Glycans on Secretory Component Participate in Innate Protection against Mucosal Pathogens*

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In mucosal secretions, secretory component (SC) is found either free or bound to polymeric IgA within the secretory IgA complex. SC displays numerous and various glycans, which are potential ligands for bacterial compounds. We first established that human SC (hSC) purified from colostrum (hSCcol) or produced in Chinese hamster ovary cells (hSCrec) exhibits the same lectin reactivity. Both forms bind to Clostridium difficile toxin A and functionally protect polarized Caco-2 cell monolayers from the cytopathic effect of the toxin. The interaction is mediated by glycans present on hSC and involves galactose and sialic acid residues. hSCcol and hSCrec were also shown to bind enteropathogenic Escherichia coli adhesin intimin and to inhibit its infectivity on HEP-2 cells in a glycan-dependent manner as well. SC remained operative in the context of the whole secretory IgA molecule and can therefore enhance its Fab-mediated neutralizing properties. On the contrary, hSC did not interact with three different strains of rotavirus (RF, RRV, and SA11). Accordingly, infection of target MA104 cells with these rotavirus strains was not reduced in the presence of either form of hSC tested. Although not a universal mechanism, these findings identify hSC as a microbial scavenger contributing to the antipathogenic arsenal that protects the body epithelial surfaces.

Mucosal surfaces cover the respiratory, urogenital, and gastrointestinal tracts and thus represent a large area, which is constantly exposed to an abundant variety of antigens. It must therefore be efficiently protected against colonization and subsequent possible infection due to pathogens (1, 2). This difficult task is achieved through a combination of innate or constitutive immunity (e.g. mucus, lysozyme, defensins, and lactoferrin) and by an induced specific immune response, which mainly relies at the antibody level on the secretion of secretory IgA (SIgA) (3). SIgA, the major immunoglobulin found in secretions, consists of two or four monomeric IgA linked through a J chain in association with secretory component (SC) (4). SC is derived from the polymeric immunoglobulin receptor (pIgR) that ensures selective transport of polymeric IgA (pIgA) across the epithelium to the luminal side, where the complex is released through proteolytic cleavage (5). Once insecretions, SIgA binds to antigen(s) and thus prevents their attachment to epithelial surfaces, a process known as immune exclusion. SC plays a pivotal role in this process by conferring to SIgA a remarkable stability to acid and proteases (6) and by ensuring correct localization of the antibody within the lumen (7). Besides SC comprised within the SIgA complex, a significant amount of SC is released as such in secretions (up to 25 μM in human milk) (8). SC is constituted of five immunoglobulin-like domains decorated by carbohydrate residues, which constitute between 15 and 24% of the whole molecular mass and are dispatched among seven N-glycosylation sites of the polypeptide (9). The composition of these glycans includes bi- and triantennary or Lewis type structures constituting binding epitopes for bacterial adhesins (10). The highly glycosylated nature of SC suggests that it might play an important role in the protection of the mucosal surfaces through nonspecific interactions with microbial antigens. The first evidence of such a role for SC was given by the observation that human SC (hSC) purified from colostrum binds to Clostridium difficile toxin A and limits its adherence on hamster brush border membranes (11). Additionally, glycans associated to SIgA have been shown to interact with Helicobacter pylori (12). However, little is known as to the underlying mechanisms and whether this holds true for other gastrointestinal pathogens. Moreover, the relevance of binding data with respect to protection was not examined.

Here, we provide biochemical characterization of the interaction between highly purified hSC, either obtained from colostrum or produced in Chinese hamster ovary (CHO) cells, and (i) C. difficile toxin A, (ii) enteropathogenic Escherichia coli (EPEC) adhesin intimin, and (iii) rotavirus triple layer particles. Further, using established in vitro infection models for each pathogen tested, we evaluate the biological significance of the observed interactions in terms of protective activity, substantiating the role of SC in innate immunity.

MATERIALS AND METHODS

Cells and Culture Conditions—Human colonic adenocarcinoma Caco-2 cells (HTB-37) were grown in Dulbecco’s modified Eagle’s medium (4.5% glucose) (Sigma) supplemented with 10% fetal bovine serum (Sigma), 10 mM HEPES (pH 7.0), 1% nonessential amino acids, 1% sodium pyruvate, 2 mM glutamine, 0.1% transferrin, and 100 units/ml penicillin and 100 μg/ml streptomycin. For establishment of polarized monolayers, Caco-2 cells cultured to 80% confluence were seeded on Snapwell filters (diameter, 12 mm; pore size, 0.4 μm; Corning Costar Corp., Cambridge, MA) at a density of 2 × 10⁶ cells/cm². The formation of a polarized Caco-2 cell monolayer at week 3 was established by morphology and monitoring of the transepithelial electrical resistance (TER) using a Millicell-ERS apparatus (Millipore Corp., Bedford, MA) (13). HEp-2 cells (CCL-23) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, 2 mM glutamine, and 100 units/ml penicillin and 100 μg/ml streptomycin. Embryonic

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2 The abbreviations used are: SC, secretory component; hSC, human SC; hSCcol, hSC purified from colostrum; hSCrec, recombinant hSC; pIgA, polymeric IgA receptor; pIgR, polymeric immunoglobulin A; CHO, Chinese hamster ovary; EPEC, enteropathogenic E. coli; TER, transepithelial electrical resistance; MEM, Earle’s minimum essential medium; HSCdg, deglycosylated hSCrec; GST, glutathione S-transferase; PBS, phosphate-buffered saline; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; A/E, attachment and effacement; TLP, triple layer particle; PIPESE, piperazine-N,N’-bis(2-ethanesulfonic acid); mAb, monoclonal antibody.
rhesus monkey kidney MA104 cells (CRL-2378.1) were grown in Earle’s minimal essential medium (MEM) (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM HEPES (pH 7.0), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. All cell lines were maintained at 37°C in a 5% humidified CO2 incubator.

**Proteins and Antibodies**—Purified colostral hSC (hSCcol) was isolated from human milk as described (14), and recombinant hSC (hSCrec) was produced in a CHO cell clone (7). Complete deglycosylation of hSCrec (hSCdeg) was accomplished using N-Glycosidase F (EC 3.5.1.52; Roche Applied Science) as described previously (7). Polymeric Ig receptor-containing Caco-2 whole cell extracts were prepared as described in Ref. 15. C. difficile toxin A was purchased from Calbiochem. Hybridoma PCG4 (anti-toxin A IgG mAb) (16), chimeric mouse-human polymeric IgA PCG4 (IgA PCG4) produced in CHO cells as described in Ref. 17, hybridoma 7D9 (anti-VP6 rotavirus IgA mAb) (18), and hybridoma 2A10 (anti-VP4 RRV toxin A IgG mAb) (16), chimeric mouse-human polymeric IgA PCG4 (IgA PCG4) produced in CHO cells as described in Ref. 17, hybridoma 7D9 (anti-VP6 rotavirus IgA mAb) (18), and hybridoma 2A10 (anti-VP4 RRV rotavirus IgA mAb) (19) were grown in a Celline-350 cartridge (Vitaris, Baar, Switzerland) in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 2 mM sodium pyruvate, 10 mM HEPES (pH 7.0), 0.1 mM folate acid, and 100 units/ml penicillin and 100 μg/ml streptomycin. The IgG antibody was purified by affinity chromatography on protein G-Sepharose beads (Amersham Biosciences), whereas purified plgA was prepared as described (20).

Fusion protein was generated by fusing the coding sequence for intimin from EPEC strain E2348/69 to glutathione S-transferase (GST). Following isolation byalkali lysis, bacterial DNA was used for PCR amplification. Primers were designed to hybridize to the bacteria pathogenicity island (GenBankTM number AF0022236) between nucleotides 26,610 and 26,635 (eaeA codons 588–596) and 27,845 and 27,873 (between eaeA and EscD). The PCR product was integrated into the plasmid vector pDEST™15 (Invitrogen) using the gateway cloning technology (Invitrogen). Protein expression in the form of an N-terminal GST fusion was carried out in E. coli BL21-SI using 300 mM NaCl to induce gene expression. Recombinant protein was purified on Glutathione-Sepharose 4B beads according to the manufacturer’s instructions (Amersham Biosciences).

**Radiolabeling of C. difficile Toxin A**—Toxin A was labeled by alkylolation-reduction based on the protocol of Wallace and Corthésy (21). Briefly, 50 μg of toxin A was dissolved in 200 μl of 0.2 mM sodium borate buffer (pH 9.0) containing 10 μg of NaBH₄[¹⁴H] (specific activity 1,000 μCi/mmole; PerkinElmer Life Sciences). Five additions of aqueous formaldehyde (Fluka, Wohlen, Switzerland) were made over a period of 10 min. The reaction was stopped by the addition of an equal volume of 0.1 M HCl. Excess of reagent was removed by passage on a PD-10 desalting prepacked column (Amersham Biosciences) equilibrated and run in phosphate-buffered saline (PBS).

**Differential Deglycosylation of hSCrec**—Treatment of hSCrec with carbohydrate-specific enzymes, including Clostridium perfringens neuraminidase (EC 3.2.1.18; Roche Applied Science), β-galactosidase (EC 3.2.1.23; Roche Applied Science), and α-1-(2,3,4)-fucosidase (EC 3.2.1.51; Sigma) was performed according to the manufacturer’s instructions with the exception that the denaturation step was omitted, and exposure of hSCrec to the different enzymes was for 4 h.

**Lectin Blotting**—200 ng of purified hSCcol, hSCrec, and hSCdeg were subjected to PAGE on 8% resolving gels under denaturing (0.1% SDS) and reducing (0.1M dithiothreitol (DTT)) conditions. Analysis of carbohydrate structures on hSCcol, hSCrec, and hSCdeg was done using the DIG glycans differentiation kit from Roche Applied Science, which comprises lectins Datura stramonium agglutinin, Galanthus nivalis agglutinin, Maackia amurensis agglutinin, Sambucus nigra agglutinin, and peanut agglutinin. Reactivity toward biotinylated Ulex europaeus agglutinin-1 (Vector Laboratories, Burlingame, CA) was assayed according to the manufacturer’s instructions. After transfer onto polyvinylidene difluoride membranes (Millipore), glycan detection was performed using the procedures and reagents provided in the kit.

**Exposure of Polarized Monolayers to Toxin A and SC**—The sensitivity of Caco-2 monolayers to C. difficile toxin A was compared with that of T84 monolayers (17) by incubating Transwell filters with 3 × 10⁻¹⁰ M toxin A for 3 h and measuring TER (13). In order to assess SC-mediated protection as a function of the amounts and presence of glycans, 3 × 10⁻¹⁰ M toxin A was preincubated with a range (1 mM to 1 μM) of hSCcol, hSCrec, or hSCdeg for 30 min at room temperature in a final volume of 50 μl. Following apical administration in a final volume of 0.8 ml, TER were measured at 3 h using three independent replicate filters per experiment. To evaluate binding inhibition, radiolabeled toxin A was preincubated with various amounts of hSCcol, hSCrec, or hSCdeg and added to the apical cell compartment, and filters were recovered after 3 h and finally placed in vials for scintillation counting (model 2500TR; Packard Instrument Co.). Controls were performed using protective plgA PCG4 specific for toxin A (17). The shielding effect of hSC preparations was examined upon the addition of increasing concentrations of the proteins to Caco-2 cell monolayers for 1 h, three washes with PBS, and subsequent incubation with 3 × 10⁻¹⁰ M toxin A.

To evaluate the binding potential of hSC on polarized Caco-2 cells, 1 × 10⁻⁸ M hSCrec and hSCdeg was incubated for 60 min at 37°C on the cells in a final volume of 0.5 ml. Supernatants and sequential washes were recovered, and cells were collected in lysis buffer (15 mM KCl, 2 mM MgCl₂, 10 mM HEPES (pH 7.6), 0.1 mM EDTA, 0.2% Nonidet P-40, and 5% protease inhibitors Complete™ (Roche Applied Science)). The concentration of hSC was then determined by enzyme-linked immunosorbent assay (ELISA) as previously described (22), and the integrity of the protein was verified by Western blot analysis.

**SC Immunoblotting and Overlay Assay**—Native or glycosidase-treated hSC (200 ng) as well as plgR (70 ng) in Caco-2 cell extracts were subjected to PAGE on 8% resolving gels under denaturing (0.1% SDS) and nonreducing conditions, these latter allowing the recovery of both proteins with preserved plgA binding properties after transfer onto polyvinylidene difluoride membranes (15). Nonspecific binding sites were saturated by incubation in blocking buffer (5% (w/v) nonfat dry milk in PBS-T containing 0.05% Tween 20 (Bio-Rad); PBS-T), and the membrane was probed for 1 h at room temperature with the following reagents: toxin A at 500 ng/ml in PBS-T and GST-intimin at 200 ng/ml in PBS-T. Membranes were washed four times for 5 min in PBS-T. Toxin A was detected using PCG4 mAb (1:2,000 dilution in 0.5% nonfat dry milk in PBS-T) (17) followed by goat anti-mouse IgG, horseradish peroxidase-conjugated (1:5,000; Sigma). GST-intimin was detected using mAb against GST (1:1,000 dilution in 0.5% nonfat dry milk in PBS-T; Zymed Laboratories Inc., South San Francisco, CA), followed by goat anti-mouse IgG, horseradish peroxidase-conjugated (1:500; Sigma). Membranes were treated with the chemiluminescence reagent from Amersham Biosciences and exposed to autoradiography films (Konica, Hohenbrunn, Germany).

**Microwell Binding Assay**—The wells of Nunc MaxiSorp ELISA plates were coated with 200 ng of hSCrec or hSCdeg in 100 μl of 50 mM sodium bicarbonate (pH 9.6) for 1 h at room temperature. Wells were blocked with 200 μl of PBS-T containing 5% (w/v) nonfat dry milk. Toxin A (500 ng/ml) or GST-intimin (200 ng/ml) in 100 μl of PBS were incubated for 2 h at room temperature. Following three washes with PBS-T, binding was detected using specific antibodies as for the overlay assay above and 1,2-phenylenediamine as a chromogene. Reactions were stopped with one volume of 2 M H₂SO₄, and the optical density was measured at 492
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FIGURE 1. Mapping of sugar moieties present in various preparations of hSC. Samples were separated by SDS-PAGE under reducing conditions, blotted onto membranes, and incubated with anti-hSC serum (lanes 1 and 2) or lectins D. stramonium agglutinin (DSA), G. nigripes agglutinin (GNA), M. amurensis agglutinin (MAA), S. nigra agglutinin (SNA), and U. europeaus agglutinin-1 (UEA-1). Lanes 3, 6, 9, 12, and 15, hSCcol; lanes 4, 7, 10, 13, and 16, hSCrec; lanes 5, 8, 11, 14, and 17, hSCdg.

nm using 620 nm as reference wavelength. For reduction of coated proteins, DTT at the indicated final concentrations (Figs. 4 and 5) was added for 15 min prior to the blocking step.

Bacterial Adhesion Assay—Confluent HEp-2 cells grown as mono-layers in 6-well culture dishes were exposed overnight to 1 × 10^6 wild type or the intimin deletion mutant EPEC (CVD206 (23); intE EPEC) in the presence of 6 μM hSCrec or without hSCrec in controls. Following four washes with PBS, HEp-2 cells were lysed in distilled water, and associated bacteria were plated onto LB-agar plates at serial dilutions for colony counting.

Attachment/Effacement (A/E) Assay—HEp-2 cells (10^5 cells/ml) were seeded into 4-well microscope slides (Chamber SlideTM, LabTek, Nalge Nunc International) and grown for 16 h. EPEC strain E2348/69 was cultivated overnight in brain-heart infusion medium and washed in PBS, and 1 × 10^5 colony-forming units were added to 0.5 ml of Dulbecco’s modified Eagle’s medium buffered with 10 mM HEPES (pH 7.0). EPEC were preincubated for 30 min with hSC or hSCdg (1.2–12 μM) at 37 °C, 5% CO2, 95% relative humidity prior to the addition to HEp-2 cells. HEp-2 cells were further incubated for 3 h, washed three times with PBS, and fixed with 4% paraformaldehyde (Sigma) in PBS for 1 h at 4 °C. Fixed cells were washed with PBS and permeabilized for 5 min with 0.1% Triton X-100 (Bio-Rad) in PBS, and actin was stained with phalloidin coupled with tetramethyl rhodamine isothiocyanate (Sigma) to quantify A/E lesions (24) by microscopic observation. Cells harboring at least one colony with established A/E were counted as a positive A/E event. Microscopic observations were done with a Zeiss Axiovert 100 equipped with a Zeiss ×63 LD Achromplan objective (Zeiss, Jena, Germany). Data are expressed as a percentage of untreated control for ease of comparison.

Virus Propagation and Purification—The bovine RF strain, the simian SA11 strain, and the rhesus RRV strain were propagated in MA104 cells in the presence of trypsin as described previously (25). Virus titers present in the lysed cell supernatant were determined by plaque assay and expressed as plaque-forming units/ml. For purification, the viral suspensions were frozen once at ~20 °C and then submitted to centrifugation at 180,000 × g for 1 h at 4 °C. The virus pellets were resuspended in 20 mM PIPES buffer (pH 6.6), and the remaining lipids were extracted with 1,1,2-trichloro-1,2,2-trifluoroethane (Freon 113). The viral solution was complemented with CsCl to achieve a homogeneous density of 1.369 g/ml and centrifuged at 140,000 × g for 15 h at 4 °C. The band located at 1.36 mg/ml corresponding to triple layer particles (TLP) was recovered from the CsCl gradient and conserved at 4 °C until further use. For biochemical analyses, purified rotavirus solutions were desalted immediately before use by gel filtration on Sephadex G-25 coarse beads (Amershams Biosciences), and the protein content was measured using the bichinonic acid protein assay (Uptima; Interchim, Montluçon, France).

ELISA and Overlay Assay for SC-Virus Interaction—For ELISA, 96-well plates (Nunc Maxisorp) were coated overnight at 4 °C with 200 ng of purified TLP rotavirus strains (RF, SA11, and RRV) in a final volume of 200 μl of 0.1 M carbonate-bicarbonate buffer (pH 8.3). Wells were blocked with 250 μl of PBS-T containing 2% bovine serum albumin for 30 min at room temperature. Diverse dilutions of hSCrec or hSCdg (60–600 nM) were incubated for 1 h at room temperature in 100 μl of PBS-T. The VP6-specific IgA 7D9 was used as a positive control. After extensive washing in PBS-T, bound ligands were detected with antibodies to hSC (22) or mouse pIgA (20).

In the overlay assay, 50 ng of purified TLP rotavirus strains (RF, SA11, and RRV) were blotted onto polyvinylidine difluoride membranes and blocked overnight at 4 °C in PBS-T containing 2% bovine serum albumin. The membranes were then incubated for 4 h at room temperature with various concentrations (0.1–1 μM) of hSCrec or hSCdg or pIgA 7D9 (2 nM). After extensive washing in PBS-T, bound ligands were detected as in ELISA, and the presence of adsorbed rotaviruses was confirmed using a sheep anti-rotavirus serum (a gift from Dr. I. Schwartz-Cornil, Institut National de la Recherche Agronomique, France).

Virus Infectivity Assay—The virus infectivity assay was adapted from the cytopathic effect assay described by Marchetti et al. (26). Briefly, MA104 cells were grown in 96-well tissue culture microplates for 2 days, washed twice with PBS, and then incubated for 3 h with Earle’s MEM supplemented with 20 mM HEPES (pH 7.0), 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin, 0.5 μg/ml trypsin, and 60 nM hSCrec or 60 nM hSCdg (hSC-EMEM). Cells were then infected with serial dilutions of the viral suspension for 1 h at 37 °C in hSC-EMEM. After removal of the viral inoculum, cells were incubated for 48 h in hSC-EMEM, and the cytopathic effect induced by rotavirus was measured by neutral red dye uptake. The measurement of infection was defined as the ratio of experimental optical density (OD) to noninfected OD.

RESULTS

In order to benefit from a continuous source of pure SC capable of replacing hSCcol that can be obtained in limited amounts only, we produced hSCrec in CHO cells. We have previously shown at the biochemical level that hSCrec binds equally well to hSC as hSCcol (6). When used in in vitro and in vivo models of infection, hSCrec proved a valid tool to unravel several novel facets of the protein associated with pIgA or as a free polypeptide (7, 27, 28). However, a comparison of the respective glycosylation profiles was not performed. Since the aim of the work reported herein is to evaluate the intrinsic protective properties of SC and explore whether glycosylation plays a role in the process, we first mapped the lectin reactivity of hSC-col and hSCrec. Using a battery of lectins, we found that all major glycans that have previously been reported to be present on native hSC (9) could be identically detected on hSC-col and hSCdg. When used in ELISA and overlay assays for SC-virus interaction, SC was able to inhibit rotavirus binding to MA104 cells. The inhibition was concentration-dependent and maximal at 60–600 nM with hSCrec or hSCdg (data not shown).
To investigate new properties of hSC and further compare both sources of protein, *in vitro* models were used. As a mimic of the intestinal epithelium, Caco-2 cells were grown on Transwell filters to form monolayers whose TER indicates both integrity and polarization. Upon incubation of cells with 0.3 nM *C. difficile* toxin A, a drastic and irreversible drop of TER values was observed within the first 3 h of challenge, indicating disruption of the tight Caco-2 cell monolayer. This cytotoxic effect could be neutralized in a dose-dependent manner upon preincubation with hSCcol, hSCrec, or hSCdg and incubated as in A. Transwell membranes were recovered, and associated radioactivity was measured by scintillation counting. C, preserved TER using equimolar amounts of free hSCrec (lane 1) or associated with specific (pIgA PCG4) or nonspecific (pIgA C5) pIgA (lanes 2 and 3). Controls include hSCdg associated with specific pIgA PCG4 and irrelevant pIgA C5 (lanes 4 and 5).

FIGURE 2. Blocking of toxin A-mediated cytotoxic effect is dependent on the presence of glycans on hSC. A, constant amounts of toxin A (0.3 nM) were mixed with hSCcol, hSCrec, or hSCdg and incubated for 3 h in the apical compartment of polarized Caco-2 cell monolayers in Transwell chambers. The shaded area represents the limit of confidence of the TER values of individual control filters incubated in the absence of toxin A. Toxin A-specific chimeric pIgA PCG4 served as an experimental positive control. B, increasing concentrations of hSCcol, hSCrec, or hSCdg were mixed with radiolabeled toxin A and incubated as in A. Transwell membranes were recovered, and associated radioactivity was measured by scintillation counting. C, Western blot analysis of the integrity of hSCrec and hSCdg after 1-h exposure on epithelial cells at 37 °C.

To exclude that the protection observed was due to the possible ability of hSC to cover the Caco-2 cell monolayer and hence mask toxin A receptor(s), monolayers were pretreated with increasing concentrations of hSCrec or hSCdg, washed, and then incubated with radiolabeled toxin A. No change in terms of toxin A binding to epithelial cells was observed (Fig. 3A), even at the highest concentration of hSCrec that conferred protection upon preincubation with toxin A. To directly assess the capacity of hSC to bind to epithelial cells, monolayers were incubated with 10 nM hSCrec or hSCdg. No hSC was bound to the cell surface or taken up by the cells. Accordingly, all hSC remained free in culture medium, as measured by ELISA in serial dilutions of supernatants, washes, and cell lysates. BD, below the level of detection. Western blot analysis of the integrity of hSCrec and hSCdg after 1-h exposure on epithelial cells at 37 °C.

FIGURE 3. Assessment of the shielding effect of SC on polarized epithelial cells. A, preincubation of Caco-2 cell monolayers with hSCrec and hSCdg preparations does not prevent subsequent binding of toxin A to cells. B, hSCrec and hSCdg do not bind to polarized Caco-2 cell monolayers. The amount of hSC was quantified by ELISA in supernatants, washes, and cell lysates. BD, below the level of detection. C, Western blot analysis of the integrity of hSCrec and hSCdg after 1-h exposure on epithelial cells at 37 °C.

Data presented so far show that (i) the effects of toxin A on epithelial cells are neutralized by SC, (ii) SC glycosylation status is a key factor, and (iii) protection is not based on a shielding of the cell surface by SC. Therefore, the capacity of SC to neutralize the toxin following direct interaction was investigated by different biochemical approaches. Using an ELISA-based approach, the interaction between unreduced hSCrec
not affect the interaction between hSCrec and the toxin A, indicating that fucose residues are not involved in the association. Control lanes showed that toxin A bound to hSCrec and also to plgR (Fig. 4C).

Since the neutralizing properties of SC were demonstrated for C. difficile toxin A, we investigated whether SC can also act as a microbial scavenger on other bacterial components, such as adhesins and other virulence factors. Because EPEC adhesion molecule intimin possesses Ig-like domains and a terminal C-type lectin-like domain (29, 30), we postulated that (i) such domains might associate with similar domains (31–33) and/or glycans displayed by hSC and (ii) such an interaction would alter the intimin-dependent EPEC adhesion as reflected by the formation of A/E lesions (34). In preliminary in vitro binding experiments, we observed that in the presence of hSCrec, the adhesion of wild type EPEC to target HEp-2 cells was reduced to levels comparable with those found with the intC/EPEC mutant (Fig. 5A). To strengthen the biological significance of the binding experiments, the A/E lesion assay reflecting intimin-dependent EPEC invasion was performed (35). In this assay, hSCrec reduced the occurrence of A/E lesions triggered upon challenge of HEp-2 cells with EPEC (Fig. 5B, arrowheads). Comparative observation of slides carrying control and infected HEp-2 cells showed a drop in the percentage of damaged cells in the presence of increasing concentrations of hSCrec (Fig. 5C). hSCdg did not confer equivalent protection against intimin-mediated A/E lesion formation, thus suggesting that the glycan residues are instrumental features for protection. Reduced EPEC binding and A/E lesion formation were not due to impaired EPEC viability, since growth of the bacteria was not altered in the presence of hSC (data not shown). We then analyzed biochemically whether this protection was indeed mediated by binding of hSCrec to intimin, as initially postulated. In ELISA, we found that hSCrec interacts with intimin-GST, a capacity progressively lost as a function of the concentration of DTT known to disrupt Ig-like domain folding (Fig. 5D). As for toxin A, the glycan moieties important for the interaction were identified with differentially deglycosylated hSCrec subjected to an overlay assay with intimin-GST (Fig. 5E); galactose and sialic acid residues were involved in the interaction, whereas fucose was not. These data suggest that SC can serve as a microbial scavenger helping to protect the surface of epithelial cells from bacterial attack in a carbohydrate-dependent fashion.

To further challenge the scavenger properties of SC, the viral gastrointestinal pathogen rotavirus was examined. Three different strains of rotaviruses were analyzed, namely the bovine RF strain, the simian SA11 strain, and the rhesus RRV strain. Four different approaches were followed to test the possible inhibitory properties of hSC on rotavirus infectivity. ELISA and overlay assay were performed as described under “Materials and Methods” using highly purified TLP rotaviruses to guarantee specific interactions between partners (Fig. 6, A and B). Both experiments demonstrate that none of the rotaviruses tested interact with hSCrec or hSCdg. To ensure that disruption of the three-dimensional conformation of the virus during coating and blotting did not affect binding artifically, two additional assays were performed in solution. Electron microscopy analyses using a mixture of purified TLP and hSCrec present no evidence of interaction with the outermost layer of the virus (data not shown). Additionally, no protection was observed when 60 nM (104-fold molar excess with respect to virus) hSCrec or hSCdg was added before, during, and after infection of MA104 cells with serial dilutions of the three rotavirus strains (Fig. 6C). Thus, our results show that hSC is unable to protect MA104 cells by either (i) shielding their cell surface, (ii) neutralizing the virus in solution before its attachment, or (iii) inhibiting infectivity following virus attachment.

DISCUSSION

The protection of mucosal surfaces is achieved through a combination of innate and adaptive immunity. To draw a distinct border
between the two is becoming more and more illusive, since the multiple partners involved increasingly show overlapping properties (36). SC, a protein with defined roles in the transport, protection, and anchoring of IgA and IgM at mucosal surfaces (37), is shown here to have strong bacterial scavenger properties as a free polypeptide. Previous data have shown that SC from human colostrum can limit the binding of toxin A to hamster brush border membranes and bind to the toxin in an overlay assay (11). Human milk-derived lactoferrin and SC reduce heamagglu-

**Figure 5.** The neutralizing property of hSC toward EPEC is mediated by direct interaction with the adhesion molecule intimin. A, binding experiments performed with HEP-2 cells incubated with wild type (wt) EPEC and the intimin deletion mutant (Int- EPEC). After overnight infection with 1 × 10^7 EPEC in the presence or absence of 6 μM hSCrec, cell-bound bacteria were plated for numbering. B, imaging by fluorescence microscopy of HEP-2 cells obtained after infection with 1 × 10^7 EPEC mixed with hSCrec or hSCdg for 3 h. The arrowheads indicate A/E lesions typical of infection. C, quantification in 50 different fields of the effect of hSCrec and hSCdg on A/E lesion occurrence. Data are expressed as a percentage of the value obtained without hSC (100%). D, hSCrec or hSCdg coated in wells of microtiter plates was treated with increasing concentrations of DTT prior to the addition of intimin-GST. SC-intimin-GST interaction was detected with anti-GST antiserum. Heat-treated hSCrec or hSCdg was coated in ELISA plates, and binding of intimin was tested as before. E, hSCrec and plgR preparations were separated by SDS-PAGE under nonreducing conditions, blotted onto membranes, and incubated with E. coli intimin-GST, followed by detection with anti-GST. NGF, N-glycosidase F; Gal, β-galactosidase; Fuc, α-1-(2,3,4)-fucosidase; Neu, C. perfringens neuraminidase.

**Figure 6.** SC does not interact with rotavirus or protect MA104 cells from infection. A and B, purified TLPs from RF, SA11, and RRV rotaviruses were coated in wells of microtiter plates (A) or blotted as dots onto membranes (B) and incubated with hSCrec, hSCdg, and the specific anti-rotavirus plgA 7D9 as positive control. Bound molecules were detected as described under “Materials and Methods.” C, MA104 cells were exposed to various amounts (plaque-forming units (p.f.u.)) of RF, SA11, or RRV rotaviruses in the presence of 60 nM hSCrec, hSCdg, or PBS. Infection of MA104 is expressed as the ratio between experimental OD and noninfected OD; data are presented for three nonsaturating viral loads. As a positive control, specific neutralization by anti-RRV plgA 2A10 is depicted on the right.
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tination of human erythrocytes by enterotoxigenic E. coli (38). This study suffered from the limitation that human SC and lactoferrin isolated from colostral milk might have cross-contaminated each other and that no cytotoxic effect was studied in appropriate target epithelial cells. Further, the biological effect of the interaction was not evaluated at the biochemical level and in cellular models of infection. The current work addresses the "innate-like" properties of SC toward three different gastrointestinal pathogens and underscores the essential role of glycans residues in conferring intrinsic protective function to the protein.

We show herein that both hSCrec and hSCcol appear equivalent in terms of glycosylation profile and biological effect, as concluded from lectin binding and protection of polarized epithelial cells from the action of C. difficile toxin A. Dallas and Rolfe (11) showed that preincubation of hamster brush border with colostral hSC did not subsequently inhibit the binding of the toxin A. This is in agreement with our findings that protection of in vitro polarized Caco-2 cells is not mediated by shielding of cells from toxin A binding but rather results from trapping of toxin A by hSC. Hence, the neutralizing action of SC toward toxin A exerts itself through the direct contact between the two proteins that subsequently prevents toxin A from recognizing its receptor on epithelial cells. In the physiological context, however, we cannot exclude the possibility that the secretion of free SC and SC-bound to its pIgA cargo might endow the epithelium with a sort of SC "shield."

Molecular mapping of the association established that it is mediated through glycans and more specifically through terminal galactose and sialic acid residues. Disruption of the three-dimensional conformation of hSC upon reduction of disulfide bridges abolished the interaction with toxin A, indicating that the carbohydrate residues need to be arranged correctly in space in order to create specific docking sites for toxin A. Therefore, glycans confer to free SC strong microbial scavenger properties, which add to the specific action of IgA in the context of SIgA. Glycans are exposed when SC is associated with pIgA, thus explaining the finding that SIgA with no antigenic specificity for toxin A protects epithelial monolayers from the action of toxin A.

In addition to blocking the action of toxin A, we found that SC exhibits scavenger properties in the context of EPEC intimin. The initial interaction between EPEC and target HEp-2 cells involves, among other adhesins, intimin. Eventually, interaction of intimin with the bacterially produced translocated intimin receptor causes the characteristic intimate EPEC adhesion that results in A/E lesion formation (39). Clearly, produced translocated intimin receptor causes the characteristic intimin properties, which add to the specific action of IgA in the context of SIgA. Glycans of the endogenous microbiota (58). As far as the whole SIgA molecule is concerned, this confers further antigen binding sites in addition to the endogenous microbiota (58). Among the three strains of rotaviruses we tested, two are neuraminidase-dependent (SA11 and RRV), yet none of them bound to SC known to have many accessible sialic acid residues at its surface (10). Similarly, galactose is implicated in the entry process of rotavirus (52), yet its distribution on hSC was without effect on infectivity. Although present on hSC, the identity and spatial arrangement of glycans appear not to be optimal enough to permit interaction with rotavirus; this is consistent with the observation that specific rotavirus-glycan interaction is indeed highly constrained (53). Other milk proteins, including lactoferrin and lactadherin, appear to protect epithelial cells from rotavirus infection irrespective of the presence of carbohydrate residues (54–56). We conclude that neither glycan structures present on SC nor the protein backbone can serve as a decoy to abrogate binding to rotavirus particles. This adds to the specificity of data collected with toxin A, intimin, and numerous other glycans structures as reviewed in Ref. 57.

In summary, this study demonstrates that the abundant and various glycans carried by SC contribute to the protein antimicrobial scavenger properties, although this does not represent an universal mode of action. In the context of commensal homeostasis, SC can add to the function of low affinity IgA, protecting the host from the penetration of the endogenous microbiota (58). As far as the whole SIgA molecule is concerned, this confers further antigen binding sites in addition to the four Fab sites, making the antibody an active entity bridging adaptive and innate immunity.

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