

Human Milk Glycosaminoglycans Inhibit HIV Glycoprotein gp120 Binding to Its Host Cell CD4 Receptor^{1,2,3}

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ABSTRACT The binding of the HIV envelope glycoprotein, gp120, to its host cell receptor, CD4, is inhibited in a solid phase assay by a glycosaminoglycan of human milk; this binding is the essential first step in HIV infectivity. The human milk glycosaminoglycans were identified in this study. Pooled, fractionated human milk contained dermatan sulfate, heparin, heparan sulfate, and chondroitin sulfate. The ability of this glycosaminoglycan fraction to inhibit binding was unaffected by digestion with lytic enzymes specific for heparin, heparan sulfate and dermatan sulfate, but was lost when the milk fraction was treated with lytic enzymes specific for chondroitin sulfate. Furthermore, a purified milk fraction with high specific inhibitory activity contained chondroitin sulfate but not other glycosaminoglycans. This indicates that the ability of human milk to inhibit gp120 binding to CD4 may be attributed to chondroitin sulfate or to a chondroitin sulfate-like moiety rather than to other components of human milk. We speculate that this human milk glycosaminoglycan could limit the rate of postnatal vertical transmission of HIV in breast-fed infants of HIV-infected mothers. *J. Nutr.* 125: 419-424, 1995.

INDEXING KEY WORDS:

- humans • milk • HIV
- glycosaminoglycans • chondroitin sulfate

The issues surrounding public health recommendations on breast-feeding have become complicated by the increasing prevalence of human immunodeficiency virus (HIV) infection among women of reproductive age. Human milk can be the agent of vertical transmission of some viruses, including retroviruses. In the case of HIV, controversy exists regarding the efficiency of human milk as an agent of vertical transmission and possible mechanisms by which this infection might occur. However, with any of the proposed mechanisms, the essential first step in

infection is the binding of HIV to the target cell via gp120. We found that gp120 binding to the host cell receptor, CD4, was inhibited by human milk (Newburg et al. 1992a) and continued our studies to characterize the human milk components thought to be responsible for this activity, with particular attention to the human milk glycoconjugates.

The human milk glycoconjugates, or carbohydrate-containing molecules, are of increasing interest as more nonantibody glycoconjugates from human milk are found that inhibit binding and replication of a range of pathogenic microorganisms. Examples include the human milk glycolipid GM1, a ganglioside that inhibits in vitro binding of cholera toxin and labile toxin of *Escherichia coli*, and inhibits toxicity in vivo (Lægread et al. 1986). A mucin-associated glycoprotein from human milk inhibits binding by rotavirus to the host receptor, and inhibits rotaviral infectivity in vitro and in vivo (Yolken et al. 1992). The mucin fraction of milk also inhibits the adherence of S-fimbriated *E. coli* to their receptors (Schroten et al. 1992). A mannosylated glycoprotein

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inhibits the adherence of enterohemorrhagic *E. coli* to its receptor (Ashkenazi et al. 1991). Human milk oligosaccharides inhibit the adherence of *Streptococcus pneumoniae* and *Haemophilus influenzae* (Andersson et al. 1986) and enteropathogenic *E. coli* (Cravioto et al. 1991). A fucosylated oligosaccharide isolated from human milk inhibits the ability of stable toxin of *E. coli* to produce diarrhea in vivo (Newburg et al. 1990). We previously found that a macromolecular factor in human milk inhibits the binding of the HIV envelope glycoprotein gp120 to the cellular CD4 receptor (Newburg et al. 1992a).

Specific polysulfated molecules, including some glycosaminoglycans, inhibit the binding and replication of HIV (Baba et al. 1988, Ito et al. 1987). Most milk glycosaminoglycans are presumed to be associated with the milk fat globule membrane. The presence of glycosaminoglycans in human milk fat globule membrane had been reported by Shimizu and associates (1981) who found approximately 150 μg glycosaminoglycans/mg membrane. The human milk glycosaminoglycans separated into two components, one of which was heparan sulfate, and the other was undefined; no evidence for chondroitin-like molecules was found.

The issues addressed in this study include: the classes of glycosaminoglycans present in human milk, the ability of milk glycosaminoglycans to account for the inhibition by human milk of gp120 binding to CD4, and the classes of glycosaminoglycans responsible for any such inhibition.

MATERIALS AND METHODS

Milk was obtained from thirty healthy HIV-seronegative women living in central Massachusetts (Central Massachusetts Regional Milk Bank, Worcester, MA). All protocols were approved by the Committee for Protection of Human Subjects at the Shriver Center for Mental Retardation. The milk samples were pooled and separated by anion exchange over Dowex macroporous resin (MSA-2, Sigma Chemical, St. Louis, MO) into the fraction that flowed through the column with distilled water (the nonacidic fraction), and, following a 10 g/L NaCl wash to remove weakly adherent glycoproteins, a fraction which eluted with 160 g/L NaCl (the glycosaminoglycans fraction). A saturated solution of sodium chloride was used to wash the column of any residual sample material; all fractions were dialyzed for at least one week before further analysis.

To identify the glycosaminoglycans of human milk, aliquots of the glycosaminoglycans fraction that eluted from the ion-exchange column with 160 g/L NaCl were digested with a series of specific glycosaminoglycan cleaving enzymes (lyases). Samples of 2.4 mg, each representing 3.75 mL of the

original human milk pool, were dissolved in 100 μL of sodium phosphate (5 mmol/L) in saline (200 mmol/L), pH 7.0. Each aliquot was incubated for 18 h with 10 mU of heparinase (heparinase I, EC 4.2.2.7), heparitinase (heparinase III, EC 4.2.2.8), chondroitinase ABC (EC 4.2.2.4), chondroitinase B (EC 4.2.2.2), or chondroitinase AC (EC 4.2.2.5) at 30, 43, 37, 37 and 37°C, respectively. The products of digestion were separated and visualized by polyacrylamide gel electrophoresis (Al-Hakim and Linhardt 1991). The release of a homologous series of oligosaccharides following digestion of a sample with a specific glycosaminoglycan lyase is evidence for the presence of the glycosaminoglycan substrate of the lyase in that sample. These methods routinely detect glycosaminoglycans at the low nanogram range.

To determine the chemical nature of the gp120-CD4 binding inhibitor, the column fractions were tested for inhibitory activity by the solid phase assay described below, as were the enzymatic hydrolyzates of the active fraction. Loss of activity following digestion with a specific glycosaminoglycan lyase gives evidence for the chemical nature of the inhibitory material. As controls the various incubation buffers were used, none of which contained inhibitory activity, and the flow-through fraction of human milk, which also contained none of the inhibitory activity either before or after incubation with the glycosaminoglycan lyases. Dextran sulfate was used as a positive control. Hydrolyzate of 600 μg of the active glycosaminoglycan fraction (the fraction which eluted with 160 g/L NaCl, above), representing 0.94 mL of milk, was dissolved in 10 mL of buffer, and 100 μL aliquots were tested for CD4-gp120 inhibitory activity in the following assay.

Microtiter wells were coated with recombinant, soluble CD4 peptide encompassing the gp120 binding domain (amino acids 1-370). Milk fractions were added to the solid phase CD4 along with 1 ng of recombinant HIV gp120 (reagents from Du Pont NEN, Boston, MA). Following incubation overnight at 4°C, unbound reagents were removed by washing. The amount of gp120 bound to CD4 was measured by sequential reactions with peroxidase-labeled monoclonal antibody to gp120 (2 h, 4°C), H₂O₂-*o*-phenylene diamine peroxidase substrate (30 min, 4°C), acidification, and measurement of the antigen-antibody reaction at 490 nm. For each sample, the indicated percentage inhibition of CD4-gp120 binding was calculated with the following formula:

$$\% \text{ Inhibition} = 1 - \frac{[(\text{OD}_{\text{sam}} - \text{OD}_{\text{b1}})]}{(\text{OD}_{\text{gp120}} - \text{OD}_{\text{b1}})} \times 100$$

where OD_{sam} is the optical density of the sample tested, OD_{gp120} is the mean optical density of wells in which gp120 was tested without added inhibitor, and OD_{b1} is the optical density of CD4-coated wells

reacted with anti-gp120 antibody in the absence of either gp120 or inhibitor. In this assay, gp120 binding is linear from 100 pg to 1 ng of gp120 per well, with 8% CV.

To provide an independent analysis of the chemical nature of the inhibitory molecule, a purified fraction of milk was prepared by preparative isoelectric focusing, as described previously (Newburg et al. 1992a), such that the specific inhibitory activity was increased 300-fold. Aliquots of this active fraction (600 μ g) were digested with a series of specific glycosaminoglycan lyases (glycosaminoglycan hydrolyzing enzymes). The partial digestion (depolymerization) products were separated and visualized by polyacrylamide gel electrophoresis utilizing a 12–22% gradient gel followed by silver staining (Al-Hakim and Linhardt 1991).

RESULTS

The glycosaminoglycan fraction of human milk macromolecules eluted from the ion exchange column with 160 g/L NaCl. Aliquots were digested with various glycosaminoglycan lyases. Oligosaccharides were released by heparinase I, chondroitinase AC, chondroitinase B, and by heparinase III (heparitinase), but no such digestion products were seen in the control lane of the untreated aliquot of the glycosaminoglycan fraction. Thus, human milk contains heparin, chondroitin sulfate, dermatan sulfate, and heparan sulfate-like macromolecules.

The fractions from the ion exchange column were tested for their ability to inhibit the binding of recombinant gp120 to CD4 in the solid phase binding assay. As shown in Table 1, the untreated glycosaminoglycan fraction inhibited binding of gp120 to CD4. Eluants from the ion exchange column other than the 160 g/L fraction displayed no evidence of the presence of glycosaminoglycans, and no inhibitory activity was noted in these fractions; thus, these were used as negative control preparations.

The chemical nature of the inhibitory macromolecule was further investigated by monitoring loss of inhibitory activity following enzymatic digestion with a series of specific glycosaminoglycan lyases. Neither heparinase I nor heparinase III (heparitinase) digestion of the glycosaminoglycan fraction resulted in a loss of this inhibitory activity (Table 1), indicating that neither heparin nor heparan sulfate is likely to be the major inhibitory molecule. In contrast, digestion with chondroitinase ABC virtually eliminated the CD4-gp120 inhibitory activity, indicating that the inhibitor has characteristics of chondroitin 4-sulfate, chondroitin 6-sulfate, or dermatan sulfate. Digestion with chondroitinase B did not reduce inhibitory activity, eliminating the possibility that the inhibitor is a dermatan sulfate. In contrast, incubation with chondroitinase AC resulted in a marked reduction in inhibitory activity, confirming

TABLE 1

Inhibition of gp120 binding to CD4 receptor by human milk fractions before and after treatment with glycosaminoglycan (GAG) lyases¹

Enzyme	Ion exchange fraction		
	Buffer	Flow-through	GAG
	% inhibition		
None	<5	<5	83
Heparinase I (Heparin)	<5	<5	79
Heparinase III (Heparan sulfate)	<5	<5	66
Chondroitinase ABC (Chondroitin 4-sulfate) (Chondroitin 6-sulfate) (Dermatan sulfate)	<5	<5	<5
Chondroitinase B (Dermatan sulfate)	<5	<5	85
Chondroitinase AC (Chondroitin 4-sulfate) (Chondroitin 6-sulfate)	<5	<5	29

¹The GAG fraction that eluted from an ion exchange column with 160 g/L NaCl was able to inhibit the binding of gp120 to CD4, indicating that the fraction of human milk which contains the GAG also blocks the binding of gp120 to CD4. The loss of activity that follows treatment by chondroitinase ABC and chondroitinase AC suggests that the inhibitor of gp120 binding to CD4 in human milk has the characteristics of a chondroitin 4-sulfate or a chondroitin 6-sulfate.

that the inhibitor of gp120 binding to CD4 in human milk has the characteristics of a chondroitin 4- or 6-sulfate.

An active fraction was prepared from milk by preparative isoelectric focusing. We have previously shown that this fraction has a CD4-gp120 inhibitory activity approximately 300-fold greater than the whole milk macromolecular fraction (Newburg et al. 1992a). This highly enriched fraction was also treated with glycosaminoglycan lyases, and the presence of cleaved oligosaccharides was detected by polyacrylamide gel electrophoresis. As depicted in Figure 1, oligosaccharides were released from this fraction by digestion with chondroitinase ABC, but not by heparinase I nor by heparinase III (heparitinase), further supporting the conclusion that the inhibitor is a chondroitin sulfate-like molecule.

DISCUSSION

Shimizu et al. (1981) reported the presence of glycosaminoglycans in human milk fat globule membrane. Because glycosaminoglycans are usually associated with plasma membrane, and because milk fat

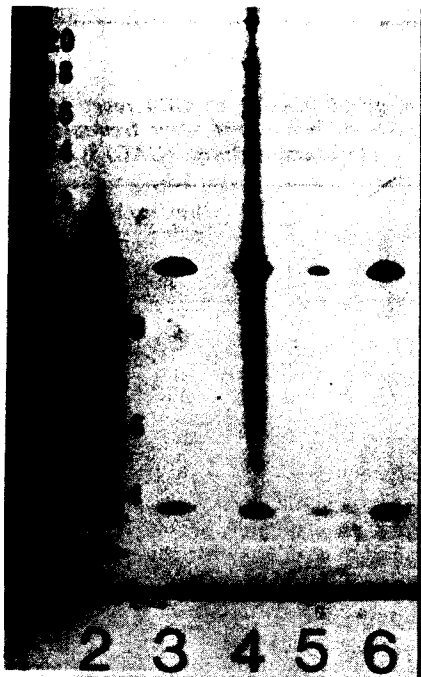


FIGURE 1 PAGE analysis of purified inhibitor of gp120 binding to CD4 from human milk. Bromophenol blue and bromophenol red (arrows) are tracking dyes present in all lanes. The lanes contain 1) bovine lung heparin oligosaccharide standard, 2) partially depolymerized porcine heparin standard (major bands in the standards are labeled with the degree of polymerization, 3) inhibitor purified from human milk by isoelectric focusing, 4) purified inhibitor treated by chondroitinase ABC, 5) purified inhibitor treated with heparitinase, and 6) purified inhibitor treated with heparinase. The presence of a regular series of oligosaccharides in lane 4 indicates that the inhibitory fraction contains material which is digested with chondroitinase ABC, and thus this fraction contains chondroitin sulfate. The lack of such oligosaccharides in lanes 5 and 6 indicates the absence of heparan sulfate and heparin, respectively, in this purified inhibitory fraction.

globule membranes are derived from the apical membrane of the mammary epithelial cell, the milk fat globule membrane is generally thought to be a rich source of milk glycosaminoglycans. Assuming a uronic acid content of 40% for milk glycosaminoglycans, human colostrum (less than 1 wk of lactation) contained ~ 150 μg glyco-saminoglycans/mg membrane, more mature pooled milk (1–3 mo) contained 160 μg glycosaminoglycans/mg membrane, whereas an individual sample from the sixth month of lactation contained 250 μg glycosaminoglycans/mg membrane. The glyco-saminoglycans of mature milk contained heparan sulfate and an unknown component while human colostrum contained only the unknown glyco-saminoglycan.

Using sensitive enzymatic and electrophoretic assays on pooled milk samples, we found strong evidence for the presence of glycosaminoglycans in whole human milk, including dermatan sulfate, heparin, heparan sulfate, and chondroitin sulfate. The

source of these glycosaminoglycans is not known with certainty, but may be related to membrane fragments derived from the milk fat globule membrane, the cell surface of the mammary epithelium, or other cellular or membranous components of the milk. Two independent isolation schemes, one using ion exchange chromatography and another isoelectric focusing, indicate that the human milk factor that inhibits the binding of gp120 to CD4 is a glycosaminoglycan and, specifically, that the inhibitory molecule is a chondroitin sulfate. In solid-phase assays, this milk glycosaminoglycan clearly inhibits binding of gp120 to CD4. Such binding is thought to be an essential first step in HIV infection (McClure et al. 1992). These data provide convergent evidence that the binding inhibition by human milk is related specifically to a chondroitin sulfate or chondroitin sulfate-like moiety. Synthetic sulfated polymers which share some of the gross features of glycosaminoglycans also have been documented to possess anti-HIV activity.

The possibility that glycosaminoglycans have other anti-microbial activities in addition to those against HIV is supported by reports that sulfated molecules can serve as receptors for several pathogens and that glycosaminoglycan-like sulfated molecules can inhibit the cellular binding and infection of several enveloped viruses (McClure et al. 1992). This indicates that the molecules responsible for blocking gp120 binding to CD4 may also provide a broad range of biologically active anti-infectious properties in the milk, or may be one of a family of such compounds. Suzu et al. (1992) have described a highly sulfated glycosaminoglycan which has the activity of the cytokine M-CSF, and have reported that cytokine activities have been found in human milk.

Preliminary studies indicate that the level of inhibitory activity is lower in mature milk of HIV infected women compared to the mature milk of uninfected controls; such differences were not observed in colostrum (Newburg et al. 1992b), perhaps reflecting fundamental differences in the glycosaminoglycans of immature and mature human milk.

The report of Shimizu et al. (1981) suggests that bovine milk has an order of magnitude less glycosaminoglycan than human milk: 15–30 $\mu\text{g}/\text{mg}$ of bovine milk fat globule membrane compared with 150–250 $\mu\text{g}/\text{mg}$ milk fat globule membrane in human milk. Bovine milk glycosaminoglycans are also qualitatively different from those of human milk with bovine milk and colostrum each containing both heparan sulfate and a chondroitin sulfate. Most infant formulas based on bovine milk use processes which exclude the milk fat globule membrane from their final product. Thus the common artificial diets for infants would not be expected to contain

glycosaminoglycans qualitatively or quantitatively comparable to those of human milk.

These findings raise several related issues regarding human milk, including: 1) the distribution of glycosaminoglycans among milk compartments, both quantitatively and qualitatively; 2) levels of milk glycosaminoglycans as a function of maternal nutritional status, disease, and stage of lactation, including the relationship between milk lipid levels and milk glycosaminoglycan levels; 3) individual variation of glycosaminoglycan levels in a healthy lactating population; 4) any modification of milk glycosaminoglycans during transit through the infant's gastrointestinal tract; and, 5) mechanisms associated with vertical transmission of HIV in breastfeeding populations.

The recent worldwide increase of HIV infection among women of childbearing age is resulting in escalating numbers of HIV infections in infants and children. The factors that modulate the transmission of HIV from mothers to their offspring have not been completely defined (Oxtoby 1988).

Breast-feeding has been postulated to be a risk factor for the perinatal transmission of HIV infection (Van de Perre et al. 1991) based on the finding of HIV virions and nucleic acids in milk samples, case reports of postnatal seroconversion in nursing mothers followed by seroconversion of the breast-feeding infant, and by some of the more recent epidemiological studies (Dunn et al. 1992). However, breast-feeding has been shown to lower the incidence of other infectious diseases during the first year of life. The withholding of breast milk can have serious consequences for infants living in areas of the world where enteric disease is endemic (Feachem and Koblinsky 1984, Grulee et al. 1935), for both HIV-infected and HIV-uninfected infants. Thus, in areas where both HIV and enteric disease are prevalent, a dilemma exists regarding breast-feeding recommendations.

Although milk may contain components of HIV, our studies indicate that it also contains glycosaminoglycan moieties that inhibit the binding of HIV envelope glycoprotein to the host cell receptor. Note that other body fluids such as saliva also contain HIV nucleic acid (Yeung et al. 1993) but generally are not considered to be important vehicles for HIV transmission, perhaps due to the presence of similar inhibitory macromolecules (Fox et al. 1989). Thus, the viral and anti-infective parameters involved in transmission of HIV from mothers to infants should be carefully evaluated. Independent of the public health controversies, the data reported herein indicate that the glycosaminoglycans represent a novel class of naturally occurring glycoconjugates of human milk capable of inhibiting viral binding to host cell receptors.

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