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Human Plasma-derived Polymeric IgA and IgM Antibodies Associate with Secretory Component to Yield Biologically Active Secretory-like Antibodies*

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Background: Production of SIgA or SIgM for therapeutic application remains an unsolved issue.
Results: Human plasma-derived polyclonal, polymeric IgA and IgM associate with recombinant or colostrum-derived human secretory component to form digestion-resistant, functionally active SIgA- and SIgM-like molecules.
Conclusion: SIgA and SIgM can be rebuilt *ex vivo* from plasma-derived IgA/IgM.
Significance: This would enable development of SIgA/SIgM-based mucosal therapeutics.

Immunotherapy with monoclonal and polyclonal immunoglobulin is successfully applied to improve many clinical conditions, including infection, autoimmune diseases, or immunodeficiency. Most immunoglobulin products, recombinant or plasma-derived, are based on IgG antibodies, whereas to date, the use of IgA for therapeutic application has remained anecdotal. In particular, purification or production of large quantities of secretory IgA (SIgA) for potential mucosal application has not been achieved. In this work, we sought to investigate whether polymeric IgA (pIgA) recovered from human plasma is able to associate with secretory component (SC) to generate SIgA-like molecules. We found that ~15% of plasma pIgA carried J chain and displayed selective SC binding capacity either in a mixture with monomeric IgA (mIgA) or after purification. The recombinant SC associated covalently in a 1:1 stoichiometry with pIgA and with similar efficacy as colostrum-derived SC. In comparison with pIgA, the association with SC delayed degradation of SIgA by intestinal proteases. Similar results were obtained with plasma-derived IgM. *In vitro*, plasma-derived IgA and SIgA neutralized *Shigella flexneri* used as a model pathogen, resulting in a delay of bacteria-induced damage targeted to polarized Caco-2 cell monolayers. The sum of these novel data demonstrates that association of plasma-derived IgA or IgM with recombinant/colostrum-derived SC is feasible and yields SIgA- and SIgM-like molecules with similar biochemical and functional characteristics as mucosa-derived immunoglobulins.

Mucosal surfaces of the digestive, respiratory, and urogenital tracts, as well as the ducts of exocrine glands are lined by layers of epithelial cells that form a tight barrier separating the internal compartments of the body from the outside environment. In humans, these vast surfaces cover 400 m^2, an area that is permanently exposed to exogenous pathogens (1). The combination of innate and inducible cellular and molecular mechanisms ensures protection against colonization and entry/invasion by microbes (2). In healthy individuals, secretory IgA (SIgA)[3] is the most abundant antibody (Ab) fulfilling the function of immune exclusion on the luminal side of mucosal surfaces (3), whereas secretory IgM (SIgM) Abs take over in IgA-deficient patients. To exert their specific protective function in mucosal secretions, polymeric IgA (pIgA) and IgM produced at effector sites are transported across the epithelium by the polymeric immunoglobulin receptor (pIgR) expressed on the basolateral side of epithelial cells (4). During transport, the pIgR is proteolytically cleaved, and the extracellular portion of the molecule, referred to as the secretory component (SC), is released in association with pIgA and IgM to form SIgA and SIgM (5, 6). Both secretory Abs are thus essential to maintain epithelial integrity.

As an alternative to vaccination, protective levels of Abs might directly be delivered to mucosal surfaces by passive immunization. In nature, this occurs physiologically in many mammalian species by transfer of maternal Abs to their offspring via milk (7, 8). Human and animal studies dealing with passive mucosal immunization have demonstrated that pIgA and SIgA Ab molecules administered by oral, intranasal, intrauterine, or lung instillation can prevent, diminish, or cure bacterial and viral infections (9). However, the secretory form naturally found at mucosal surfaces was rarely used, and purification/production of SIgA still remains a challenging task (10). In this study, we sought to determine whether polyclonal plasma-derived pIgA and IgM Abs can be transformed into secretory-like Abs *in vitro*. We found that IgA- and IgM-en-

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[3] The abbreviations used are: SIgA, secretory IgA; pIgA, polymeric IgA; SC, secretory component; mIgA, monomeric IgA; Ab, antibody; SIgM, secretory IgM; pIgR, polymeric immunoglobulin receptor; hSC, human SC; hSCrec, recombinant hSC; hSCcol, colostrum-derived hSC; mSC, mouse SC; TER, transepithelial electrical resistance; LB, Luria-Bertani; LSCM, laser-scanning confocal microscopy.

riched plasma preparations, or purified plasma pIgA and IgM, are able to associate with recombinant or colostrum-derived human SC (hSC). As in nature, disulfide bridges between hSC and the Abs formed with a 1:1 stoichiometry. Association with hSC delayed degradation of either pIgA or IgM upon exposure to intestinal washes rich in proteases. Biological activity of plasma-derived molecules was demonstrated in neutralization assays based on the protection of polarized Caco-2 epithelial cells used as a mimic of the intestinal epithelium against invasion by *Shigella flexneri*. Together, the data demonstrate that both reconstituted SIgA and SIgM display many biochemical features of secretory Abs and open the path to explore their protective function in *in vivo* models of infection.

EXPERIMENTAL PROCEDURES

Preparation of Human Plasma IgA- and IgM-enriched Fractions—IgA and IgM were purified from process intermediates of immunoglobulins manufactured from human plasma (11) by affinity chromatography using CaptureSelect Human IgA and CaptureSelect Human IgM resins (Bioaffinity Company BAC). Three different starting materials were used: 1) cryo-poor human plasma (termed "plasma"); 2) immunoglobulin-enriched cold ethanol precipitate (termed "paste"), a process intermediate obtained during large scale ethanol fractionation of human plasma proteins; 3) a chromatography side fraction (termed "column strip"), consisting of the strip fraction from an ion-exchange chromatography column used in the large scale manufacture of IgG from human plasma. The different starting materials were diluted in PBS to a target protein (IgA or IgM) concentration of ~1 mg/ml and then loaded onto a CaptureSelect Human IgA or IgM column pre-equilibrated with PBS, without exceeding the IgA- or IgM-binding capacity of the column. After loading, the column was washed with PBS, and IgA or IgM was eluted with glycine buffer at pH 3.0. The eluate was adjusted with 0.5 M Tris-base (pH 8.0) to pH 4.5 and concentrated up to 16 mg/ml protein.

Production/Purification of Recombinant Proteins and Colostral Human SC—Recombinant hSC (hSCrec) was produced from a CHO clone stably transfected with an expression cassette coding for the protein (12). Colostrum-derived hSC (hSCcol) was obtained as described (13). Mouse IgAC5 specific for *S. flexneri* LPS serotype 5a and recombinant mouse SC (mSC) were produced and purified as described (12, 14).

Western blot analysis—SDS-PAGE and transfer onto PVDF membranes was carried out as described (15). The membranes were then blocked for 30 min in PBS-0.05% Tween 20 solution (PBS-T) containing 1% BSA. Detection of the polypeptides in IgA- and IgM-enriched or purified IgA and IgM preparations was carried out with: 1) rabbit IgG anti-human alpha chain, HRP-conjugated (Dako, 1/5,000 dilution); 2) rabbit IgG anti-human mu chain, HRP-conjugated (Dako, 1:5,000 dilution); 3) goat anti-human kappa chain (Cappel, 1/3,000 dilution), followed by secondary anti-goat HRP-conjugated antiserum (Pierce, 1/5,000 dilution); 4) rabbit anti-J chain antiserum (1/3,000 dilution) (16), followed by secondary anti-rabbit HRP-conjugated antiserum (Sigma, 1/3,000 dilution). In reconstituted SIgA or SIgM, the presence of hSC was assessed using rabbit anti-hSC antiserum (1/3,000 dilution) (17), followed by secondary anti-rabbit HRP-conjugated antiserum (Sigma, 1/10,000 dilution). In reconstituted SIgAC5, the presence of mouse SC (mSC) was assessed using rabbit anti-mSC antiserum (1/3,000 dilution) (14), followed by secondary anti-rabbit HRP-conjugated antiserum (Sigma, 1/5,000). All incubations were performed in PBS-T containing 0.1% BSA at ambient temperature for 1–2 h. After final washing with PBS-T, immune complexes on membranes were detected by chemiluminescence and exposure on autoradiographic films.

Dot Blot Reassociation Assay—Dot blot reassociation assays were essentially carried out as described (17) with the following modifications: blotting membranes consisted of PVDF; blocking solution was PBS-T containing 1% BSA; IgA- and IgM-enriched preparations were used for overlay incubation in 200 μl of PBS-T containing 0.1% BSA; and detection Abs were directly coupled to HRP.

Separation of Plasma-derived pIgA and mIgA and Purification of Plasma-derived IgM—IgA-enriched preparations containing a mixture of mIgA and pIgA were diluted in PBS to a final volume of 10 ml suitable for injection onto the ÄKTA*prime* chromatography system (GE Healthcare). The flow rate was set at 1 ml/min with PBS as the mobile phase for all runs. To resolve the two molecular forms of IgA from other plasma components, the material was initially applied onto a 1-meter-long column filled with Superdex 200 resin (GE Healthcare). Separation of the two molecular forms of IgA was also performed on two serially coupled 1-meter-long columns filled with Sephacryl S-300 HR beads (18). The IgA content of 3.5-ml fractions was verified by immunodetection, and pools of mIgA and pIgA were obtained. IgM-enriched preparations run under identical conditions yielded a single fully excluded peak. Concentration was performed using the Labscale system (Millipore) connected to a 100-kDa cut-off cartridge and stored at 4 °C until further use.

In Vitro Association of Polymeric Ig and hSC—SIgA molecules were obtained by combining *in vitro* 10 μg of purified pIgA molecules with 2.5 μg of either hSCrec or hSCcol. SIgM molecules were obtained by combining *in vitro* 10 μg of purified IgM molecules with 1.5 μg of either hSCrec or hSCcol. SIgAC5 molecules were obtained by combining *in vitro* 10 μg of purified pIgAC5 molecules with 2 μg of mSC. Association was performed in PBS for 30 min at ambient temperature as described previously (19). Integrity and correct assembly of the molecules into possible covalent complexes was examined by SDS-PAGE under non-reducing and reducing conditions, followed by Western blotting and immunodetection with antiserum specific for SC as indicated above.

FPLC Sizing Column Chromatography—To further assess the assembly of purified pIgA with hSCrec or hSCcol, complexes with a 1:1 and 1:2 stoichiometry were prepared in a final volume of 100 μl (total amount of protein: 10 μg) and passed over a 1 × 30-cm Superose 12 HR prepacked column (GE Healthcare) hooked to the ÄKTA*prime* chromatography system at a constant flow rate of 0.2 ml/min. Co-elution of bound hSC with pIgA, reflecting covalent association, was verified by immunodetection specific for hSC, and quantification of IgA and hSC in pooled fractions was carried out by ELISA (20).

Digestion of Abs with Mouse Intestinal Washes—Collection of intestinal washes form BALB/c mice (4–6 weeks old) was done according to the published procedure (19). For *in vitro* digestion, 125 ng of purified pIgA and reconstituted SIgA, or purified IgM and reconstituted SIgM, were mixed or not with 1 or 2 μl of intestinal washes in a final volume of 20 μl of PBS and incubated at 37 °C for various periods of time. Reactions were stopped by the addition of 2 μl of Complete^{TM} protease inhibitor mixture (Roche Applied Science) and kept frozen prior to analysis by immunoblot detecting the reduced form of the heavy chain of the antibody.

Caco-2 Cell Culture and Growth as a Polarized Monolayer—The human colonic adenocarcinoma epithelial Caco-2 cells (American Type Tissue Collection) were grown in complete DMEM consisting of DMEM-Glutamax (Invitrogen) supplemented with 10% FCS (Sigma), 10 mM HEPES (Invitrogen), 1% non essential amino acids (Invitrogen), 1% sodium pyruvate (Invitrogen), 1% L-glutamine (Sigma), 1% penicillin/streptomycin (Sigma), and 0.1% transferrin (Invitrogen), and used between passages 32 and 40. Cells cultivated up to 80% confluency were seeded on polyester Snapwell filters (diameter, 12 mm; pore size, 0.4 μm; Corning Costar) at a density of 0.8×10^5 cells/cm^2. At week 3, the Caco-2 cell monolayer integrity was checked by measuring the transepithelial electrical resistance (TER) using the Millicell-ERS device (Millipore) (21). TER values of well differentiated monolayers were in the range of 380–550 ohms $\times$ cm^2.

Bacterial Strain and Culture Conditions—Bacteria used were the virulent strain of serotype 5a LPS *S. flexneri* M90T constitutively expressing GFP (22). Bacteria from frozen stock were grown in Luria-Bertani (LB) agar plate containing 0.1‰ Congo Red (Applichem) and 50 μg/ml ampicillin (Sigma-Aldrich), for 30 h at 37 °C. Three red colonies were amplified in 10 ml of LB liquid broth supplemented with 50 μg/ml ampicillin (LB liquid/ampicillin) for 16 h at 37 °C and 200 rpm. The culture was centrifuged at 2,000 $\times$ *g* for 5 min, resuspended in PBS, diluted 1/10 in 10 ml LB liquid/ampicillin and then cultured for 2 h at 37 °C with shaking (200 rpm). Finally, bacteria in the exponential phase were washed twice in PBS by centrifugation at 2,000 $\times$ *g* for 5 min and resuspended in PBS. Assessment of cfu/ml was carried out by measurement of the optical density at 600 nm with the knowledge that 1 optical density unit at 600 nm corresponds to 5×10^8 cfu/ml.

Incubation of Bacteria with Different Ab Preparations—2×10^7 bacteria were mixed with 0.049 μM of SIgAC5 specific for *S. flexneri* LPS serotype 5 or with human plasma-derived pIgA (0.61 μM), SIgA (0.61 μM), or mIgA (0.61 μM), respectively, in a final volume of 500 μl of plain DMEM (DMEM complemented with 10 mM HEPES (Invitrogen), 1% non essential amino acids (Invitrogen), 1% sodium pyruvate (Invitrogen), 1% L-glutamine (Sigma), and 0.1% transferrin (Invitrogen)). The mixtures were incubated for 1 h at ambient temperature under gentle agitation.

Protection Assay—1 h before the use of polarized Caco-2 cell monolayers, complete DMEM was replaced by plain DMEM in both the apical and basolateral compartments. Polarized Caco-2 cell monolayers were infected apically with *S. flexneri* serotype 5a alone or in combination with the Ab preparations. Exposure of Caco-2 cells to *S. flexneri* or the various immune complexes was performed for up to 13 h, and bacteria-induced damage was tracked by measuring TER decrease over time.

Laser-scanning Confocal Microscopy (LSCM) Observation of Caco-2 Cell Monolayers—To examine the integrity of Caco-2 cell monolayers, Snapwells were washed twice with PBS, prior to fixation overnight with 5 ml of 4% paraformaldehyde at 4 °C. After washing, filters were permeabilized, and nonspecific binding sites were blocked using PBS containing 5% FCS and 0.2% Triton X-100 for 30 min at ambient temperature. All Abs were diluted in PBS-T. Filters were incubated with rabbit anti-human Zonula occludens-1 (ZO-1) (1/200 dilution, Invitrogen) for 2 h at ambient temperature, washed in PBS, followed by goat anti-rabbit IgG conjugated with Alexa Fluor® 647 (1/100 dilution, Invitrogen) for 90 min at ambient temperature. To visualize cells, filters were finally incubated with 200 ng/ml of 4′,6′-diamidino-2-phenylindole (DAPI; Invitrogen) in PBS for 30 min. Filters were cut out of their holders and mounted in Vectashield solution (Vector Laboratories) for observation using Zeiss LSM 710 Meta confocal microscope (Carl Zeiss) equipped with a 40$\times$ objective. Three-dimensional reconstructions along the xy plans were performed with the Zeiss ZEN 2009 light software.

Quantification of the Infected Areas—Observation of whole filters was carried out with the 10$\times$ objective using Zeiss ZEN 2009 light software. The sum of infected areas was determined using the particle analysis tool of ImageJ software applied onto the channel associated with the bacteria.

Statistical Analysis—Results were expressed as means $\pm$ S.E. of the mean. Student's *t* test analysis was performed using GraphPad Prism software (version 5). Differences were considered as significant when $p < 0.05$.

RESULTS

Plasma-derived IgA and IgM Contain J Chain and Assemble as Polymers—Human blood is known to contain mostly mIgA, with a minor proportion of pIgA (12–15% range) (23). We first analyzed whether plasma-derived, polyclonal pIgA contained J chain, as this is a prerequisite for subsequent assembly with SC (24). Immunoblot analysis of various plasma IgA-enriched preparations confirmed that in addition to the major mIgA fraction, an SDS-resistant polymer-fraction was present that carried all three expected polypeptides, *i.e.* the α and κ chains, and the J chain (Fig. 1*A*). Interestingly, the immunoreactivity toward the J chain depended on the starting material used to obtain the IgA-enriched fractions, suggesting varying content of pIgA in the different source materials. Incorporation of J chain in monomeric forms resulting from partly covalently assembled pIgA was also observed (20). Most of the pIgA in all three preparations contained covalently bound κ chain. As expected, plasma IgM Abs contained J chain, a feature of the pentameric molecular form, as well as the μ and κ chains (Fig. 1*B*).

Plasma-derived IgA and IgM Both Bind to Immobilized hSC in Vitro—We have demonstrated previously that dot blot reassociation assay is an appropriate assay to test interaction of polymeric Abs with SC, even when relatively crude material is used (17). The multistep assay (Fig. 2*A*) was thus used to assess

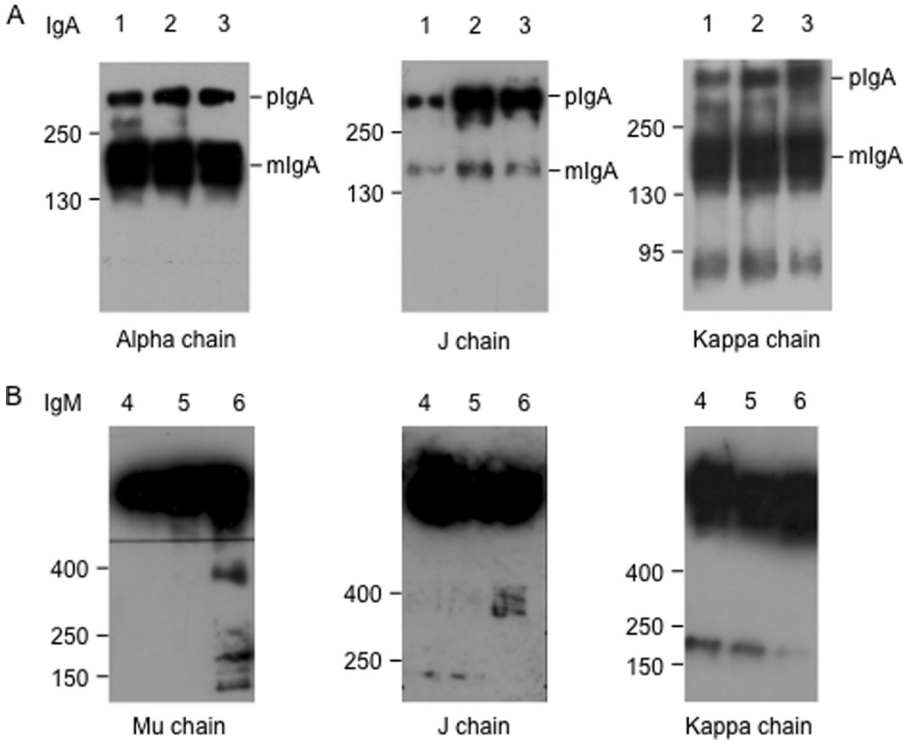


FIGURE 1. **Biochemical characterization of plasma IgA- and IgM-enriched preparations.** Immunoblot analysis under non-reducing conditions of IgA (*A*; *lanes 1–3*) and IgM (*B*; *lanes 4–6*) recovered from various IgA- and IgM-enriched preparations obtained from plasma (*1* and *4*), paste (*2* and *5*), or column strip (*3* and *6*) as described under "Experimental Procedures." The specificity of the antisera used is indicated *below* the respective panels. *A*, 200 ng of IgA was used for detection with anti-α chain antiserum, 500 ng for detection with anti-J chain antiserum, and 400 ng with anti-κ chain antiserum. *B*, 250 ng of IgM was loaded per lane, and signals were obtained using detection with anti-μ chain antiserum, and anti-J chain and κ chain antisera as for IgA. The position of migration of molecular weight markers is indicated alongside the panels.

the ability of purified hSCrec to bind with pIgA and IgM present in IgA- and IgM-enriched preparations, respectively. Immobilization of hSCrec on membranes followed by overlay incubation with preparations enriched in plasma IgA led to specific detection of IgA-positive signals only when all partners were incubated sequentially (Fig. 2*B*). The same was true when preparations enriched in plasma IgM were added in the overlay phase, yielding positive signals only in the presence of all components (Fig. 2*C*). In control experiments with human plasma-derived IgG, no signal was obtained, even at a 10-fold molar excess of the IgG or SC (data not shown). Binding to immobilized hSCcol yielded the same results, confirming the validity of hSCrec as a surrogate for the natural protein (data not shown) (25). Specific interaction between hSCrec or hSCcol and IgA- and IgM-enriched preparations prompted us to separate pIgA from mIgA and to purify IgM to more precisely analyze the association.

Fractionation of pIgA and mIgA Present in Plasma-derived Preparations—Size exclusion chromatography is a robust technique to separate pIgA from mIgA recovered from hybridoma cell supernatants (15) and from cell clones engineered to produce IgA (26). Two different resins and column sizes were used in the present study. Superdex 200 with a fractionation range of 10–600 kDa yielded several fractions containing J chain-reactive material indicative of the presence of pIgA (Fig. 3*A*). α chain-positive bands migrating at the position of mIgA co-eluted with pIgA, due to either insufficient resolution or the presence of non-covalently associated pIgA molecules that dissociate during non-reducing SDS-PAGE. To resolve this issue, Sephacryl S-300 HR (fractionation range, 10–1,500 kDa) was tested. J chain-positive pIgA was recovered in fractions in the first elution peak, whereas mIgA lacking J chain (data not shown) represented the major species in the second peak (Fig. 3*B*). Again, some apparent mIgA co-eluted with the pIgA fraction, likely representing non-covalently associated mIgA molecules that dissociated during SDS-PAGE. In support of this hypothesis, the non-covalent nature of human pIgA1 has been reported after analysis by SDS-PAGE under reducing and non-reducing conditions (27). In sizing chromatography run under native conditions, we found that the mixture of pIgA1 and pIgA2 naturally present in the plasma-derived preparations co-eluted.

Purified pIgA and IgM Assemble in Covalent Complexes with SC—In mucosal secretions and following *in vitro* association between monoclonal pIgA and hSC or mSC, SIgA exists as a covalent complex involving single disulfide bridges that can be identified by SDS-PAGE under non-reducing conditions. Equimolar amounts of purified pIgA and either hSCrec or hSCcol were allowed to associate for 30 min. They were then run on a denaturing polyacrylamide gel, transferred onto PVDF membranes, and immunodetected with anti-hSC antiserum. As for SIgA isolated from colostrum (13) and reconstituted recombinant SIgA Abs (15), we found partial covalency to occur, as indicated by the detection of SC signals at the position of migration of pIgA (Fig. 4*A*). Incubation of mIgA or IgG with hSC did not result in a covalent association, as there was no shift

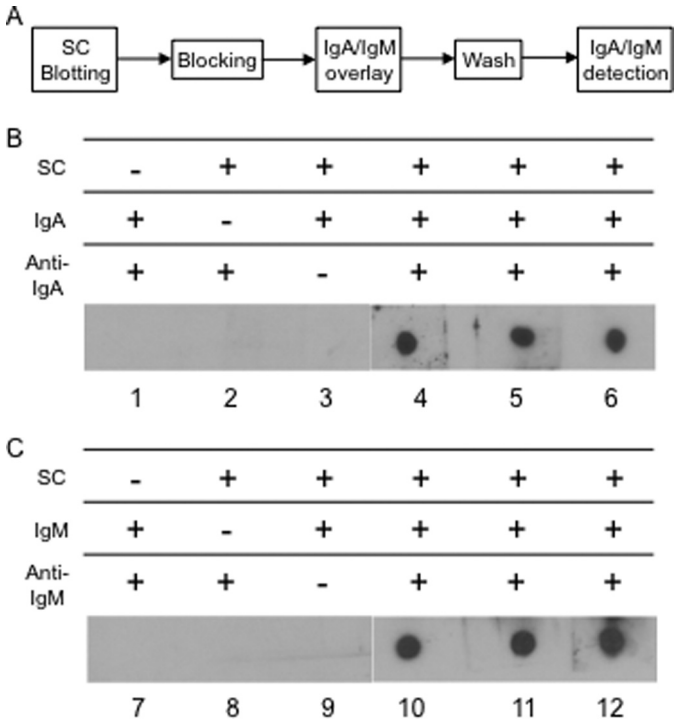


FIGURE 2. **Dot blot association assay of plasma-derived IgA and IgM with hSCrec.** *A*, schematic representation of dot blot reassociation assay. The successive incubation steps are depicted. *B* and *C*, binding of the various preparations enriched in plasma-derived IgA (*lanes 4–6*; *B*) and IgM (*lanes 10–12*; *C*) to immobilized hSCrec. *Lanes 4* and *10*, Ig from plasma; *lanes 5* and *11*, Ig from paste; *lanes 6* and *12*, Ig from column strip, as described under "Experimental Procedures." Control conditions (*lanes 1–3* and *7–9*) include lack of hSCrec ligand, lack of IgA or IgM, or lack of detection antiserum.

in position of hSC on the blot (data not shown). The same analysis of purified IgM associated with hSC resulted in the formation of covalent SIgM (Fig. 4*A*). This confirmed that pentameric IgM containing J chain is the major molecular form of the Ab in plasma (28). Of note, the immunoreactivity of bound hSC in SIgA is known to be strongly reduced, and thus, the true percentage of covalent binding is much higher than it appears by hSC-specific immunodetection (15). The involvement of disulfide bridges in covalent association between hSC and either IgA or IgM was confirmed by treatment with DTT, resulting in the detection of free hSC only (Fig. 4*A*).

Stoichiometry of Association between hSC and Plasma-derived pIgA or IgM—One can argue that the partial covalency observed upon association of the IgA/IgM and hSC is due to a portion of binding-incompetent, possibly denatured, molecules. To exclude this hypothesis, reconstituted SIgA prepared from a 1:1 and 1:2 ratio of Ab to hSC was separated on a sizing column (fractionation range, 10–2,000 kDa) using fast-protein liquid chromatography (Fig. 4*B*). Determination by ELISA of the IgA content of the fraction yielded identical values for 1:1 and 1:2 associated preparations, indicating an equimolar stoichiometry of association (Fig. 4*C*). Use of a 2-fold molar excess of hSC resulted in the additional appearance of late-eluting free hSC (Fig. 4*B*, *inset*), further indicating a specific, saturable level of interaction. Both recombinant and colostrum-purified hSC exhibit the same binding properties (Fig. 4*C*) (19). Thus, incomplete covalent association in SIgA most likely reflects intrinsic properties of the proteins and not major differences between the natural hSCcol and recombinant hSC. This also demonstrates that plasma-derived pIgA display all of the necessary structural features to serve as a ligand to hSC. The same set of

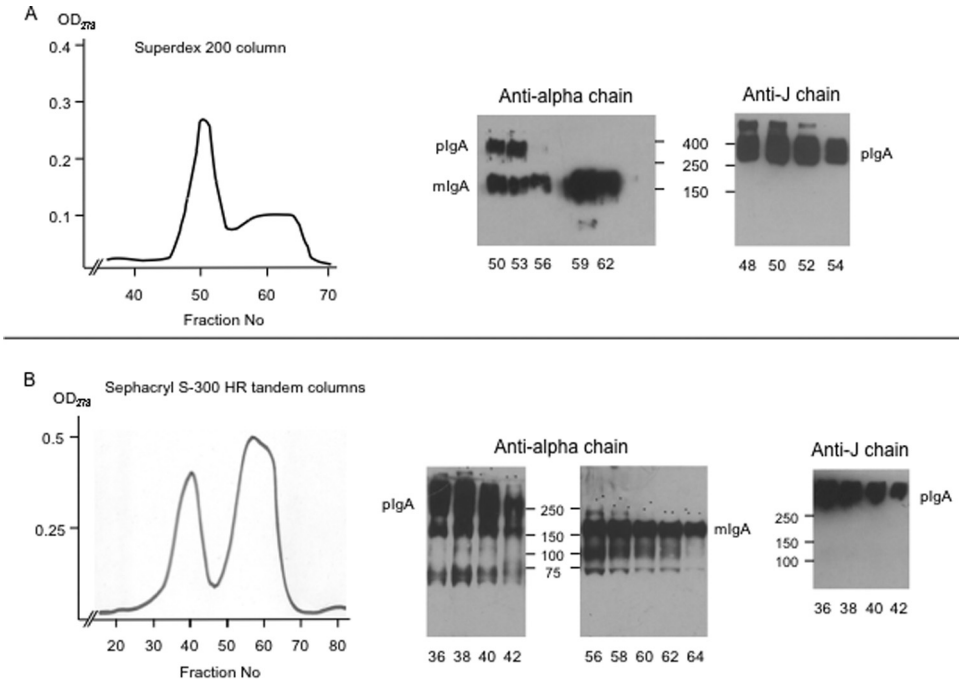
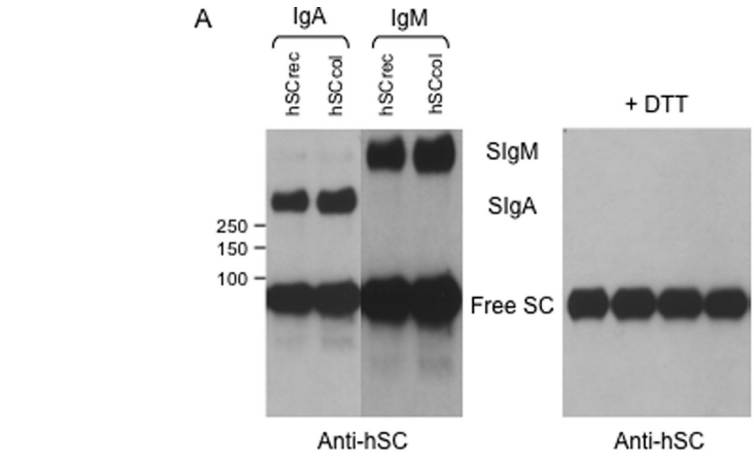
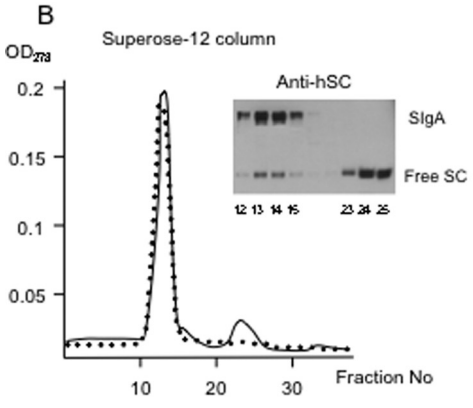


FIGURE 3. **Fractionation of polymeric *versus* monomeric plasma IgA.** *A*, elution profile of the 500-ml Superdex 200 column run in PBS. Immunoblot analysis of a selection of fractions confirmed the presence of J chain-containing pIgA (purified from column strip) in the first elution peak, whereas mIgA was eluted later. *B*, elution profile of the tandem (2 × 500-ml) Sephacryl S-300 HR columns run in PBS. Immunoblot analysis performed as for *A* demonstrates a better resolution of (J chain-containing) polymeric and mIgA. Pooled fractions of pIgA were used for *in vitro* association experiments with hSC.





C

	Peak SIgA		Peak hSC	
	1:1	1:2	1:1	1:2
pIgA	19.8±0.3	19.7±0.3	--	--
hSCrec	5.1±0.2	5.0±0.3	--	4.9±0.1
hSCcol	5.2±0.2	5.0±0.3	--	4.8±0.2

D

	Peak SIgM		Peak hSC	
	1:1	1:2	1:1	1:2
IgM	51.0±0.5	49.9±0.4	--	--
hSCrec	4.8±0.1	4.9±0.2	--	5.0±0.1
hSCcol	4.9±0.1	4.7±0.3	--	5.0±0.2

FIGURE 4. **Analysis of *in vitro* reconstituted SIgA and SIgM.** *A*, covalent association of purified pIgA and IgM (purified from column strip) with hSCrec and hSCcol. The formation of covalent complexes was detected by immunoblot using anti-hSC antiserum. After treatment of covalent complexes with the reducing agent DTT (+DTT) only the free hSC was detected on the immunoblot. The position of migration of molecular weight markers is indicated alongside the panels. *B*, elution profiles after molecular sieve chromatography in PBS solution of reconstituted SIgA in 1:1 (*dotted line*) and 1:2 (*black line*) ratio of pIgA to hSC; the *inset* shows immunodetection of hSC in a mixture at a 1:2 ratio, demonstrating the presence of co-eluting covalent and noncovalent hSC with pIgA. The excess of free hSC is eluted as an independent peak in late fractions. *C*, quantification by ELISA of pIgA, hSCrec and hSCcol (expressed in μg) in pooled fractions corresponding to peaks containing reconstituted SIgA or excess of free hSC. −, below level of detection.

experiments was carried out with reconstituted SIgM, yielding identical association results (Fig. 4, *A* and *D*).

Reduced Sensitivity of SIgA and SIgM to Proteases Indicates Correct Assembly as Secretory Abs—To test whether association of plasma pIgA or IgM with hSCrec would confer increased stability for potential mucosal application, the susceptibility of pIgA, SIgA, IgM, and SIgM to digestion with intestinal washes was examined as described previously (19). Changes in the migration profile of the α and μ heavy chains reflecting degradation were assessed by immunoblot after separation by SDS-PAGE under reducing conditions. Initially, conversion of the 62-kDa α chain into a band migrating at 40 kDa was observed at 2 h (Fig. 5*A*). This intermediate degradation product disappeared at 4 h, an effect due to the stringent conditions encountered in intestinal washes (19). At 6 h, most of the α chain in IgA was degraded, whereas the SIgA material displayed preserved integrity. In contrast to IgA, the SIgA counterpart survived overnight digestion (16-h time point, Fig. 5*A*). Generally, IgM appeared to be less sensitive to the action of intestinal washes (Fig. 5*B*). Nevertheless, as revealed in Fig. 5*B*, appearance of degraded mu chain fragments occurred more rapidly and more extensively for IgM compared with SIgM. To confirm this, densitometric analyses of the immunoblot films were performed. The sum of signals resulting from the degraded μ chain divided by the sum of signals corresponding to all immunoreactive species in the individual lane (×100) yielded the percentage reported in Fig. 5*C*. We conclude that although the phenomenon is not as marked as for SIgA, the sensitivity of SIgM to intestinal washes is reduced in comparison with IgM.

Human Plasma-derived IgA Protects Polarized Caco-2 Cell Monolayers from Damage by S. flexneri—To study the protective potential of human plasma-derived IgA, an *in vitro* model of reconstituted intestinal epithelial cell monolayers infected with a virulent strain of *S. flexneri* was used. The advantage of this model is that it reflects epithelial cell infection resulting from exposure to bacteria, viruses, and bacterial toxins and has proven very valuable to examine the neutralizing properties of Abs of various isotypes and in particular IgA (26, 29–31). The extent to which plasma-derived polyclonal mIgA, pIgA, or SIgA confer protection of epithelial cells was evaluated in comparison with an anti-*Shigella* protective mAb (IgAC5). TER values, LSCM observations, and quantification of infected areas were independently assessed to determine the integrity of Caco-2 cell monolayers. TER reflects the increased passage of ions and

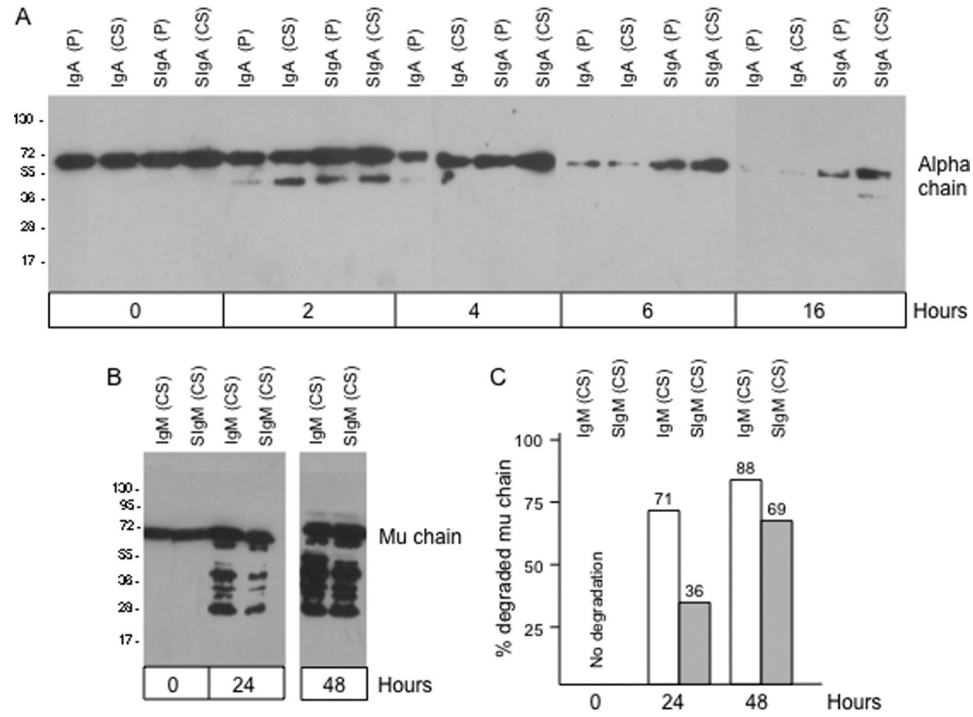


FIGURE 5. **Pattern of digestion of pIgA, SIgA, IgM, and SIgM incubated with intestinal washes.** Immunoblot analysis under reducing conditions of various preparations of purified IgA (purified from paste (*P*) or column strip (*CS*) as indicated in the figure; *A*) and IgM (purified from column strip; *B*) left as such or reconstituted into secretory Ab exposed to intestinal washes for increasing periods of time. Disappearance of α chain and degradation of μ chain reflects the action of proteases in the intestinal washes. The nature of digested proteins and the time course of incubation are indicated *above* the lanes. The position of migration of molecular weight markers is indicated alongside the panels. *C*, percentage of degraded μ chain in *B* analyzed by densitometry of the immunoblot. Calculated values are indicated on the *top* of columns.

indirectly damage to the epithelial monolayer. A similar weak reduction in TER was measured when either the IgAC5 mAb or a 10- to 12.5-fold higher concentration of plasma-derived pIgA or SIgA were examined, whereas bacteria alone or in combination with mIgA led to a marked drop within 13 h (Fig. 6*A*). Additionally, LSCM observations were performed 13 h post-infection. *S. flexneri* alone induced extensive damage reflected by widespread areas of the Transwell membrane devoid of cells (Fig. 6*B*, representative pattern). Maintenance of the cell monolayer integrity with human plasma pIgA and SIgA was very close to that obtained with SIgAC5, with occasional holes forming (Fig. 6*B*, representative pattern). Strikingly, mIgA does not offer the same degree of protection (Fig. 6*B*). In a more quantitative approach, the sum of infected areas of all sizes was determined for each individual whole filter ($n = 4$) at 13 h and is expressed as the surface (in mm^2) devoid of organized Caco-2 cell monolayers (Fig. 6*C*).

DISCUSSION

For IgA Abs to be effective upon mucosal application, the association with SC is essential (9). In the airways and the gut, SC-mediated anchoring to mucosal epithelial cells ensures optimal protective function (12), whereas in the intestine, SC confers improved stability to the IgA molecule (19). Purification of SIgA from a natural source, such as milk, intestinal fluid, or saliva is possible at small scale, but these materials are not appropriate sources for IgA/IgM-production on a larger scale. The possibility to associate plasma-derived pIgA Abs with hSCrec as described in this work indicates that it might be feasible to develop a plasma IgA-based product for mucosal application.

The detection of low levels of IgA monomers by SDS-PAGE in the plasma-derived pIgA fraction after separation by size exclusion chromatography suggests that a portion of pIgA is assembled as a non-covalent species sensitive to the presence of the detergent. This material is devoid of J chain and has been reported in analyses of hybridoma cell culture supernatants (15), in samples of bile and feces (16), in CHO cell line clones expressing pIgA (20), and even in human colostrum (13).

One can argue that due to different environments of biosynthesis for mucosal and serum IgA, the structure of the polymeric form may be different, resulting in a different capacity to recognize pIgR/SC *in vivo* and *in vitro*. However, the fact that hSCrec and "natural" hSCcol both effectively associate with plasma pIgA indicates that existence of intrinsic conformational plasticity in interacting partners is adequate to promote *in vitro* reconstitution of SIgA. This is in keeping with the delivery of pIgA in the mouse circulation, which allows the recovery of SIgA in secretions (32). We have established that covalent, disulfide bridge-mediated binding between plasma pIgA and hSC occurs, further demonstrating the efficiency of the *in vitro* assembly process. Similar results were observed for SIgA recovered from human colostrum (13, 33) and may be due to the reduced capacity of SC in SIgA2 to form covalent complexes, as compared with SIgA1 (27). Alternatively, in rodent species with a single IgA subtype, partial covalency is observed systemati-

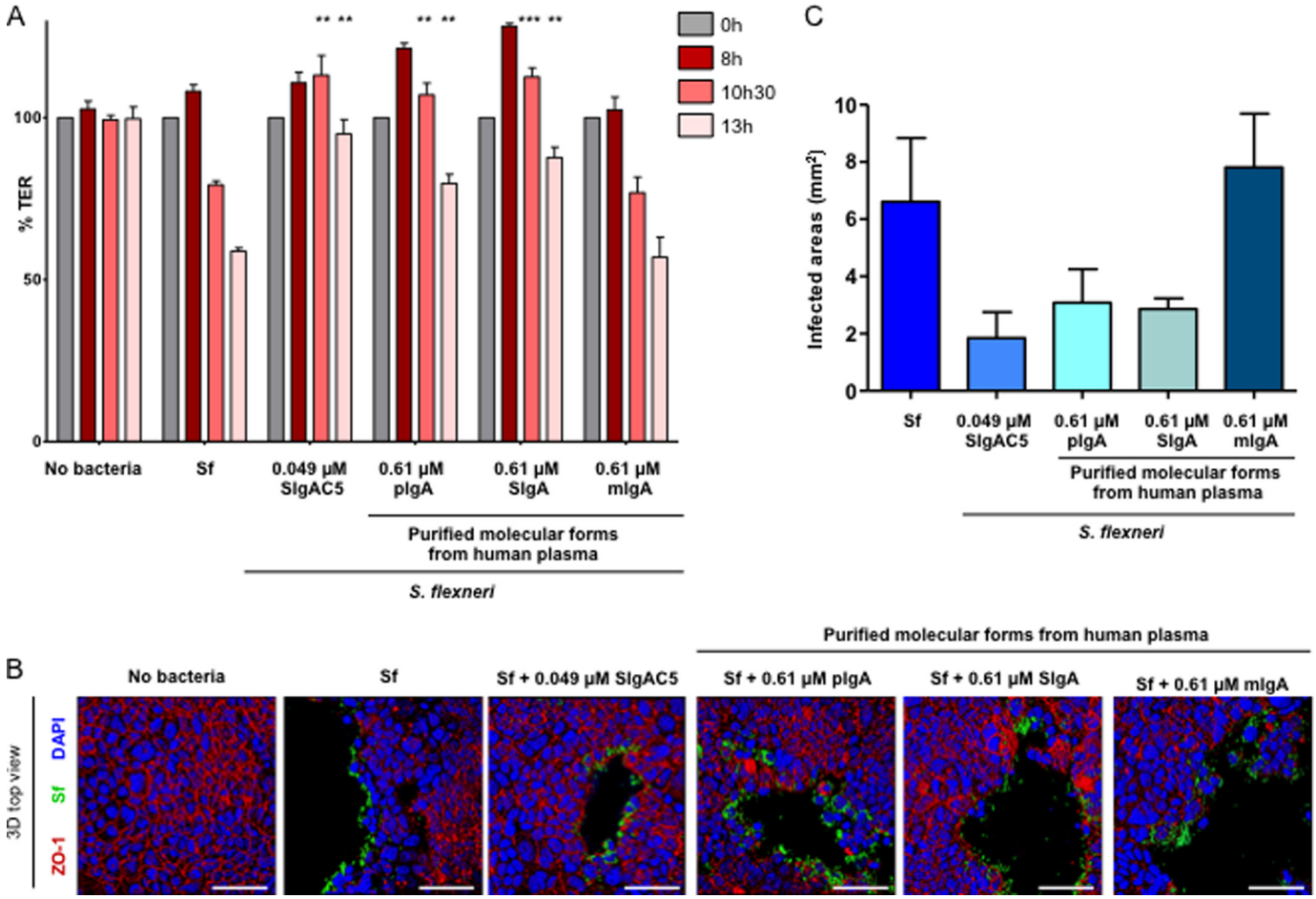


FIGURE 6. **Integrity of Caco-2 cell monolayers infected with *Shigella flexneri* alone or in combination with various IgA preparations.** *A*, TER of intestinal Caco-2 cell monolayers exposed to 2×10^7 *S. flexneri* M90T alone or in combination with purified column strip-derived pIgA, SIgA, or mIgA, determined at four time-points. The TER values for each condition and each time point were normalized with the TER values at the beginning of the experiment and are expressed in percentage. Protection offered by SIgAC5 specific for *S. flexneri* LPS and non-infected Caco-2 cell monolayers (no bacteria) serve as controls. The panel is representative of one individual triplicate experiment performed three times. Significant statistical differences calculated by comparison with the condition *S. flexneri* alone (*Sf*) are indicated above the columns: **, $p < 0.01$; ***, $p < 0.001$. *B*, LSCM three-dimensional reconstructed images (snapshot) of Caco-2 cell monolayers exposed to 2×10^7 *S. flexneri* M90T alone or in combination with human plasma-derived pIgA, SIgA, or mIgA for 13 h. Tight junctions stabilizing the monolayer are visualized with ZO-1 labeling (*red*). Caco-2 cells are visualized via nuclear staining with DAPI (*blue*) and bacteria constitutively expressing GFP stain (*green*). Control monolayers are the non-infected Caco-2 cell monolayers (no bacteria). *Scale bars*, 50 μm. *C*, for each condition, the sum of infected areas was determined from LSCM pictures of whole filters using ImageJ software. *B* and *C* are representative of two independent experiments performed in duplicates.

cally (34) and may reflect intrinsic exchange between disulfide bonds in the α chain and domain 5 in SC (19).

In secretions, only pentameric, J chain-containing IgM is released as SIgM. It is therefore highly likely that this molecular form in blood is responsible for the interaction we have detected with hSC. Our data are the first to show that partially covalent, disulfide bridge-mediated interaction indeed takes place between IgM and hSC. Reconstitution as SIgM allowed us to address the positive impact SC has on the stability of SIgM when exposed to proteases. Hence, the "sacrificial" transport of IgM by pIgR may be similarly beneficial to the half-life of the Ab, as already known for SIgA. In conclusion, although it has never been formally demonstrated that pIgR and SC bind identically to pIgA/IgM (35), and despite the presence of mIgA in the plasma preparation, *in vitro* association with SCrec and SCcol is able to take place with both IgA and IgM.

The sum of these data indicates that essential biochemical properties, including J chain-dependent binding specificity to SC (recombinant as well as colostrum-derived), increased stability in protease-rich intestinal washes, and covalent association occur after association between SCrec/SCcol and plasma-derived IgA/IgM. In addition, the remarkable protective potential against *S. flexneri* with human plasma-derived reassociated SIgA but also pIgA using an *in vitro* model of intestinal Caco-2 cell monolayers shows that the polyreactive Abs are functional. Remarkably, only a 10- to 12.5-fold higher concentration was required to reach similar degrees of protection as the specific SIgAC5 mAb via a mechanism of action relying on Ab-mediated bacterial aggregation. Both pIgA and reconstituted SIgA exhibit a similar neutralizing capacity at concentration (0.61 μM) close to that measured in human gut lavage fluids (36), thus opening up a therapeutic possibility for passive immunization with the optimal molecular form, *i.e.* reconstituted SIgA. As plasma-derived Abs are intrinsically polyreactive, future work will evaluate their protective capabilities against a large panel of pathogens infecting mucosae, using

both *in vitro* models (21, 37, 38) and more demanding *in vivo* models of infection (39–41).

Plasma is an appropriate source of rare proteins with a tremendous field of application in human medicine. Clotting factors, albumin, IgG Abs are already used compounds with established health benefits. In patients with primary or secondary immunodeficiency, replacement therapy with IgG effectively prevents severe systemic infection. However, despite appropriate IgG supplementation, frequently occurring chronic infection/inflammation of the respiratory and gastrointestinal mucosae has been linked to low serum IgA levels (42–44). The inherent structural features of SIgA would make it a logical agent to fill this therapeutic gap in combination with standard therapy (*e.g.* antibiotics, anti-virals, and IgG). Importantly, the combination of two crucial functional properties, namely antigen recognition via Fab (similar to IgG), and anti-inflammatory effector function via Fc is an additional argument to consider IgA in prevention/treatment of mucosal infection and particularly of inflammation associated with chronic infection (45–47). Starting from 1 liter of plasma, current purification procedures allow to recover 40–50 mg of J chain-containing pIgA and up to 100 mg of IgM. Production from 10,000 liters as this is classically performed will yield Abs in sufficient amounts to consider clinical applications. In conclusion, passive topical administration of polyreactive SIgA as well as SIgM may represent a valuable therapeutic approach to control infection before the immune system of the host can take over.

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