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Bile Salt-Stimulated Lipase from Human Milk Binds DC-SIGN and Inhibits Human Immunodeficiency Virus Type 1 Transfer to CD4$^+$ T Cells

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A wide range of pathogens, including human immunodeficiency virus type 1 (HIV-1), hepatitis C virus, Ebola virus, cytomegalovirus, dengue virus, *Mycobacterium*, *Leishmania*, and *Helicobacter pylori*, can interact with dendritic cell (DC)-specific ICAM3-grabbing nonintegrin (DC-SIGN), expressed on DCs and a subset of B cells. More specifically, the interaction of the gp120 envelope protein of HIV-1 with DC-SIGN can facilitate the transfer of virus to CD4$^+$ T lymphocytes in *trans* and enhance infection. We have previously demonstrated that a multimeric Le^X component in human milk binds to DC-SIGN, preventing HIV-1 from interacting with this receptor. Biochemical analysis reveals that the compound is heat resistant, trypsin sensitive, and larger than 100 kDa, indicating a specific glycoprotein as the inhibitory compound. By testing human milk from three different mothers, we found the levels of DC-SIGN binding and viral inhibition to vary between samples. Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western blotting, and matrix-assisted laser desorption ionization analysis, we identified bile salt-stimulated lipase (BSSL), a Lewis X (Le^X)-containing glycoprotein found in human milk, to be the major variant protein between the samples. BSSL isolated from human milk bound to DC-SIGN and inhibited the transfer of HIV-1 to CD4$^+$ T lymphocytes. Two BSSL isoforms isolated from the same human milk sample showed differences in DC-SIGN binding, illustrating that alterations in the BSSL forms explain the differences observed. These results indicate that variations in BSSL lead to alterations in Le^X expression by the protein, which subsequently alters the DC-SIGN binding capacity and the inhibitory effect on HIV-1 transfer. Identifying the specific molecular interaction between the different forms may aid in the future design of antimicrobial agents.

Dendritic cells (DCs) express, among other C-type lectins, the DC-specific ICAM-3 grabbing nonintegrin (DC-SIGN) receptor (28, 46). DC-SIGN has been shown to interact with a wide array of pathogens, including hepatitis C virus, Ebola virus, cytomegalovirus, dengue virus, *Mycobacterium*, *Leishmania*, and *Helicobacter pylori* (12). The gp120 envelope glycoprotein of human immunodeficiency virus type 1 (HIV-1), HIV-2, and simian immunodeficiency virus (8–10, 32, 47, 48) can interact with DC-SIGN and has been implicated as playing an important role in HIV-1 transmission and disease progression through infection or propagation of viral replication in CD4$^+$ T lymphocytes and the establishment of infection (9, 10, 16, 34, 36, 38). DC-SIGN is also expressed by a subset of B cells in the tonsils and blood; these cells also transfer HIV-1 to CD4$^+$ T lymphocytes in culture, indicating a role for B cells in HIV-1 transmission and disease progression (35).

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We previously demonstrated that Lewis X (Le^X), 3-fucosyl-*N*-acetyllactoseamine, in human milk can inhibit DC-SIGN-dependent transfer of HIV-1 to CD4$^+$ T lymphocytes by binding to DC-SIGN and blocking the viral interaction with the receptor (30). We also demonstrated that the sugar epitope in milk is likely part of a larger molecule, either as an oligosaccharide or protein associated. The sugar epitope has been identified in many bodily fluids, including saliva, blood, and human milk, and has also been shown to be present in a number of pathogens (2) and pathogen extracts (44).

Bile salt-stimulated lipase (BSSL) is a Le^X-carrying glycoprotein (27) secreted by the pancreas and activated by bile salts in the intestine. BSSL is also expressed by the mammary gland and present in human milk (4) at a concentration of between 100 and 200 μg/ml (42). Neonates normally secrete only small amounts of colipase-dependent pancreatic lipase into the duodenum (14), so gastric lipase as well as BSSL in human milk markedly enhances fat digestion in the newborn (3). Pancreatic BSSL and human milk BSSL were shown to be identical at the amino acid level (19, 31) while substantially varying in their carbohydrate contents (26, 43), with Le^X being present at the

C terminus of BSSL (20, 27). BSSL is also present in testes and adrenals (21) and blood plasma of humans (25). BSSL is produced by stimulated macrophages (22) and eosinophils (15), and its activity has been detected in endothelial cells (23), aortic homogenate (39), and human placenta (7).

Here we demonstrate that the compound in human milk that binds DC-SIGN and inhibits HIV-1 transfer is heat resistant, trypsin sensitive, and over 100 kDa in size. Human milk samples from different mothers were shown to possess various levels of inhibitory activity associated with altered Le^X expression and DC-SIGN binding of a specific protein. Matrix-assisted laser desorption ionization (MALDI) analysis led to the identification of BSSL as the variant protein. We illustrate that BSSL isolated from human milk can bind DC-SIGN and inhibit HIV-1 transfer to $CD4^+$ T lymphocytes and that binding can be alleviated with an antibody (Ab) against Le^X, demonstrating the significance of the Le^X epitope. We have identified BSSL as a major glycoprotein in human milk that has the capacity to bind DC-SIGN and have shown differences in BSSL from different mothers in terms of binding capacity.

MATERIALS AND METHODS

Cells. The Raji and Raji-DC-SIGN cell lines were obtained and cultured as previously described (9, 30). Peripheral blood mononuclear cells (PBMCs) were isolated from three Buffy coats by standard Ficoll-Hypaque density centrifugation, pooled, and frozen in multiple vials. After being thawed, PBMCs were activated with phytohemagglutinin (2 μg/ml) and cultured in RPMI medium containing 10% fetal calf serum (FCS), penicillin (100 units/ml), and streptomycin (100 units/ml) with recombinant interleukin-2 (IL-2) (100 units/ml). On day 3, the cells underwent $CD4^+$ enrichment by incubation with CD8 immunomagnetic beads (Dynal; Breda, The Netherlands) and were negatively selected according to the manufacturer's instructions and cultured with IL-2 (100 units/ml). Dendritic cells for use in the gp120 bead adhesion assay were generated from PBMCs with cells layered on a standard Percoll gradient (Pharmacia, Uppsala, Sweden). The light fraction with a predominance of monocytes was collected, washed, and seeded in 24-well or 6-well culture plates at a density of 5×10^5 or 2.5×10^6 cells per well, respectively. After 60 min at 37°C, the adherent cells were cultured to obtain immature DCs in Iscove's modified Dulbecco's medium with gentamicin (86 μg/ml) and 10% fetal clone serum (HyClone, Logan, Utah) and supplemented with granulocyte-macrophage colony-stimulating factor (500 units/ml) and IL-4 (250 units/ml).

Viruses. Replication-competent HIV-1 stocks were generated by the passage of viruses through $CD4^+$ T lymphocytes, with 50% tissue culture infectious doses ($TCID_{50}$)/ml determined by limiting dilution on $CD4^+$-enriched lymphocytes (33). Subtype B molecularly cloned virus LAI (X4) was used as the virus in all experiments.

Biochemical analyses of human milk. Human milk was incubated with trypsin-EDTA (1×) (Invitrogen, Breda, The Netherlands) or RPMI (Invitrogen, Breda, The Netherlands) for 3 h while shaking at 37°C, after which the trypsin was inactivated by heating at 95°C for 10 min.

Human milk was fractionated by use of Microcon centrifugal filter devices (Millipore, Amsterdam, The Netherlands) with sizes of 3,000, 10,000, 30,000, and 100,000 nominal molecular weight limit. First, the milk was loaded onto the 3,000-nominal-molecular-weight-limit filter device, and then the retained fraction was loaded onto the next filter while compensating for lost volume with phosphate-buffered saline (PBS). The standard manufacturer's protocol was followed to obtain the different fractions.

Human milk and BSSL. Human milk samples (S1, S2, and S3) were collected from three mothers in Amsterdam, The Netherlands. S1 and S3 were collected after 6 months of lactation, and sample S2 was taken at an unknown time point. Internal review board approval was not required, since the samplings were to be discarded material. The milk was centrifuged at $400 \times g$ for 10 min and subsequently at $530 \times g$ for 10 min to remove lipid and cells. The human milk samples were sterilized by sequential filtration through 0.45-μm and 0.2-μm syringe filters (Schleicher & Schuell, Amsterdam, The Netherlands). Milk samples were also collected from two additional mothers in Umeå, Sweden, and BSSL was isolated from these samples (S4 and S5).

BSSL was isolated from human milk as previously described (5) but by use of a second heparin-Sepharose chromatography rather than Affi-Gel blue Sepharose for final purification. Collected fractions were analyzed for BSSL by assay of lipase activity, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting (5).

DC-SIGN–Fc binding ELISA. The DC-SIGN–Fc chimera contained the extracellular portion of DC-SIGN (amino acids 64 to 404) fused at the C terminus to a human immunoglobulin G1 (IgG1) Fc fragment which has been previously described (11). Human milk or BSSL was diluted in 0.2 M $NaHCO_3$, coated onto enzyme-linked immunosorbent assay (ELISA) plates (Maxisorb plate; Nunc, Amsterdam, The Netherlands), and incubated overnight at 4°C or for 2 h at 37°C. This was followed by blocking with TSM (20 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$) containing 1% bovine serum albumin (BSA) for 30 min at 37°C before the addition of soluble DC-SIGN–Fc (5 μg/ml) for 2 h at room temperature (RT). The binding was determined by incubation of a peroxidase-labeled anti-IgG1 antibody for 30 min at RT. DC-SIGN–Fc binding specificity was determined by preincubation of the DC-SIGN–Fc with either 50 μg/ml DC-SIGN-specific mouse antibody AZN-D1 (10) or 10 mM EGTA (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 20 min before the addition of DC-SIGN–Fc to the coated human milk. Due to interassay variation, large differences in the optical density values could be observed, but each independent experiment was performed with the relevant controls to demonstrate binding specificity.

DC-SIGN-mediated HIV-1 transfer assay. The DC-SIGN-mediated HIV-1 transfer assay was performed as previously described (30). The Raji and Raji-DC-SIGN cells were plated at a concentration of 2×10^4 cells/well in a 96-well format. Dilutions of human milk or BSSL were made in PBS containing 10% FCS and spiked with 3.7-log $TCID_{50}$/ml of the appropriate virus before being added to the Raji-DC-SIGN cells. As controls, PBS containing 10% FCS was spiked with the same amount of virus before being added to the Raji or Raji-DC-SIGN cells. After a 2-hour incubation, the culture was washed with PBS prior to the addition of $CD4^+$-enriched T lymphocytes at a concentration of 1×10^5 cells/well. Capsid P24 (CA-p24) values were determined on day 7 using a standard ELISA protocol (29). In short, 96-well plates were coated with a sheep anti-p24-specific antibody (Biochrom AG, Berlin, Germany), after which the culture supernatant was added. As a conjugate, a mouse anti-HIV-1-p24 alkaline phosphatase conjugate antibody (Aalto Bio Reagents Ltd., Dublin Ireland) was used. Development was performed by use of Lumi-phos plus (Lumigen Inc., Southfield, Mich.) according to the manufacturer's instructions and measured by use of a Lumistar Galaxy instrument (BMG LABTECH GmbH, Offenburg, Germany). CA-p24 levels were determined by a standard curve present on each plate (*Escherichia coli*-expressed recombinant HIV-1-p24 [Aalto Bio Reagents Ltd., Dublin, Ireland]).

gp120 bead adhesion assay. Beads were prepared as previously described (10). In short, streptavidin was covalently coupled to carboxylate-modified TransFluoSpheres (488/645 nm, 1.0 μm; Molecular Probes). The streptavidin beads were incubated with biotinylated $F(ab')_2$ fragment goat anti-human IgG (6 μg/ml; Jackson Immunoresearch, West Grove, PA) and subsequently incubated overnight with a gp120-Fc chimera. Fifty thousand Raji-DC-SIGN cells were preincubated with human milk or a fraction thereof, 20 μg/ml AZN-D1 (DC-SIGN-specific mouse antibody) (10), 5 mM EGTA (Sigma-Aldrich, Zwijndrecht, The Netherlands), or 5 mM mannan (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 30 min at RT. The ligand-coated beads (20 beads/cell) were added to the preincubated cells and incubated for 30 min at 37°C, after which the cells were washed with TSM containing 0.5% BSA. After being washed, the cells were resuspended in 100 μl TSM-BSA buffer, and the adhesion was measured by flow cytometry (BD Biosciences).

Western blots and Coomassie staining. The concentrations of the human milk samples were standardized to 30 μg/ml, and the samples were separated on 8% SDS-PAGE gels (Bio-Rad, Veenendaal, The Netherlands). The gel was transferred to polyvinylidene difluoride membranes (Millipore, Amsterdam, The Netherlands) and stained with a mouse anti-human Le^X Ab, C3D-1 (Santa Cruz) (0.2 μg/ml), and a mix of two different goat anti-mouse IgG antibodies (one, at 0.07 μg/ml, from Bio-Rad, Veenendaal, The Netherlands, and the other, at 1/10,000, from Biosource, Breda, The Netherlands), or the membrane was stained with DC-SIGN–Fc (600 μg/ml) and a goat anti-human IgG (Jackson Immunoresearch, West Grove, PA). Visualization was performed using enhanced chemiluminescence (Amersham Biosciences, Inc., Diegem, Belgium). For the Coomassie staining, 60 μg of each human milk sample was loaded on an 8% SDS-PAGE gel. The gel was stained with 50% methanol, 2% acetic acid, and 0.25% Coomassie blue, after which the gel was destained with 30% methanol and 2% acetic acid. The gel was stored in water with 1% acetic acid at 4°C for further analysis.

MALDI protein identification. The protein bands of interest were cut from the gel after staining. For mass spectrometry analysis, the gel slices were S alkylated with iodoacetamide and vacuum dried using a Speedvac instrument. The in-gel digestion with trypsin (sequencing grade; Roche Molecular Biochemicals, Almere, The Netherlands) and extraction of the peptides after the overnight incubation were done according to the work of Shevchenko et al. (40). The collected eluates were dried overnight in a Speedvac instrument. The peptides were redissolved in 6 μl of a solution containing 1% formic acid and 60% acetonitrile. The peptide solutions were mixed 1:1 (vol/vol) with a solution containing 52 mM α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) in 49% ethanol-49% acetonitril-2% TFA and 1 mM ammonium acetate. Prior to the dissolving procedure, the α-cyano-4-hydroxycinnamic acid was washed briefly with acetone. The mixture was spotted on a target plate and allowed to dry at room temperature. Reflectron MALDI–time of flight spectra were acquired with a M@LDI instrument (Micromass Wythenshawe, United Kingdom). The resulting peptide spectra were used to search with MassLynx ProteinProbe (Micromass Wythenshawe, United Kingdom) in a Fasta database or in the sequence databases of the Mascot search engine (http://www.matrixscience.com).

Statistics. All statistical comparisons were performed using analysis of variance. P values of <0.01, <0.02, and <0.05 were considered statistically significant.

RESULTS

Biochemical analysis of the DC-SIGN binding compound in human milk. In order to determine whether the previously identified inhibitory activity of human milk is protein associated or not, we trypsin treated milk sample S3 and tested it in the DC-SIGN–Fc binding ELISA (Fig. 1A). Human milk incubated with medium showed DC-SIGN-specific binding, whereas the trypsin-treated milk showed a significant reduction in binding to DC-SIGN ($P < 0.01$), although not all activity was lost. This result was confirmed in the DC-SIGN-mediated HIV-1 transfer assay, which demonstrated a reduced inhibition of HIV-1 infection with the trypsin-treated human milk in comparison to the untreated sample (Fig. 1B). We could not alleviate the activity entirely, highlighting the fact that the inhibitory activity is robust. These results reiterate that the inhibitory compound in human milk is protein or protein associated.

We next tested whether heating the human milk sample S3 could alleviate DC-SIGN binding or HIV-1-inhibitory activity. Heating the milk to 99°C induced no loss of DC-SIGN–Fc binding either in the ELISA (Fig. 1C) or in the gp120 bead adhesion assay with either Raji-DC-SIGN cells or immature DCs. Heating the milk sample also did not diminish inhibition in the HIV-1 viral transfer assay (data not shown), indicating that the protein does not lose its antiviral function when the native structure is lost. Interestingly, for the heated human milk sample, significant enhancement ($P < 0.001$) of DC-SIGN binding was observed in the binding ELISA (Fig. 1C), as was increased inhibition in the Raji-DC-SIGN transfer assay observed (data not shown), which was reproducible in four separate experiments.

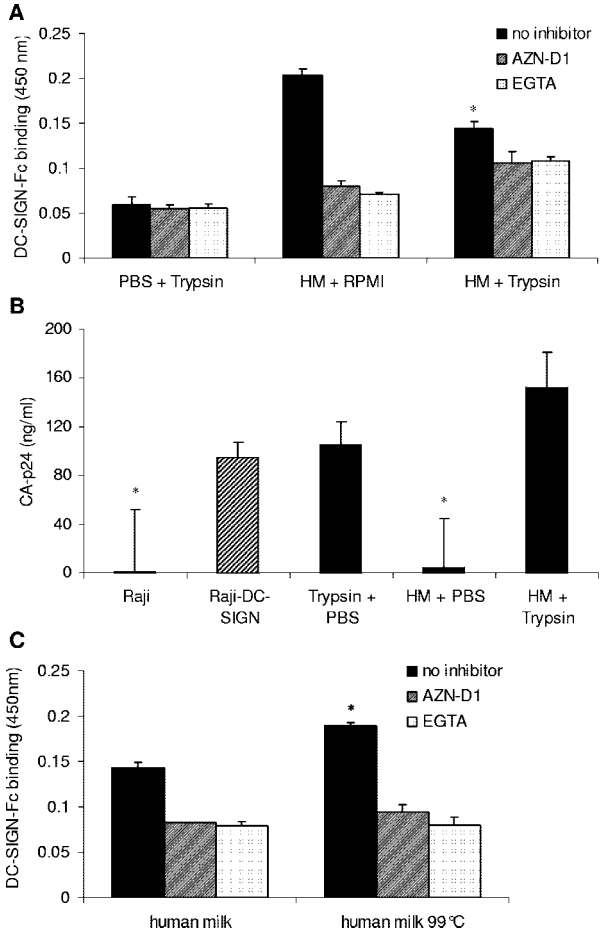

FIG. 1. Biochemical analysis of DC-SIGN binding component in human milk. (A) The DC-SIGN–Fc binding capacity of trypsin-treated human milk (HM) (end concentration, 1/40) was measured by DC-SIGN–Fc binding ELISA. The DC-SIGN–Fc binding background level was obtained by preincubation with AZN-D1 (DC-SIGN-specific blocking antibody) and EGTA. * represents a P value of <0.01 compared to the binding of the human milk incubated with RPMI. (B) Raji-DC-SIGN cells were incubated with the trypsin-treated human milk or controls in the presence of HIV-1 before being washed and before $CD4^+$ T lymphocytes were added. As a control, Raji or Raji-DC-SIGN cells were incubated with PBS and virus before the addition of $CD4^+$ T lymphocytes. CA-p24 production was measured at day 7 by standard ELISA. * represents a P value of <0.01 for inhibition compared to that of Raji-DC-SIGN. (C) Human milk (dilution, 1:2) was heated at 99°C for 10 min before the determination of the DC-SIGN–Fc binding capacity. AZN-D1 and EGTA were used as controls to show DC-SIGN–Fc-specific binding. * represents a P value of <0.001 in comparing heat-treated milk to a nontreated sample. Standard deviations are depicted in all graphs.

The >100-kDa fraction contains the active compound in human milk. To gain an indication as to the size of the protein responsible for the inhibitory activity, we performed size fractionation of human milk. S3 was fractionated, and the obtained fractions were tested by means of the DC-SIGN–Fc binding ELISA (Fig. 2A). Specific binding to DC-SIGN was observed only with the >100-kDa fraction; however, nonspecific binding was seen with the 30- to 100-kDa fraction. We also performed a gp120 bead adhesion assay for the same fractions and observed that only the >100-kDa fraction was able to inhibit the interaction of DC-SIGN with gp120 (Fig. 2B), indicating that the binding observed with the 30- to 100-kDa fraction is indeed nonspecific (Fig. 2A). The gp120–Raji–DC-SIGN interaction could be inhibited with AZN-D1, mannan, and EGTA, illustrating the DC-SIGN specificity of the binding. Incubation of immature DCs with the different fractions before the addition of fluorescent gp120 beads (data not shown) also confirmed that the >100-kDa fraction contains the inhibitory compound(s). In the Raji-DC-SIGN culture assay, both the unfrac-

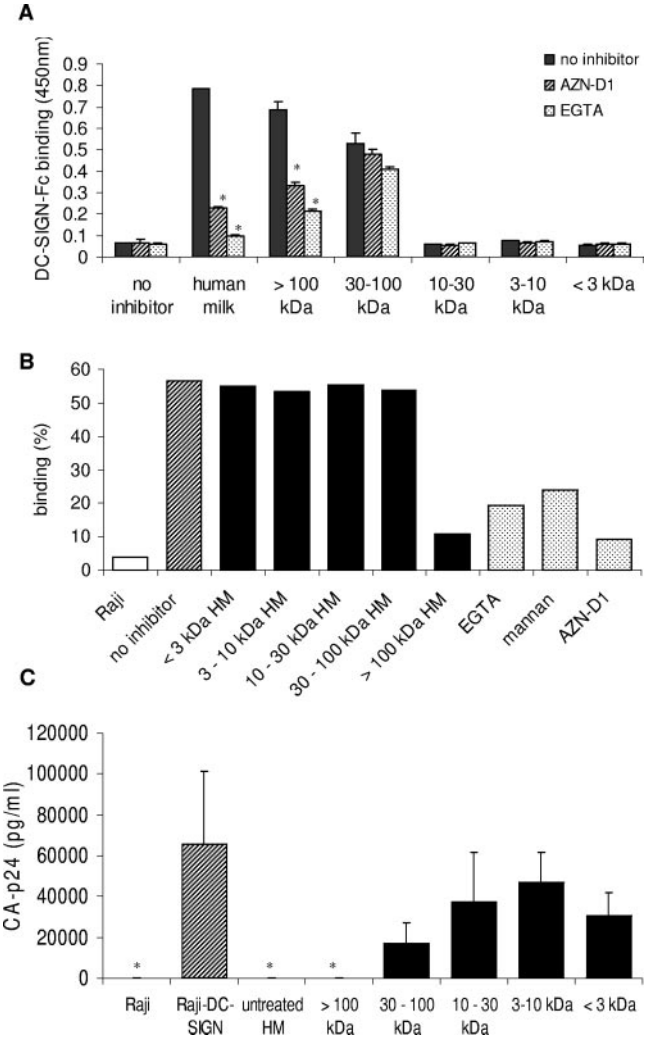

FIG. 2. The inhibitory component of human milk is present in the >100-kDa fraction of human milk. (A) Levels of DC-SIGN–Fc binding of the differently sized fractionations were determined for the human milk fractions (1:100) in the DC-SIGN–Fc binding ELISA, with AZN-D1 and EGTA controlling for DC-SIGN binding specificity. * represents a P value of <0.01 compared to the relevant control. Standard deviations are depicted. (B) Raji-DC-SIGN cells were incubated with the differently sized fractions before being washed and before the addition of fluorescent gp120 beads. To control for DC-SIGN-specific binding, the cells were also incubated with EGTA, mannan, and AZN-D1. HM, human milk. (C) The Raji-DC-SIGN cells were incubated with the different human milk size fractions (1/4) and virus before the addition of $CD4^+$ T lymphocytes. As a control, Raji or Raji-DC-SIGN cells were incubated with PBS and virus before the addition of $CD4^+$ T lymphocytes. CA-p24 production was measured at day 7 by standard ELISA. * represents a P value of <0.01 compared to the PBS control. Standard deviations are depicted.

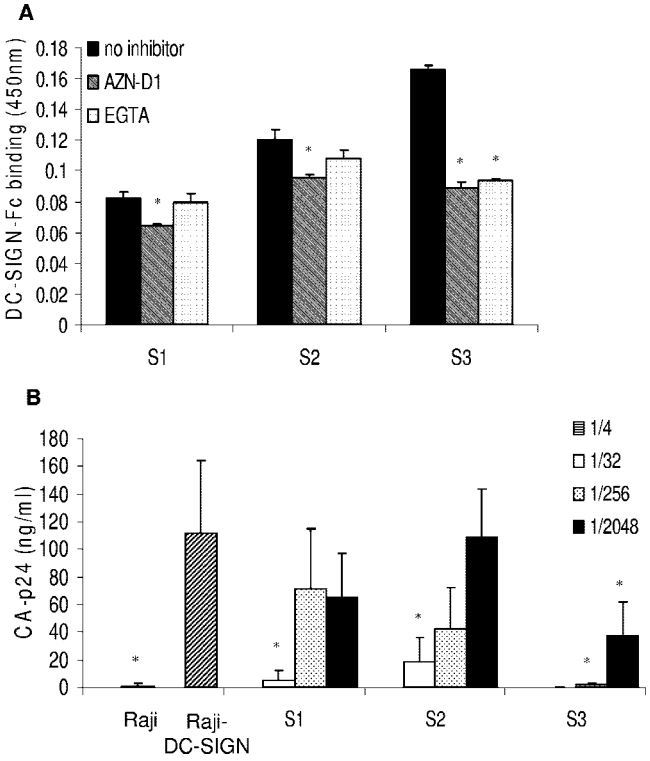

FIG. 3. Differences in the DC-SIGN binding capacities of human milk samples from three mothers (S1 to S3). (A) The DC-SIGN–Fc binding capacity was measured for three different human milk samples (1:200). Preincubation of DC-SIGN–Fc with AZN-D1 and EGTA controlled for DC-SIGN-specific binding. * represents a P value of <0.01 compared to normal DC-SIGN–Fc binding. (B) Different dilutions of the milk samples (S1 to S3) were tested in the Raji-DC-SIGN transfer culture assay. To control for infection, Raji or Raji-DC-SIGN cells were incubated with PBS and virus before the addition of $CD4^+$ T lymphocytes. CA-p24 production was determined on day 7 by standard ELISA. * represents a P value of <0.02 compared to the PBS control. Standard deviations are depicted in both graphs.

tionated milk and the >100-kDa fraction of the human milk show significant ($P < 0.01$) inhibition compared to the PBS control (Fig. 2C). The reduced transmission observed with the 30- to 100-kDa fraction is not statistically significant and likely not due to factors binding DC-SIGN, since the fraction was negative in the gp120 bead adhesion assay (Fig. 2B).

Variation in binding activity between human milk samples from three different mothers. To determine whether variability in the DC-SIGN binding capacity of human milk from different mothers exists, we tested milk samples (S1, S2 and S3) from three individuals. The three samples were tested in the DC-SIGN–Fc ELISA (Fig. 3A) and the DC-SIGN transfer culture assay (Fig. 3B). In the DC-SIGN–Fc ELISA, both S1 and S2 demonstrated low to no binding differences from the relevant AZN-D1 and EGTA controls, whereas S3 showed increased binding ($P < 0.01$) in comparison to AZN-D1 and EGTA (Fig. 3A). The DC-SIGN–Fc ELISA results were confirmed by the DC-SIGN transfer culture assay (Fig. 3B), which showed a significant loss of inhibition at a dilution of 1:256 for S1 and S2 in comparison to S3, which still showed significant inhibition ($P < 0.02$) of HIV-1 transfer at a dilution of 1:2,048. These results demonstrate that the inhibitory activity is significantly different for samples from different mothers.

Identification of BSSL as an inhibitory glycoprotein. Since we previously demonstrated that Le^X is involved with the inhibitory activity of milk (30), we performed Western blot analysis with αLe^X Ab as well as the DC-SIGN–Fc product to try to identify the factor. We performed Western blot staining of human milk samples S1, S2, and S3 with a Le^X-specific antibody (Fig. 4A). Upon comparing of the different samples, we

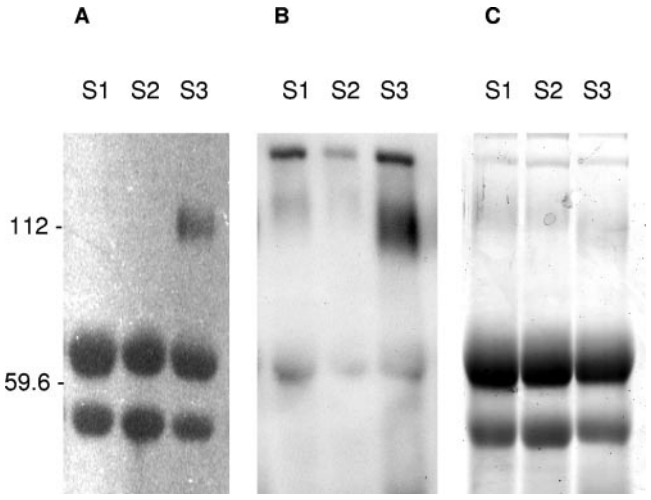

FIG. 4. Western blotting and Coomassie staining of three human milk samples with different DC-SIGN binding capacities. (A) Western blot of human milk samples S1, S2, and S3 stained with Le^X-specific antibody. (B) Western blot stained with DC-SIGN–Fc. (C) Coomassie-stained SDS-PAGE gel. Molecular weights are indicated to the left.

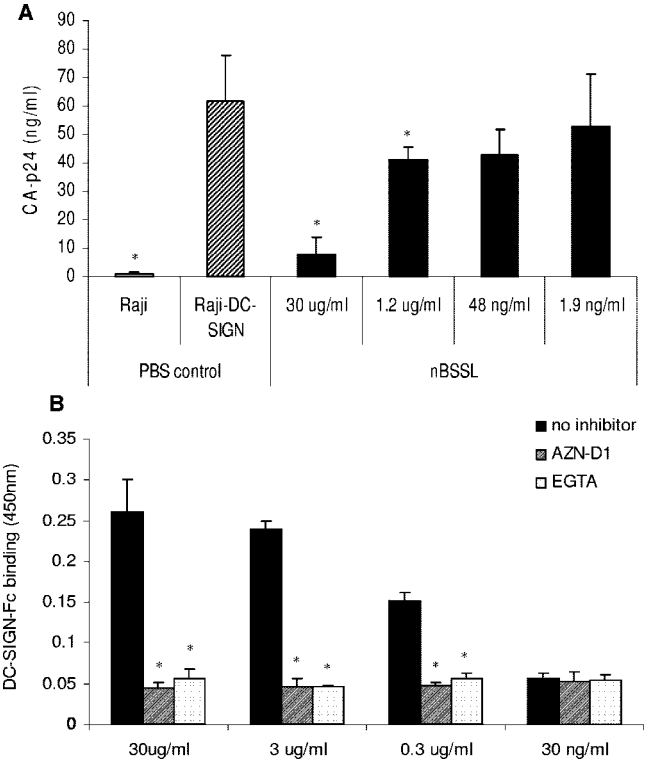

FIG. 5. BSSL binds DC-SIGN and prevents the transfer of HIV-1 to $CD4^+$ T lymphocytes. (A) Raji-DC-SIGN cells were incubated with different dilutions of native BSSL (nBSSL) isolated from human milk (S4) and virus before the addition of $CD4^+$ T lymphocytes. As a control, Raji and Raji-DC-SIGN cells were incubated with PBS instead of BSSL. CA-p24 production was determined on day 7 by standard ELISA. * represents a P value of <0.05 compared to the PBS control. (B) DC-SIGN–Fc binding capacity was determined by ELISA for different dilutions of BSSL isolated from human milk (S4). To control for DC-SIGN specificity, DC-SIGN–Fc was preincubated with AZN-D1 and EGTA to allow comparison to the relevant binding without inhibitor. * represents a P value of <0.01. Standard deviations are depicted in both graphs.

observed a band above 100 kDa for S3, which was not detected for S1 or S2 for equal total protein amounts, indicating a difference in Le^X expression. The Le^X staining result also demonstrates that the lower-molecular-weight proteins are efficiently glycosylated to contain Le^X epitopes with no major quantitative difference between the samples from three mothers, suggesting that these Le^X-associated lower-molecular-weight proteins are not involved in the observed inhibitory activity, which is in accordance with the size fractionation experiment showing the factor as being of >100 kDa. In the DC-SIGN–Fc-stained Western blot (Fig. 4B), the S3 sample contains a clear band at a similar molecular weight, albeit much more pronounced than those for S1 and S2. We also observe that the equivalent bands for S1 and S2 run higher in the gel than the one for S3. These results are in accordance with the differences in inhibitory activity observed for the samples. The Coomassie-stained SDS-PAGE gel (Fig. 4C) also shows a band at the corresponding molecular weight and demonstrates even more clearly that the bands from human milk samples S1 and S2 run higher than the band of interest from S3.

We subsequently extracted the protein from the appropriate SDS-PAGE bands from S1, S2, and S3. The peptide mass fingerprint analysis of the selected protein bands identified it as human bile salt-stimulated lipase (AAA63211) with 20 peptides out of 35 matching (at 30 ppm or below). The sequence coverage was 32% and the probability-based molecular weight search score 199 (with protein scores greater than 76 considered significant). The identification of BSSL is in accordance with the observation that the protein expresses Le^X (27).

BSSL can inhibit DC-SIGN binding and DC-SIGN-mediated transfer of HIV-1 to $CD4^+$ lymphocytes. To confirm that BSSL can serve as an inhibitory compound in human milk, we isolated BSSL from S4. The isolated BSSL was tested in the DC-SIGN transfer culture assay (Fig. 5A) and showed significant inhibition at 30 μg/ml and 1.2 μg/ml ($P < 0.05$). The addition of BSSL (30 μg/ml) to Raji-DC-SIGN cells did not affect cell counts or viabilities as tested by trypan blue exclusion (data not shown), suggesting that the protein is not toxic to the cells. The DC-SIGN–Fc ELISA (Fig. 5B) showed binding at concentrations of 30 μg/ml, 3 μg/ml, and 0.3 μg/ml BSSL ($P < 0.01$), illustrating that BSSL can indeed bind to DC-SIGN and inhibit DC-SIGN-mediated transfer of HIV-1 to $CD4^+$ T lymphocytes. In order to show that BSSL has the same characteristics as the human milk inhibitory factor, we performed trypsinization and heat treatment of purified BSSL and determined it to be heat resistant and trypsin sensitive (data not shown), in accordance with the results obtained with human milk. Interestingly, as with the human milk sample, the BSSL binding is not fully abrogated by trypsin treatment, again suggesting a robust activity against the binding of HIV-1 to DC-SIGN.

BSSL binding to DC-SIGN can be blocked with Le^X antibodies. To confirm our hypothesis that Le^X is (or is part of) the active component of the glycoprotein BSSL, we preincubated BSSL with Le^X IgM before adding DC-SIGN–Fc to the DC-SIGN–Fc binding ELISA. The DC-SIGN binding capacity of

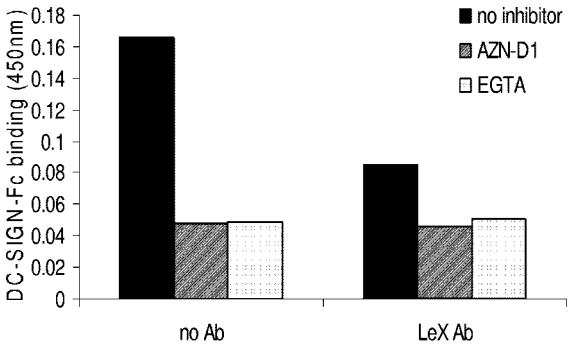

FIG. 6. DC-SIGN–Fc binding can be blocked with a Le^X-specific antibody. BSSL was plated at a concentration of 0.3 μg/ml, and before the addition of DC-SIGN–Fc, the coated BSSL was preincubated with the Le^X-specific antibody. DC-SIGN–Fc was also preincubated with AZN-D1 and EGTA to determine the specificity of binding.

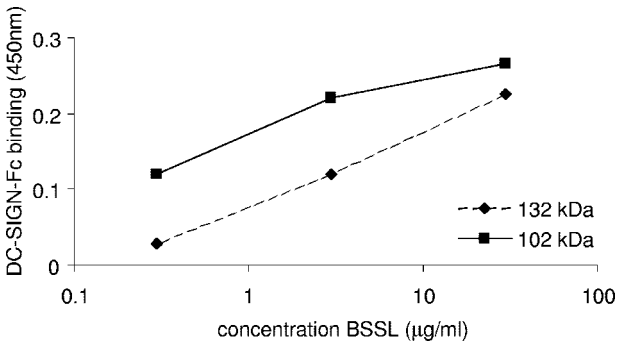

FIG. 7. Variant BSSL isoforms of different sizes show differential binding to DC-SIGN. Two BSSL isoforms isolated from the same human milk sample (S5) demonstrate a difference in DC-SIGN–Fc binding capacity. Different dilutions of the BSSL were plated, and the DC-SIGN–Fc binding was determined by ELISA. To control for the specificity of the binding, DC-SIGN–Fc was preincubated with AZN-D1 or EGTA. The highest binding value observed for these controls was subtracted from the observed value for binding without an inhibitor.

BSSL could be blocked by preincubation with the Le^X-specific Ab (Fig. 6), confirming that the Le^X expressed by BSSL is crucial for DC-SIGN binding.

Two isoforms of BSSL from the same human milk sample show a difference in DC-SIGN binding capacity. To further analyze the correlation between the size of BSSL and the DC-SIGN binding capacity, we isolated two isoforms, variant in size, from the same mother. The larger isoform (132 kDa) demonstrated low DC-SIGN binding compared to the smaller form (102 kDa) of BSSL (Fig. 7), indicating that the differences in binding between the mothers is most likely due to the difference in the BSSL isoforms. Since the two bands are isolated from a single milk sample from the same mother, the different binding patterns are not likely to be due to differences in Le^X secretor status or difference in the activity of fucosyltransferases.

DISCUSSION

DC-SIGN binding differences in milk from different mothers were observed and analyzed by Western blotting and MALDI technology, which enabled us to identify BSSL as a DC-SIGN binding glycoprotein found in human milk that can efficiently block the transfer of HIV-1 to $CD4^+$ T lymphocytes. We have previously demonstrated that a Le^X saccharide containing human milk compound could bind to DC-SIGN and thereby prevent the interaction of the receptor with the gp120 molecule of HIV-1 (30). We demonstrate here that the inhibitory activity of human milk resides within the >100-kDa size fraction, even though Le^X motifs are also linked to proteins of lower molecular weight, corresponding to our previous observation that not all Le^X-containing compounds could mimic the inhibitory effect of human milk (30). We show that the inhibitory activity of purified milk BSSL shares properties with whole milk (30) in being trypsin sensitive and heat resistant. We also demonstrate that as with whole milk, pretreatment of purified native BSSL with an antibody against Le^X interferes with both the binding activity and the block of viral transfer. Collectively, these results indicate that BSSL in human milk is one of the main glycoproteins or the only glycoprotein binding to DC-SIGN, providing the viral inhibitory activity of human milk.

BSSL is expressed in human milk at a concentration of 100 to 200 μg/ml. In our experiments, 30 μg/ml of BSSL showed inhibition of viral transfer and high binding to DC-SIGN. The observed BSSL concentration corresponds to a 1/3 to 1/6 dilution of the human milk, even though we previously showed complete inhibition of viral transfer at a 1/128 dilution (30). This discrepancy could be the result of the presence of an additional inhibitory factor in milk, even though we observe a complete correlation between the DC-SIGN binding capacity and the Le^X and DC-SIGN–Fc staining of the BSSL band in the Western blot analysis. More likely, the DC-SIGN binding activity of BSSL is diminished by the purification from human milk. No proenzyme of BSSL exists, and BSSL is always present in its active form in human milk. Breakdown products of the BSSL protein could be present in human milk, but we demonstrated no DC-SIGN binding activity in the smaller fractionations, suggesting that the larger protein is required to provide for the inhibition.

Variations in DC-SIGN binding and viral transfer inhibition capacities were observed in milk from different mothers in which variations in BSSL protein size and Le^X expression patterns were apparent. Differences were also identified between two isoforms of BSSL isolated from the same milk sample. The BSSL gene is located on chromosome 9 and contains 11 exons. Exon 11 encodes on average 16 repeat domains of 11 amino acids (amino acids 536 to 711) at the C terminus, which contains a high number of O glycosylation sites, while the N terminus contains only one potential N glycosylation site (Asn 187) (1, 37). Variant isoforms of BSSL have been shown to differ in the number of repeats at the C terminus of the molecule (24, 41), which has been shown to be the domain with a high expression of Le^X (27), likely explaining the observed correlation between Le^X expression and BSSL size. In accordance with our observation, it has been shown that the expression of Le^X can vary between mothers (20).

BSSL has been characterized in terms of its lipase activity, which is lost after heating at 50°C (13). Here we show that the DC-SIGN binding capacity is not lost after boiling, indicating

that the enzymatic function and the tertiary structure of the protein are not required for DC-SIGN binding or inhibition of viral transfer. It has been postulated that the high proline content in the C-terminal region prevents this protein from folding into a compact secondary structure, resulting in a flexible, open configuration (37) that could explain the heat-resistant property of the repeat section. We are currently in the process of analyzing further the enhancing effect to inhibition upon heating of the milk and determining whether heating the weakly binding S1 and S2 samples provides for a similar enhancement.

Interestingly, BSSL is found in the blood plasma of a number of mammals, including humans (25); has been postulated to be derived from the pancreas (6); has been shown to be produced by stimulated macrophages and human eosinophils (15); and is present in testes and adrenals (21), endothelial cells (23), aortic homogenate (39), and human placenta (7). Given the wide array of anatomical sites and cell types producing BSSL, it will be interesting to determine whether expression of BSSL can influence microbial transmission, dissemination, and pathogenesis of a number of infectious agents. Differential expression of BSSL or indeed of its variant isoforms may prove to have significant implications for HIV-1 pathogenesis in general by relating to the overall viral loads obtained in individuals after infection. This type of association will be identified through screening HIV-1-infected cohorts for the presence or expression levels of the variant BSSL isoforms in plasma. Another immunomodulatory molecule, Mac-1, has been shown to interact with DC-SIGN (45). Mac-1 is expressed on neutrophils; therefore, identifying natural proteins that can block DC-SIGN may have implications for modulating immune responses involving this cell type.

Fucosyltransferases are responsible for the complex sugar additions and modifications of proteins. Mutations in fucosyltransferase genes have been previously identified (18) and could result in the variant expression of Le^x on BSSL and other proteins. Individuals can be categorized as secretors or nonsecretors of specific sugar epitopes based on the expression patterns of such proteins. Our results show that lower-molecular-weight proteins are equally stained in Western blots with the Le^x Ab when milk samples from different mothers are used, suggesting that the difference observed for BSSL is not due to alterations in such fucosyltransferases and that the Le^x glycosylation machinery is functional for all mothers tested.

Here we identify a Le^x-expressing glycoprotein, BSSL, which binds to DC-SIGN and can prevent its interaction with HIV-1, leading to the blocking of HIV-1 *trans* infection of $CD4^+$ T lymphocytes. It remains to be determined whether BSSL is the only DC-SIGN binding glycoprotein present in human milk or whether other glycoproteins can provide binding and inhibitory functions. Linking variant BSSL isoforms or levels of expression with viral transmission rates via breastfeeding may provide evidence for DC-SIGN involvement in the mucosal transmission of HIV-1. Interestingly, a molecule present in cervicovaginal lavages has also been demonstrated to bind to DC-SIGN but is unidentified as of yet (17). Elucidating the precise molecular interactions of the different BSSL forms, with their differing inhibitory activities, may lead to the development of a DC-SIGN binding molecule that can be incorporated into future antimicrobial or immunomodulatory agents.

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