lower anti-DNA antibodies, and decreased immunoreactants in glomeruli (117). As with rabbit rheumatoid arthritis, the mechanisms for this deleterious effect of bovine casein are unknown. Casein-fed mice display compromised humoral immunity (118); specifically, splenocytes exhibited a marked suppression of their antibody response to the T-dependent antigen, sheep red blood cells, and the T-independent antigen, dinitrophenyl-Ficoll.

In contrast to casein, dietary whey proteins appear

to be immunoenhancing, as evidenced by plaque-forming cell response to sheep red blood cells (119); an effect not evident with casein or soy protein. The immunoenhancing properties of dietary whey relative to casein were also confirmed in dimethylhydrazine-induced colon carcinogenesis in A/J mice (119a). Dietary whey increased three-fold the number of plaque-forming cells in the spleen and reduced the incidence and size of tumors induced by dimethylhydrazine, although body weight curves were comparable in whey- and casein-fed groups.

Rats fed casein-predominant diets die 13.5% sooner than rats fed a comparable soy diet, primarily from chronic nephropathy (120). Similarly, increasing the casein content in the diets of mice (CBL/65) is associated with decreased longevity (121), whereas diet-enriched lactalbumin, a whey protein, is associated with increased longevity in Syrian hamsters (122). The renal stress associated with reduced renal mass, which mimics reduced glomerular reserve, also is exacerbated by a diet of 24% casein. This diet is associated with more proteinuria, greater effective renal plasma flow and glomerular filtration rate, greater blood urea and serum cholesterol, reduced survival, and more extensive histological damage and renal hypertrophy than rats fed a 24% soy protein diet (123, 124). It has been well understood that low protein diets can retard renal damage due to reduced renal mass or glomerular number (125), but these results demonstrate that the form of protein is also important. Casein in particular appears to overtax compromised kidneys, causing greater morbidity and mortality.

Atherosclerosis

While atherosclerosis represents an alteration in vascular wall immunology, the focus of casein's contribution to the pathology of atherosclerosis has been on cholesterol absorption and lipid turnover. In light of reports of casein-induced modification of leukocyte function, especially monocytes, direct effects on foam cell formation may be possible.

Casein promotes atherosclerotic disease experimentally and may have similar effects in humans (126, 127). The mechanisms by which this occurs are not clear. However, casein is associated with hypercholesterolemia, decreased binding of β very low-density lipoprotein to hepatic receptors and a diminished very low-density lipoprotein catabolism (127–130). Experimentally, casein appears to enhance the intestinal absorption of cholesterol (131, 132). Although casein does not interfere with the biliary efflux of steroids and bile acids, this increased cholesterol absorption is linked to diminished bile acid excretion. Casein also directly binds bile acids, disturbing the micellular solubilization of fatty acids by bile acids, prior to their absorption (133). This effect was not observed with elemental diets, which may account for their effectiveness in the treatment of patients with steatorrhea.

There are marked species differences in the hypercholesterolemic effect of dietary casein. The rabbit appears to be particularly susceptible; in rabbits dietary casein can establish atherosclerosis in cholesterol-free diets. The rat requires both cholesterol and casein feeding in order to elicit marked hypercholesterolemia. This may be due to the marked differences between the rat and rabbit in their ability to regulate low-density lipoprotein receptor activity. Alternative suggestions are that the rabbit, in contrast to rat and man, has low levels of intestinal phosphatase and more glycine conjugation of bile acids. Casein is highly phosphorylated, and, in the rabbit, intact casein or phosphopeptide fragments will interfere with the binding of insoluble calcium phosphate to bile acids. As a result more bile acids, and hence more cholesterol, are absorbed and less excreted (126, 132, 134). The relatively lower sensitivity of the rat to casein may also be due to the extraordinary capacity of rats to metabolize casein in the stomach, with negligible intact casein entering the duodenum to affect bile acid absorption (135–137).

Supplementation of fish meal diets with tyrosine elevated serum cholesterol in rats to a level similar to casein-fed rats (casein is particularly rich in tyrosine), suggesting that tyrosine may also contribute to the hypercholesterolemic effects of casein (138). Generally, reports indicate that intact casein is more effective.

Does casein have similar effects in primates and in particular, humans? Rhesus monkeys alternatively fed diets of soy protein or casein for 13 to 17 weeks consistently displayed elevations of serum cholesterol with casein diets, mostly due to elevated low-density lipoprotein (131). Manipulation of dietary protein alone with low cholesterol diets was reported not to elevate serum cholesterol in humans (139). However, the same authors, in a follow-up cross-over design study, demonstrated that normolipidemic subjects consuming 500 mg/day of cholesterol and a 20% casein diet exhibited a 10% higher concentration level of low-density lipoprotein cholesterol and a 10% lower concentration of high-density lipoprotein cholesterol (140). Thus, casein may adversely alter serum lipoproteins in normal humans, but relative to species like the rabbit, it is only evident if dietary cholesterol is also raised. However, educational programs continue to focus on the fat content of dairy products, ignoring the potential contributions of casein on the development of atherosclerosis and the other disease states which might be adversely influenced by casein.

Effects of Biologically Active Fragments

There is growing appreciation that the biologic effects of casein may be mediated by fragments generated during digestion or directly administered in the

Table III. Biologic Effects of Casein Fragments

Fragment	Effect	Source	Reference
Val-Glu-Pro-Ile-Pro-Tyr Gly-Leu-Phe Leu-Leu-Tyr	Stimulates binding and phagocytosis of senescent red blood cells by human monocytes	Human casein	(86, 87) (171–173)
β-Casomorphins (β-CM)			
D-Ala-β-CM7	Opioid (μ receptor) agonist	Bovine β-casein	(142, 145, 149, 150)
β-CM7 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile)			
β-CM5	Inhibition of intestinal propulsion		(159)
Morphiceptin (Tyr-Pro-Phe-Pro-NH_2)			
B-[D-$Ala^{2,4}$$Tyr^5$]CM5	Inhibition of cholera toxin induced intestinal secretion	Bovine β-casein analogue	(72)
	Reduction of short-circuit current (inhibition of electrolyte secretion)		(158)
Human β-CM (Tyr-Pro-Phe-Val-Glu-Pro-Ile)	Weak μ opioid agonist	Human β-casein	(144)
Morphiceptin (IV, ICV) β-CM7, β-CM5 (ICV only)	Depressed ventilation in newborn rabbits, adult rats	Bovine β-casein	(169)
β-CM5	Stimulation of somastotatin release in dogs	Bovine β-casein	(161)
β-casomorphins Casopeptone	Stimulation of postprandial insulin release	Bovine casein	(164)
β-casomorphins Exorphins (gluten-derived)	Correlated with incidence of schizophrenia	Bovine casein Gluten	(187)
β-casomorphins	Promotes quiet sleep in newborn rats	Bovine casein	(141)
Unknown	Decreased cardiac glycogen stores	Bovine casein hydrolysate	(163, 165)
Unknown	Bradykinin potentiation (inhibition of Kininase II and other kininases)	Trypsin-hydrolysate of bovine casein	(183)
Histidy-L-proline diketopiperazine (cyclo-(His-Pro))	TRH metabolite, central nervous system depression, reduced food intake, opioid agonist, natriuresis	Bovine casein hydrolysate infant formulas	(176–182)
Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn-Gln-Asp-Lys (undecapeptide)	Inhibits platelet aggregation and binding of fibrinogen to platelets	Bovine κ-casein	(175)
Unknown from tryptone (di- or tripeptide)	Enhanced carbohydrate fermentation in *K. pneumoniae* isolates from necrotizing enterocolitis	Bovine casein tryptic hydrolysate (tryptone)	(67, 92)
Unknown	Stimulation of β-lactamase in *E. cloacae*	Bovine casein tryptic hydrolysate	(66)

form of acidic or enzymic hydrolysates (Table III). Reported effects of these fragments are diverse, encompassing immunomodulation, inflammation, opioid effects, metabolic, endocrine, as well as modification of gut flora and function. In some instances the structure of the biologically active products is known. Little is known about the generation of these products during digestion, their bioavailability and metabolism, their interactions with other components in the diet as well as species differences in generation, handling, and responsiveness. Although food has been often jokingly referred to as a drug, there is now sufficient evidence to ponder hormone or drug-like actions of some foods. For example, does the warm cup of milk that aids the induction of sleep act via the generation of potent opioids like β-casomorphin? A recent report noted that β-casomorphin administered intraperitoneally enhances the time spent in quiet sleep (141). This effect can be reversed by the opiate antagonist, naloxone, and is consistent with the calming effect of milk on the newborn.

β-Casomorphins. Of all of the fragments of casein which may act as food hormones, β-casomorphins are the best characterized. An excellent review of this growing field has been recently published by Hamosh *et al.* (142). Briefly, β-casomorphins are a class of opioid peptides generated from bovine or human β-casein, which are structurally distinct from the endogenous opioids, endorphins, or enkephalins. β-Casomorphins act via the μ receptor; they are of seven amino acids in length or less. They are generated from β-casein; those from bovine casein are more potent than β-casomorphins from human milk (143, 144). Similar opioid agonists called exorphins may also be generated during the digestion of the wheat protein, gluten (145, 146).

Evaluation of the potential role of casomorphins as food hormones must weigh their potency, generation, and bioavailability. They can be generated by cleavage of β-casein, by proteases in the gastrointestinal tract, by proteolytic microorganisms (147), and by plasmin, which generates an α-amidated derivative of β-casomorphin 4, called morphiceptin, a potent μ-receptor agonist (84, 148). The β-casomorphin structure is resistant to degradation by gastric and pancreatic enzymes (149). Following milk consumption, β-casomorphins have been detected in the intestinal contents (150, 151); this can be duplicated *in vitro* by replication of the digestive process (152).

If β-casomorphins are generated during digestion, are they absorbed and where do they act? Following milk ingestion, a precursor of β-casomorphin was detected in plasma, although β-casomorphin itself was not detected (153, 154). This may be due to assay sensitivity and the rapid metabolism of β-casomorphin in plasma, whereas the precursor form is resistant to degradation by plasma peptidases (155). There is *in vitro* evidence that a modified form of β-casomorphin 7 with a D-alanine substituted for proline in position 2, traverses the epithelial barrier of rabbit ileum and activates mucosal and muscularis opioid receptors (156, 157). On the other hand, natural β-casomorphins appear to be fully degraded by intestinal brush border enzymes. Thus, under normal conditions, access to these receptors from the intestinal lumen may be limited. β-casomorphins are effective agonists when directly applied to the serosal surface (158), where they can stimulate electrolyte absorption and inhibit intestinal propulsion (159). The latter effect is primarily due to a reduction in periodicity rather than the amplitude of peristaltic waves (160), effects which are similar to other opioid agonists used in the treatment of diarrhea (157). β-Casomorphins have also been found to negate the secretory effects of cholera toxin (73).

Although the gastrointestinal tract is likely to be the major target organ of β-casomorphins, there is considerable evidence suggesting that other organs may be involved. Ingestion of milk, casein hydrolysates, or casomorphins have been reported to increase the release of somatostatin (161) and pancreatic polypeptide (162), impair cardiac glycogen stores, and stimulate insulin release (163–165). Hamosh *et al.* (142) proposed that the higher incidence of obesity in bottle-fed children may be related to the generation of the more potent bovine β-casomorphins, as endogenous opiates are important regulators of food intake (166).

The central nervous system is also a potential target. β-Casomorphins have been detected in the cerebral spinal fluid of lactating women where they may affect mood (167). In newborns the gastrointestinal tract is more permeable (168) and may allow an increased uptake of β-casomorphins. This may lead to respiratory depression (169) and has prompted some suggestions that sudden infant death syndrome may be related to the effects of casomorphins (170). Premature infants are often susceptible to irregular respiratory and cardiac reflexes and their immature gastrointestinal barrier may lead to a higher rate of absorption of casomorphins and other casein-derived products.

Other Casein Fragments. Research by Jolles and co-workers (85, 171, 172) has demonstrated that a number of fragments obtained from enzymic digests of human casein possess immunostimulatory properties, as evidenced by a stimulation of opsonized sheep erythrocyte phagocytosis by murine peritoneal macrophages and *in vivo* killing of *Klebsiella pneumoniae.* The biologically active fragments include a hexapeptide and two tripeptides and are derived from human casein. There is no convincing evidence that digests of bovine casein contain comparable compounds. Rather, there is evidence reviewed above that bovine casein may be associated with immunosuppression, or at least a deleterious modification of immune-related diseases. Thus,

while casein has been proposed as a prohormone with an immunomodulatory role, particularly in the newborn (173), we need to define the importance of potential differences in the source of casein (bovine versus human) as well as the effects of specific fragments. The role of casein in the many transitions occurring in the perinatal period and during infant development remains poorly understood.

Jolles *et al.* (174) have also compared blood clotting and curd formation and noted that there is a strong functional and structural analogy between fibrinogen and κ-casein. Furthermore, an undecapeptide derived from κ-casein inhibits ADP-induced platelet aggregation and fibrinogen binding to ADP-treated platelets (175). This undecapaptide was related to a dodecapeptide C-terminal fragment of human fibrinogen. Thus, it appears that blood clotting and curd formation have similar mechanisms and potentially share biologic ancestry.

Infant formulas which are based on protein hydrolysates are potentially a rich source for biologically active fragments. In addition to the casomorphins, it has been reported that casein hydrolysate formulas contain high levels of histidyl-L-proline diketopiperazine. This compound was recovered in the urine of infants only when they were fed these formulas (176); similar findings have been reported in the urine of phenylketonurics receiving Lophenalac (177). Histidyl-L-proline diketopiperazine may have marked effects on the central nervous system. It is produced endogenously as a metabolite of thyrotropin-releasing hormone (178) and may affect thermoregulation, prolactin release, natriuresis, as well as cause depression of the central nervous system through opioid-like activities (179–181). This compound is also found endogenously in the gastrointestinal tract of the rat (182).

Casein and casein digests affect bacterial function and growth (66, 70, 73, 92, 182) as discussed above. The gastrointestinal system may be particularly susceptible to compromise because it is exposed to high concentrations of casein and the modification of gut flora simultaneously.

Henrique *et al.* (183) have demonstrated that a tryptic digest of bovine α-casein potentiated the proinflammatory and musculotropic responses to bradykinin. Although this factor(s) inhibited kininase II activity, the full response was not solely due to inhibition of the degrading enzyme kininase II alone, as the effects were two- to three-fold greater in magnitude than those observed with captopril or enalapril, potent inhibitors of kininase II (angiotensin-converting enzyme). As bradykinin is a potent proinflammatory agent implicated in inflammatory bowel disease (184), it is possible that bovine casein or its tryptic hydrolysates could exacerbate intestinal inflammation through bradykinin-dependent mechanisms.

Summary

The consequences of bovine milk consumption are diverse, some of which are potentially deleterious. Although certain cultures shun cow's milk or milk-based products, Western societies consume large quantities of cow's milk. Although there are stronger similarities between bovine whey proteins and human whey proteins, the quantity and nature of casein in cow's milk differ markedly from human milk. We propose that the consequences of diets based on bovine casein should be more closely evaluated and certainly expanded beyond the simplistic approach of growth. What is good for the goose may be good for the gander, but what is good for the cow could be harmful to the human.

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