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Short Communication

Human milk oligosaccharides reduce HIV-1-gp120 binding to dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN)

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Breast-feeding is the predominant postnatal transmission route for HIV-1 infection in children. However, a majority of breast-fed infants do not become HIV-infected despite continuous exposure to the virus through their mothers' milk over many months. What protects some breast-fed infants from HIV-1 infection? HIV-1 entry across the infant's mucosal barrier is partially mediated through binding of the HIV-1 surface glycoprotein gp120 to dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN) on human dendritic cells. Lewis antigen glycans, present in human milk, bind to DC-SIGN and inhibit HIV-1 transfer to CD4 + T lymphocytes. Human milk contains a high amount of unbound, complex oligosaccharides (5–10 g/l) that carry one or more Lewis antigen glycans, and we hypothesized that they compete with gp120 for DC-SIGN binding. Here, we show in two independent assays that physiological concentrations of human milk oligosaccharides significantly reduce gp120 binding to DC-SIGN by more than 80 %. These results may provide an additional explanation for the inhibitory effects of human milk on HIV-1 mother-to-child-transmission. Identifying the specific milk oligosaccharides that interact with DC-SIGN may guide the development of glycan-based drugs that prevent transmission of HIV-1 and other pathogens that use DC-SIGN as an entry point. However, blocking DC-SIGN may be a two-edged sword.

Breast-feeding: Human milk oligosaccharides: HIV-1: Dendritic cells: DC-SIGN

The Joint UN Programme on HIV/AIDS estimates that 2·3 million children worldwide are infected with HIV-1 (UNAIDS/WHO AIDS Epidemic Update: December 2006). More than 500 000 children have been newly infected in 2006. Mother-to-child transmission accounts for more than 40 % of all HIV-1 infections in children, with breast-feeding being the predominant postnatal transmission route[(1)], especially in developing countries. However, a majority of breast-fed infants born to HIV-positive mothers remain uninfected despite continuous exposure to the virus over many months. Breast-milk contains compounds that reduce HIV-1 transfer, at least in *in vitro* models[(2)]. Identifying those protective compounds in human milk may guide us in designing drugs that help fight the AIDS epidemic.

Viral entry across the infant's mucosal barrier is partially mediated by binding of the HIV-1 envelope glycoprotein gp120 to dentritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN) on human dendritic cells (DC) (reviewed in Su *et al.* [(3)] and Wu & KewalRamani[(4)]). Normally, DC-SIGN-bound ligands (or pathogens) are internalized into DC lysosomes, processed and then presented to T cells, which triggers an immune response to eliminate the intruder. HIV-1, however, 'hides' within the DC for several days and is then transferred to CD4 + T lymphocytes where it replicates and spreads. Other viruses such as hepatitis C virus, Ebola virus, Dengue virus or Cytomegalovirus employ a similar strategy and 'subvert' DC-SIGN at their point of entry (reviewed in van Kooyk & Geijtenbeek[(5)]).

DC-SIGN is a carbohydrate binding protein of the C-type lectin family. It recognizes mannose-containing glycoconjugates such as HIV-1-gp120 but has even higher binding affinities for Lewis blood group antigens (Lewis x (Le^x; galactose β1–4[fucose α1–3]*N*-acetylglucosamine), Lewis y, Lewis a and Lewis b)[(6)]. The Lewis antigens contain *N*-acetyllactosamine (galactose β1–3/4 *N*-acetylglucosamine), which is fucosylated in α1–2 position on galactose and/or α1–3/4 position on *N*-acetylglucosamine. Although monomeric Lewis epitopes bind to DC-SIGN[(6)],

Abbreviations: DC, dendritic cell; DC-SIGN, dendritic cell-specific ICAM3-grabbing non-integrin; HMO, human milk oligosaccharides; Le^x, Lewis x.

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the presence of multivalent Lewis epitopes is required to compete with gp120 for DC-SIGN binding[2]. Naarding *et al.* [2] reported that Le^x glycans in human milk bind DC-SIGN, compete with gp120 for binding to DC-SIGN and inhibit HIV-1 transfer to CD4 + T lymphocytes. Later, bile salt-stimulated lipase was identified as one of the human milk glycoproteins that carries Le^x glycans, binds to DC-SIGN and inhibits HIV-1 transfer to CD4 + T lymphocytes[7].

Besides glycoproteins and glycolipids, human milk also contains an even higher amount (5–10 g/l) of unbound oligosaccharides that are not part of glycoconjugates (reviewed in Bode[8]). These human milk oligosaccharides (HMO) carry lactose at the reducing end and can be elongated with up to fifteen *N*-acetyllactosamine repeats at the non-reducing end. Lactose or the polylactosamine backbone can be fucosylated in α1-2, α1-3 and/or α1-4 linkages[8]. Thus, some HMO structurally resemble the Lewis blood-group antigens, which was confirmed by several groups[9,10]. Since Lewis blood-group antigens show high binding affinity for DC-SIGN and some HMO carry one or multiple of these Lewis epitopes, we hypothesized that HMO compete with gp120 for binding to DC-SIGN and reduce HIV-1 mother-to-child transmission.

Materials and methods

Human milk oligosaccharide isolation

Pooled milk was provided by the Mother's Milk Bank of San Jose, CA, and the Mother's Milk Bank of Austin, TX. Oligosaccharides were extracted and lactose removed as previously described[11]: 1 litre of milk was centrifuged at 5000 ***g*** for 30 min at 4°C, and the fat was removed. Ethanol (2 litres) was added, and the solution was incubated overnight at 4°C. The precipitate was removed by centrifugation at 5000 ***g*** for 30 min at 4°C, and the solvent was removed by rotary evaporation. In order to remove the lactose, the concentration of the solution was adjusted to 0·05 M with phosphate buffer (pH 6·8), 3000 U β-galactosidase (*Kluyveromyces fragilis*) was added, and the solution was incubated for 1 h at 37°C. Then the solution was extracted with four volumes of chloroform–methanol (2:1, v/v), and the aqueous layer was collected. Monosaccharides and disaccharides were removed by selective adsorption of HMO, using solid-phase extraction with non-porous graphitized carbon cartridges (Supelco Inc., Bellefonte, PA, USA). Retained oligosaccharides were eluted with water–acetonitrile (60:40) containing 0·01 % trifluoroacetic acid. Residual lactose and glucose contents in the eluant were determined enzymatically (R-Biopharm, South Marshall, MI, USA).

Human milk oligosaccharide characterization

A 30·5 mg HMO sample (pooled) in 1 ml deionized water was reduced by adding 1 ml of 2 M-sodium borohydride, incubated at 65°C for 1 h and purified following the previously described procedure[12]. The dried sample was reconstituted with 50 μl deionized water. A 2 μl sample was diluted with 200 μl 0·1 % formic acid in 50 % acetonitrile–water (v/v). The oligosaccharides were analysed using an Agilent 6200 Series HPLC-Chip TOF MS (Agilent Technologies) equipped with an autosampler, capillary sample loading pump, nano pump, HPLC-Chip interface and the Agilent 6210 TOF LC/MS. The HPLC-Chip was packed with porous graphitized carbon (45 × 0·75 mm internal diameter, 5 μm). Nano liquid chromatography/MS separation was run at a flow rate of 0·3 μl/min using 0·1 % formic acid in 3 % acetonitrile–water (v/v) (A) and 0·1 % formic acid in 90 % acetonitrile–water (v/v) (B) as liquid chromatography solvents with gradient from 3 to 100 % B. Injection volume was 0·2 μl. Data were acquired in the positive ionization mode with a mass range of *m/z* 500–3000 and analysed using Analyst QS 1.1 software. A list of deconvoluted masses (neutral) and corresponding retention times and abundances was generated using the Agilent Mass Hunter software.

HIV-1-gp120–dendritic cell-specific ICAM3-grabbing non-integrin binding ELISA

Ninety-six-well plates were coated with the extracellular domain of DC-SIGN, washed three times, blocked with 5 % bovine serum albumin and 0·05 % Tween 20, and preincubated with HMO at different concentrations for 15 min. Afterwards, gp120-Fc chimera was added, incubated for 1 h, washed three times, and incubated with anti-human Fc-horseradish peroxidase antibody (1:5000) for 30 min. Afterwards the plate was washed again three times and developed with TMB ELISA substrate. Since binding to DC-SIGN is Ca-dependent, assays were performed in the presence of 1 mM-$CaCl_2$.

HIV-1-gp120 binding to Raji–dendritic cell-specific ICAM3-grabbing non-integrin cells

DC-SIGN-expressing Raji cells (100 000) were incubated with HMO in different concentrations for 15 min. Afterwards gp120-Fc chimera was added and incubated for 1 h at 4°C. Cells were washed three times with PBS + 2·5 % fetal bovine serum + 1 mM-$CaCl_2$ and incubated with anti-human Fc:phycoerythrin for 30 min at 4°C. Cells were washed again three times and fixed with 2 % paraformaldehyde. Binding of gp120 to Raji–DC-SIGN cells was determined by fluorescent-activated cell sorting analysis.

Statistical analysis

Binding of HIV-1-gp120 to DC-SIGN or Raji–DC-SIGN cells in the absence of HMO is considered 100 %. Experiments were performed in triplicate. Results are given as means and standard deviations. Differences in HIV-1-gp120 binding to DC-SIGN in the absence or presence of HMO were tested by two-tailed Student's *t* test. $P<0·05$ is considered significant.

Results and discussion

Human milk can transmit HIV-1 from an infected mother to the breast-fed child, but it also contains several antimicrobial compounds such as lactoferrin[13], lysozyme[14] or long-chain *n*-6 PUFA[15] that reduce the risk of transmission. HMO may serve as an additional defence mechanism. Here, we used two independent assays to test our hypothesis that HMO compete with HIV-1-gp120 for binding to DC-SIGN, the initial step in viral entry across the mucosal barrier.

Although local HMO concentrations in the infant's intestine and especially in the DC microenvironment are unknown, the

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concentrations we chose in the present assays are likely to be within the physiological range. A litre of mature human milk contains 5–10 g HMO; the concentrations in colostrum are even higher (>20 g/l). HMO resist the low pH in the gut as well as degradation through enzymes from pancreas and brush border membrane. Intact HMO rinse the infant's laryngopharyngeal region, the oesophagus, stomach, small intestine and even the colon (reviewed in Bode[8]). Once ingested, human milk gets diluted by saliva and 'digestive fluids', but it also gets concentrated by water absorption. Although the local HMO concentration is unknown, a more than 10-fold dilution (0·5 g/l) appears unlikely. Based on these assumptions, we used HMO at concentrations between 0·5 and 0·005 g/l, spanning a 100-fold range.

Using an ELISA-based assay, HMO at 0·5 g/l reduced HIV-1-gp120 binding to DC-SIGN by more than 60 % (Fig. 1(A)). We confirmed these results in a cell-based assay with DC-SIGN-expressing Raji cells (Fig. 1(B)). Here, HMO at 0·5 g/l reduced HIV-1-gp120 binding by more than 80 %. The inhibitory effects were concentration dependent in both assays. Even at HMO concentrations of 0·05 g/l binding was reduced by more than 50 %. HMO were less effective when incubated with a Le^x antibody prior to the assay (data not shown), indicating that Le^x epitopes on the effective HMO are partially responsible for blocking HIV-1-gp120 binding to DC-SIGN. Other Lewis epitopes (Lewis y, Lewis a and Lewis b) may also contribute.

Lipopolysaccharide also binds to DC-SIGN. We used a Limulus Amebocyte Lysate Gel Clot assay (MO BIO Laboratories Inc., Carlsbad, CA, USA) and showed that the lipopolysaccharide content in our HMO sample was below detection level (0·06 EU/mg), excluding that the observed inhibitory effects on HIV-1-gp120–DC-SIGN binding were due to lipopolysaccharide contaminations.

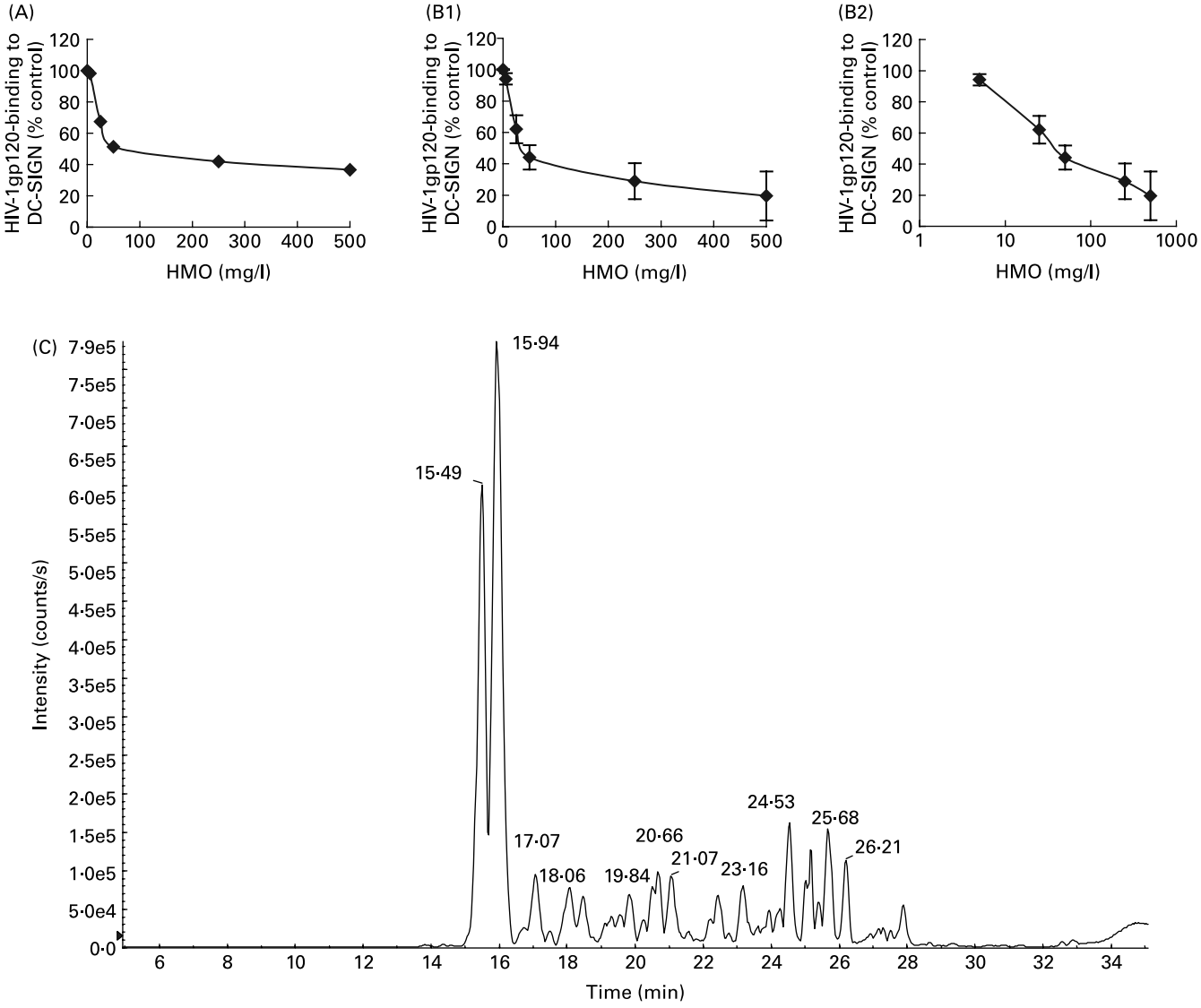

Fig. 1. Human milk oligosaccharides (HMO) reduce HIV-1-gp120–dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN) binding. HMO reduce gp120 binding to DC-SIGN in an ELISA-based assay (A) and in a cell-based assay (B; B1, linear, B2 logarithmic). HIV-1-gp120 binding to DC-SIGN in the absence of HMO is defined as 100 %. Values are means with their standard errors depicted by vertical bars. (C), Base peak chromatogram of HPLC-Chip time-of-flight MS run for pooled HMO sample.

Table 1. List of human milk oligosaccharides with potential Lewis x epitopes (detected by HPLC-Chip time-of-flight MS), their masses (M), retention times (RT), abundances and oligosaccharide compositions*

	M (measured)	M (calculated)	RT (min)	Abundance (% total)	Hex	HexNAc	Fuc	NeuAc
1	855·3327	855·3219	15·48	9·94	3	1	1	
2	855·3331	855·3219	19·85	0·10	3	1	1	
3	855·3332	855·3219	17·08	0·20	3	1	1	
4	855·3333	855·3219	18·05	0·15	3	1	1	
5	1220·4675	1220·4541	20·66	0·07	4	2	1	
6	1220·4687	1220·4541	21·45	0·16	4	2	1	
7	1220·4702	1220·4541	19·09	0·54	4	2	1	
8	1220·4706	1220·4541	22·45	0·51	4	2	1	
9	1220·4709	1220·4541	18·12	5·24	4	2	1	
10	1220·4710	1220·4541	16·80	1·96	4	2	1	
11	1220·4713	1220·4541	18·47	1·24	4	2	1	
12	1366·5302	1366·5120	21·40	0·41	4	2	2	
13	1366·5304	1366·5120	22·78	0·09	4	2	2	
14	1366·5317	1366·5120	15·35	0·28	4	2	2	
15	1366·5320	1366·5120	17·07	3·97	4	2	2	
16	1366·5324	1366·5120	13·92	0·09	4	2	2	
17	1366·5333	1366·5120	14·59	0·13	4	2	2	
18	1512·5912	1512·5699	20·97	0·57	4	3	2	
19	1512·5916	1512·5699	15·18	0·42	4	3	2	
20	1512·5918	1512·5699	14·35	0·20	4	3	2	
21	1512·5918	1512·5699	18·90	0·09	4	3	2	
22	1585·6065	1585·5863	19·71	0·37	5	3	1	
23	1585·6075	1585·5863	26·06	0·08	5	3	1	
24	1585·6080	1585·5863	20·67	2·28	5	3	1	
25	1585·6083	1585·5863	24·25	1·02	5	3	1	
26	1585·6086	1585·5863	22·26	0·69	5	3	1	
27	1585·6089	1585·5863	23·17	1·92	5	3	1	
28	1731·6685	1731·6442	20·58	0·79	5	3	2	
29	1731·6687	1731·6442	21·07	2·71	5	3	2	
30	1731·6687	1731·6442	23·57	0·12	5	3	2	
31	1731·6693	1731·6442	21·80	0·23	5	3	2	
32	1731·6694	1731·6442	22·44	1·71	5	3	2	
33	1731·6695	1731·6442	19·81	2·52	5	3	2	
34	1731·6699	1731·6442	18·64	0·39	5	3	2	
35	1877·7270	1877·7021	21·50	0·33	5	3	3	
36	1877·7274	1877·7021	19·84	0·55	5	3	3	
37	1877·7276	1877·7021	19·28	1·29	5	3	3	
38	1877·7277	1877·7021	20·51	1·87	5	3	3	
39	1877·7278	1877·7021	18·74	0·37	5	3	3	
40	1877·7288	1877·7021	17·44	0·17	5	3	3	
41	2023·7889	2023·7600	19·12	1·06	5	3	4	
42	2023·7894	2023·7600	17·28	0·13	5	3	4	
43	2168·8249	2168·7975	25·26	0·10	5	3	3	1
44	2169·8238	2169·8179	26·29	0·09	5	3	5	
45	2169·8262	2169·8179	26·78	0·13	5	3	5	

Fuc, fucose; Hex, hexose; HexNAc, *N*-acetyl-hexose; NeuAc, *N*-acetylneuraminic acid.
* For details of procedures, see Materials and methods.

MS analysis confirmed the presence of potential Lewis epitopes in the HMO sample (Fig. 1(C)). Table 1 shows that approximately 47 % of all detected HMO in the sample were fucosylated (poly-)lactosamines; 26 % of all HMO contained one fucose residue; more than 20 % carried two or more fucose residues. Some oligosaccharides contained up to five fucose resides, indicating the presence of multiple Lewis epitopes on certain oligosaccharides. Naarding *et al.* [2] showed that compounds with multiple Le^x epitopes inhibit HIV-1 transfer to CD4 + T lymphocytes more efficiently than the monovalent Le^x trisaccharide or the monofucosylated lacto-*N*-fucopentaoses (I, II, III), which are present in milk. Since multivalent binding is required, we hypothesize that high molecular weight HMO with multiple fucose residues and multiple Lewis epitopes have high binding affinity for DC-SIGN and compete with HIV-1-gp120 for binding to DC-SIGN.

HMO block HIV-1-gp120 binding to DC-SIGN similar to certain human milk glycoproteins such as bile salt-stimulated lipase[7]. However, more than 150 different HMO have been characterized so far and some investigators speculate that the actual number may exceed a thousand[9,16]. Which of these HMO are the most potent inhibitors of HIV-1-gp120–DC-SIGN binding? We now aim to develop an affinity chromatography system coupled with online MS that will allow us to identify individual HMO with high binding affinity for DC-SIGN. If multivalency is required for high affinity binding to DC-SIGN, HMO with high molecular weight (> 1300 Da) will be the ones detected in the affinity chromatography system. The results of this approach may guide the development of glycan-based drugs that inhibit DC-SIGN-mediated HIV-1 transmission. These drugs may also be effective against other pathogens that employ DC-SIGN as their

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point of entry (reviewed in van Kooyk & Geijtenbeek[5]). However, DC-SIGN and other lectin- and non-lectin receptors on DC play an important role in early defence mechanisms. Blocking DC-SIGN may be a two-edged sword. It may reduce the entrance of certain viruses such as HIV-1, but at the same time it may also reduce the ability of the infant's immune system to detect and fight other pathogens. This may potentially increase the risk of infants developing bacterial or viral gastroenteritis. Also, HMO as well as milk glycoconjugates may interact with other DC lectins and interfere with the fine-tuned DC receptor crosstalk. Once individual HMO have been identified that block HIV-1-gp120 binding to DC-SIGN, it will be important to use additional *in vitro* and *in vivo* models to assess whether these HMO trigger adverse effects.

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