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Simplified procedure to recover recombinant antigenized secretory IgA to be used as a vaccine vector

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Abstract

Induced protection mechanisms at mucosal surfaces involve secretory IgA (SIgA), a complex structure made of polymeric–dimeric IgA ($IgA_{p/d}$) antibody associated with secretory component (SC). SIgA can adhere to M cells of the intestinal and nasal epithelia, are transported across these latter, and are thus available to the immune cells underlying the epithelia. This property makes SIgA suitable as potential mucosal vaccine delivery vector. It remains that production and purification of SIgA is a complex task since $IgA_{p/d}$ and SC are naturally synthesized by two different cell types. Furthermore, only $IgA_{p/d}$ are capable to associate with SC. Thus, we sought to separate $IgA_{p/d}$ and monomeric IgA (IgA_m) antibodies secreted by hybridoma cells in CELLine bioreactors. To this aim, we connected together two 1-m long columns filled with Sephacryl S-300 beads and placed them under the control of a automatized chromatographic system. In parallel, we produced recombinant antigenized human SC (ra-hSC) in Chinese hamster ovary (CHO) cells adapted to suspension culture in CELLine bioreactors. To avoid intermediate purification of ra-hSC, culture supernatants (SN) containing this latter were combined with purified $IgA_{p/d}$, and the recombinant antigenized SIgA (raSIgA) complex was resolved on a 1-m long column filled with Superdex 200 beads. Biochemical characterization based on SDS–PAGE, silver staining, immunodetection and enzyme-linked immunosorbent assay (ELISA) indicates that highly purified raSIgA can be recovered using this simple two-step procedure. Such preparations are currently used to immunize mice to induce mucosal and systemic responses.

Keywords: Immunoglobulin A; Recombinant protein

1. Introduction

Mucosal surfaces comprising the gastro-intestinal, respiratory and urogenital mucosae represent a large port of entry (400 m^2 in humans; [1]) for most of the pathogens and thus have to be efficiently protected. This goal is achieved by a combination of constitutive, non-specific substances (e.g. mucus, lyzozyme, lactoferrin and defensins; [2]), and induced, specific immune mechanisms mediated by cellular and antibody responses [3]. In vivo, the chief antibody at mucosal surfaces is SIgA [4,5], a complex structure made of $IgA_{p/d}$ produced by activated B cells in the mucosal epithelium and of the SC, a polypeptide of 85 kDa corresponding to the extracellular part of the poly-immunoglobulin receptor (pIgR) expressed by the epithelial cells. SC results from the cleavage of

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pIgR that transports $IgA_{p/d}$ from the basolateral to the apical side of the epithelium [6]. Once in secretions, SIgA binds antigen(s), thus preventing their adhesion to the luminal epithelial surface [7,8] and facilitating their elimination by peristalsis or mucociliary movement [2], a phenomenon called immune exclusion [9].

In the intestine, SIgA have been shown to be able to specifically bind to a specialized type of enterocytes called M cells [10,11], irrespectively of the antibody specificity. These cells are able to sample antigens in the intestinal lumen and to present them in an intact form to the underlying immune structure named Peyer's patches (PP) [12]. Binding of SIgA to M cells raises the possibility to use these molecules as antigen carrier to target mucosal immune sites exemplified by PP. This concept has been validated by two experiments: (1) oral administration of heterologous SIgA was able to induce the production of IgG, IgM and IgA positive B cells specific for the SC and IgA moieties in the PP in the absence of an adjuvant [13]; (2) oral administration of antigenized SIgA carrying a nine amino acid epitope from *Shigella flexneri* within SC induced an immune response against the epitope in the presence of an adjuvant [13]. This indicates that a peptidic epitope inserted by genetic engineering within SC is protected by the SIgA complex and can be brought intact to mucosal immune sites even after having crossed the very harsh environment of the stomach.

In order to further investigate this mucosal vaccine carrier approach, we produced raSIgA complexes carrying a bacterial antigen fused at the C-terminus of human SC (hSC). This antigenization approach permits to insert more than one single bacterial epitope as previously done [13], and thus trigger improved immune responses in vaccination trials. The model antigen used in this study is the *Helicobacter pylori* GroES chaperonin [14]. Diverse approaches can be considered to produce recombinant SIgA, including co-cultures [15], expression of SIgA by one single cell line [16], or in vitro association of purified $IgA_{p/d}$ and recombinant SC [17,18]. By taking advantage of the last approach, we describe herein a way to achieve production of highly purified raSIgA molecules. Our procedure is based on both optimized cell culture conditions and a simple two-step purification protocol relying on size exclusion chromatography. The purity and amounts of the recovered material is fully suitable for mouse immunization.

2. Experimental

2.1. Material

Restriction enzymes, Klenow enzyme and T4 DNA ligase were from Roche Applied Science (Basel, Switzerland). PBS II KS+ plasmid comes from Stratagene (La Jolla, CA, USA). Oligonucleotides have been ordered lyophilized from Microsynth (Balgach, Switzerland) and resuspended in deionized water (dH_2O, 18 MΩ) at a concentration of 1 μg/μl. DNA sequencing was performed using the T7 sequencing kit from Amersham Biosciences (Uppsala, Sweden).

For cell transfection, the Gene Pulser II electroporation system was used in combination with 0.4-cm electrode gap cuvettes (Bio-Rad, Hercules, CA, USA).

Adherent CHO cells ($dhfr^-$ cells, American Tissue Culture Collection CRL 9096) were cultured in MEM-alpha medium with Glutamax-I from Invitrogen (Carlsbad, CA, USA) complemented with (final concentrations): 10% fetal bovine serum (FBS), 10 m*M* HEPES, 100 Units/ml of penicillin and 0.1 mg/ml of streptomycin (all from Sigma, St. Louis, MO, USA). Geneticin used for selection was obtained from Roche Applied Science. CHO suspension culture was carried out with CHO-S-SFM II medium from Invitrogen complemented with 100 Units/ml of penicillin and 0.1 mg/ml of streptomycin (final concentrations). Hybridoma cells (HNK20 clone, [19]) were cultured in RPMI-1640 medium from Sigma complemented with (final concentrations): 2.5% FBS, 10 m*M* HEPES, 1 m*M* sodium pyruvate, 2 m*M* L-glutamine, 100 Units/ml of penicillin, 0.1 mg/ml of streptomycin as above and 25 μ*M* folic acid (Fluka, Buchs, Switzerland).

High density cell cultures were carried out in CELLine 350 bioreactors (Integra Bioscience, Wallisellen, Switzerland).

Before loading onto the automatized chromatographic system, cell culture SN or raSIgA were concentrated to the desired volume using Centriplus 50 or 100 centrifugal filter devices from Amicon/Millipore (Bedford, MA, USA).

For IgA separation, two successive 1-m long×2.6-cm I.D. columns filled with Sephacryl S-300 high resolution beads (Amersham Biosciences) were used. For raSIgA separation, one 1-m long×1.6-cm I.D. column filled with Superdex 200 preparative grade beads (Amersham Biosciences) was used. The columns were run under the control of a ÄKTA*prime* system (Amersham Biosciences) in sterile PBS (116.3 m*M* NaCl, 10.4 m*M* Na_2HPO_4, 3.2 m*M* KH_2PO_4, pH 7.4).

For SDS–PAGE and immunoblotting onto polyvinylidine fluoride (PVDF) membranes (Millipore), the Mini-Protean II system from Bio-Rad was used as described previously [17].

Rabbit anti-hSC [17], rabbit anti-GroES [14] and rabbit anti-J chain [20] antibodies are published material. Mouse monoclonal anti-FLAG and anti-hSC antibodies (mAb) and goat anti-mouse IgA (α-chain-specific) were purchased from Sigma. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was from Pharmingen (San Diego, CA, USA) and HRP-conjugated goat anti-mouse IgG was obtained from Pierce (Rockford, IL, USA). The chemiluminescent substrate for HRP was the Uptilight HRP Blot from Uptima-Interchim (Montluçon, France). Autoradiographic films used were from Konica Medical Imaging (Wayne, NJ, USA).

For ELISA dosages, Nunc-immuno Plate Maxisorp surface from Nalge Nunc (Roskilde, Denmark) were used.

All other chemicals were purchased from Fluka (Buchs, Switzerland).

2.2. ra-hSC cloning and cellular expression

A modified version of the expression vector pCB6-hpIgR coding for human pIgR (a gift from Charlotte S. Kaetzel, University of Kentucky) served as the starting plasmid for cloning. A portion of the multiple cloning site (MCS) in pCB6-hpIgR was removed by *Sac*I and *Cla*I digestion, followed by filling with Klenow enzyme and ligation, yielding vector pCB6-hpIgR-δMCS. Then, as outlined in Fig. 1, a portion of the hpIgR sequence corresponding to

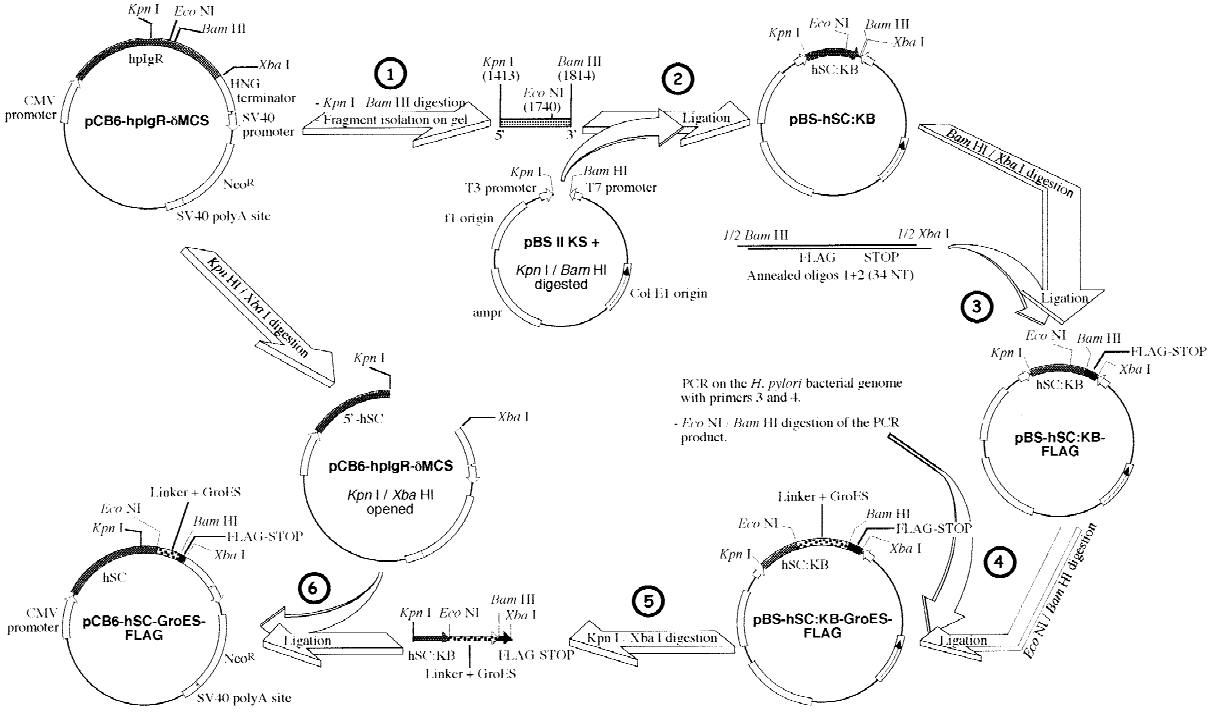

Fig. 1. Schematic representation of the cloning strategy used to elaborate recombinant antigenized human SC (ra-hSC) expression vector. Numbers in circles indicate intermediate cloning steps as described in Section 2. Only restriction sites useful for the understanding of cloning steps are depicted.

the C-terminal end of hSC was excised by *Kpn*I and *Bam*HI digestion (step 1) and ligated into pBS II KS+ plasmid digested the same way (step 2). The resulting plasmid (pBS-hSC:KB) was then digested with *Bam*HI and *Xba*I to fit hybridized oligonucleotides 1 and 2 (coding for the FLAG peptide and a stop codon, Table 1; step 3). Following *Helicobacter pylori* genome PCR amplification using GroES-specific oligonucleotides 3 and 4, the product was digested with *Eco*NI and *Bam*HI and inserted into pBS-hSC:KB-FLAG cut with the same two enzymes (step 4). The linker (coding for four glycines and one serine) in oligonucleotide 3 has been inserted at the 5′-end of the GroES sequence in order to avoid a potential negative effect on the proper folding of the C-terminus of hSC. DNA sequencing of the 3′-end of engineered SC indicated that all PCR products contained no mutation. Finally, the antigenized C-terminal portion of hSC was excised from pBS-hSC:KB-GroES-FLAG by a *Kpn*I/*Xba*I digestion (step 5) and inserted into pCB6-hpIgR-δMCS digested the same way (replacement of the C-terminus end of pIgR, step 6). Expression vector pCB6-hSC-GroES-FLAG was transfected by electroporation (10 μg of DNA for 10^7 cells, 400 V, 125 μF) into adherent CHO cells and then cultured in complete MEM-alpha medium under Geneticin selection (200 μg/ml) as the vector contains the resistance gene to this drug (Neo^R). After 6 weeks of selection, single cell cloning was performed and the best secreting clone (determined by ELISA SN quantification) was adapted to suspension culture conditions in CHO-S-SFM II medium. To ensure sufficient productivity, the clone was transferred from T-flask culture bottles to CELLine 350 bioreactors. SN were collected twice a week, filtered through 0.22-μm cartridges, and frozen at −20 °C until use.

2.3. IgA production

Mixture of the various molecular forms of IgA were recovered from hybridoma HNK20 cultured in complete RPMI-1640 medium using CELLine 350 bioreactors. SN were collected twice a week, filtered through 0.22-μm cartridges, and frozen at −20 °C until use.

2.4. Separation of polymeric and monomeric IgA

SN containing IgA were concentrated to a final volume (5 ml) suitable for injection onto the ÄKTA*prime* system using Centriplus 50 centrifugal filter devices. To resolve the various molecular forms of IgA, two 1-m long columns filled with Sephacryl S-300 beads were coupled serially and the separation was performed at a constant flow-rate of 1 ml/min with PBS as mobile phase. Protein collection started after the first 385 ml (void volume) were passed; the

Table 1
Sequences of the 4 oligonucleotides used in the cloning strategy depicted in Fig. 1. All oligonucleotides are shown in the 5′→3′ orientation

Oligonucleotide sequence	Used for
Reading frame correction 1/2 *Bam* HI — FLAG — STOP — 1/2 *Xba* I 1) 5′ GAT CCC GAC TAC AAG GAC GAC GAT GAC AAG TAA T 3′ Reading frame correction 1/2 *Xba* I — STOP — FLAG — 1/2 *Bam* HI 2) 5′ CTAGA TTA CTT GTC ATC GTC GTC CTT GTA GTC GG 3′	Annealing and insertion at the 3′-end coding sequence of SC (step 3).
Clamp — *Eco* NI — Linker : G_4S — GroES 3) 5′ GCACGT C CTA GCG AAG GGC GGC GGC GGC AGC CCA TTA GGA GAA AGG GTC 3′ Pro$_{95}$ Clamp *Bam* HI — GroES 4) 5′ CG GG ATC CTT TTT GTG ATC ATG ACA GC 3′ Lys_{111} Lys_{112}	PCR amplification on the *H. pylori* whole genome to get the GroES sequence inserted at the 3′-end of SC at step 4.

1 and 3: sense sequences; 2 and 4: anti-sense sequences.

content of 3.5-ml fractions was analyzed by immunodetection, fractions containing $IgA_{p/d}$ were pooled, concentrated using Centriplus 100 devices, and stored at 4 °C until further use.

2.5. Western blot analysis

SDS–PAGE and transfer onto PVDF membranes was carried out as described [18]. The membranes were then blocked for 20 min in PBS–0.05% Tween 20 solution (PBS-T) containing 5% non fat dry milk. Goat anti-mouse IgA antiserum (α-chain-specific; 1:5000 dilution), rabbit anti-hSC antiserum (1:5000), rabbit anti-GroES antiserum (1:5000), mouse anti-FLAG mAb (1:5000), or rabbit anti-J chain antiserum (1:3000) were used as primary antibodies. Antiserum dilutions were performed in PBS-T containing 0.5% non fat dry milk. Membranes were then extensively washed with PBS-T. As secondary antibody coupled to HRP, goat anti-rabbit IgG (1:10 000) or goat anti-mouse IgG (1:10 000) were used. After final washing with PBS-T, immune complexes on membranes were detected by chemiluminescence and exposure on autoradiographic films.

2.6. Silver staining

Samples were loaded and run on SDS–PAGE as described above. After migration, proteins were fixed by a 30-min incubation in 20% methanol–10% acetic acid solution. Following extensive washing with dH_2O, the gels were exposed to 400 μ*M* DTT for 3–4 min, rapidly washed with dH_2O and incubated for 15 min in 6 m*M* $AgNO_3$ in dH_2O. After rapid final washing with dH_2O, gels were developed with a 283 m*M* Na_2CO_3–0.03% formaldehyde solution before blocking with 10% acetic acid.

2.7. ELISA

Wells of 96 micro-well plates were coated with 50 μl of 1:500 capture antibody dilutions in 50 m*M* carbonate–bicarbonate (pH 9.6). Incubation was for 1 h at 37 °C, using goat anti-mouse IgA (α-chain specific) antibody for IgA quantitation, or mouse anti-hSC mAb for hSC quantitation, respectively. After washings with PBS-T (300 μl), wells were blocked for 20 min at 37 °C with PBS-T containing 5% non fat dry milk (50 μl). Diverse dilutions (in 50 μl) of the cell culture SN, as well as of the hSC or IgA standards, were then incubated for 1 h at 37 °C. Bound molecules were then detected by a 1-h incubation at 37 °C with 50 μl of either rabbit anti-κ chain or a rabbit anti-hSC antibody (diluted 1:1000 in PBS-T containing 0.5% non fat dry milk). After washings, 50 μl of HRP-conjugated goat anti-rabbit IgG antibody (diluted 1:3000 in PBS-T containing 0.5% non fat dry milk) were added and incubated for 45 min at 37 °C. Finally, detection was performed with 50 μl of a 0.1 *M* pH 5.0 citrate solution containing 1 mg/ml of ortho-1,2-phenylenediamine and 0.03% H_2O_2. The reaction was stopped with 50 μl of 2 *M* sulfuric acid and absorbance was measured at 492 nm with 620 nm as a reference.

2.8. In vitro raSIgA association and separation

For raSIgA association, 5 mg of purified $IgA_{p/d}$ were mixed with 2 mg of ra-hSC contained in crude SN. Association was achieved by a 2-h incubation at room temperature (RT). Then, the association mixture was concentrated to a final volume of 5 ml and injected onto the ÄKTA*prime* system. A single 1-m long column filled with Superdex 200 beads was used and separation run was performed at a constant flow-rate of 0.25 ml/min with PBS as mobile phase. Protein collection (1.5-ml fractions) started after the first 30 ml (void volume) were passed. Fractions containing raSIgA were pooled, concentrated by Centriplus 100 devices and stored at 4 °C until use.

3. Results and discussion

3.1. Expression of IgA and ra-hSC molecules

As described in Section 2, hybridoma cells were cultivated in CELLine 350 bioreactors and SN containing the IgA were recovered twice a week. Western blot and silver staining analyses of the SN show that they contain a mixture of polymeric, dimeric, monomeric IgA and large amounts of bovine serum albumin (BSA) as expected (Fig. 2A). The $IgA_{p/d}$ were characterized by the presence of the J chain in the immunoglobulin. Anti-IgA specific

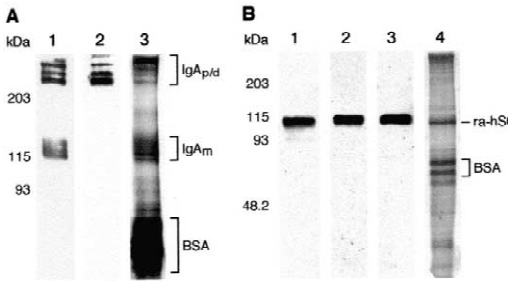

Fig. 2. Western blot and silver staining analysis of diverse crude cell culture SN. Sample SN containing IgA (A) and ra-hSC (B) were run on 6 or 8% polyacrylamide gels in SDS, respectively. The protein content was monitored by immunodetection using antisera against IgA α chain (lane A1), J chain (lane A2), hSC (lane B1), GroES (lane B2) and FLAG (lane B3), or by silver staining (lanes A3 and B4).

ELISA indicated that up to 2 mg of total IgA could be recovered in each SN corresponding to a cell pellet of about 500 μl (data not shown). SN were 0.22-μm filtered and stored at −20 °C until next step.

To permit expression of ra-hSC, CHO cells were stably transfected with expression plasmid pCB6-hSC-GroES-FLAG, then adapted to suspension culture in a low protein culture medium, and finally cultured in CELLine 350 bioreactors. SN containing the ra-hSC were collected twice a week. Western blot and silver staining analyses of the SN showed that they essentially contain the desired protein with all its engineered features, namely hSC, GroES and FLAG sequences (Fig. 2B). Only residual amounts of BSA contained in the culture medium could be observed. Anti-hSC specific ELISA proved that up to 1 mg of ra-hSC could be recovered in each SN corresponding to a cell pellet of about 1 ml (data not shown). SN were 0.22-μm filtered and stored at −20 °C until next step.

3.2. IgA preparation and separation

In order to get a volume suitable for loading onto the automatized chromatographic system, SN from individual CELLine 350 harvests were concentrated to a final volume of 5 ml using Centriplus 50 devices. Each concentrated SN were then applied onto an ÄKTA*prime* system coupled to two 1-m long columns filled with Sephacryl S-300 beads. Following chromatography, fractions were tested for their IgA content by immunoblot using anti-IgA detection (Fig. 3A). The $IgA_{p/d}$ molecules were recovered in fractions 9–30, while the IgA_m molecules eluted in fractions 33–49 (Fig. 3B), with basically no mixture of the two molecular forms. These optimal separation conditions allow recovering more than 98% of purified $IgA_{p/d}$ molecules with respect to the content of the initial load. The low molecular mass molecules reactive to the anti-IgA antibody present in the $IgA_{p/d}$ fractions correspond to dimers of heavy chains not covalently associated into polymeric complexes. Indeed, they dissociate under denaturing conditions during SDS–PAGE, but co-elute from the column with $IgA_{p/d}$, under native conditions (PBS buffer).

Fractions positive for $IgA_{p/d}$ were then pooled and concentrated up to 2 mg/ml. The high degree of purification is reflected by the mere presence of high molecular mass dimers and polymers when tested by the sensitive silver staining method (Fig. 3C).

3.3. In vitro raSIgA association and purification

It has been shown that in vitro SC-$IgA_{p/d}$ association takes place with a stoichiometric ratio of 1:1 [21]. In agreement with a molecular mass ratio of 4:1, X mg $IgA_{p/d}$ has to be mixed with $X/4$ mg of SC. Thus, in order to ensure that every $IgA_{p/d}$ was associated with one ra-hSC molecule, a slight excess of ra-hSC was used: 5 mg of purified $IgA_{p/d}$ was mixed with the equivalent of 2 mg of ra-hSC present in crude SN. Association was achieved by incubation of the mixture for 2 h at RT.

The volume of the mixture was then brought to 5 ml to be loaded onto the system coupled to a 1-m long column filled with Superdex 200 beads. Fractions containing either covalently reconstituted raSIgA or free ra-hSC were tested for their SC content using anti-hSC detection (Fig. 4A). Free ra-hSC migrates at a relative molecular mass of 110 000, but when associated with $IgA_{p/d}$ it appears as high molecular mass raSIgA complexes (Fig. 4B). Thus, fractions 14–20 contain the desired raSIgA molecules, while fractions eluted after tube 24 contain the excess of ra-hSC as well as the cell culture by-products in which crude ra-hSC was recovered. Virtually no mixture of raSIgA complexes

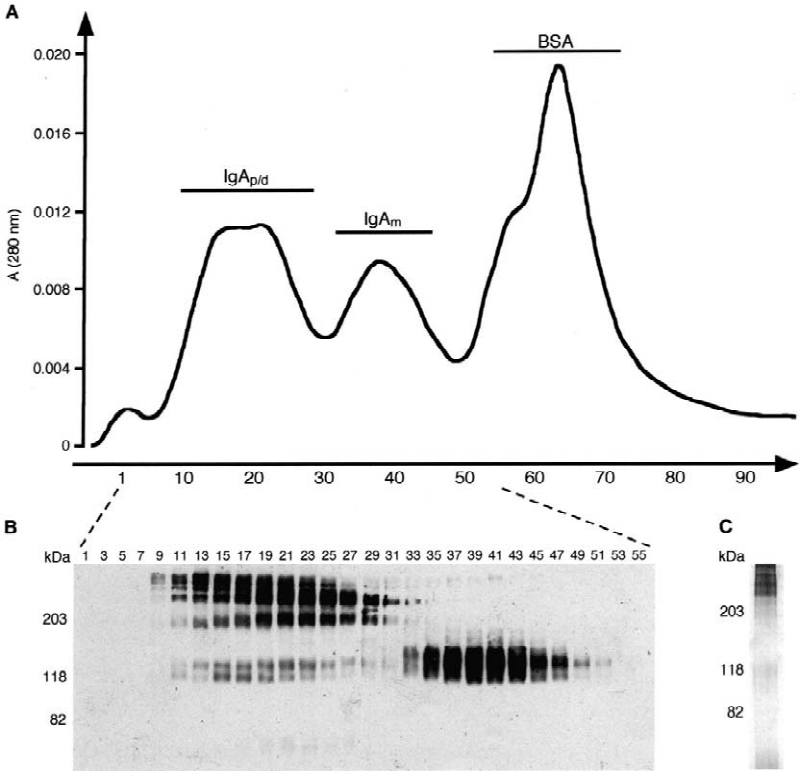

Fig. 3. Separation of the various molecular form of IgA recovered in one CELLine 350 harvest. (A) Elution profile recorded after chromatography on two 1-m long Sephacryl S-300 columns connected serially. Fraction numbers are indicated under the arrow showing the elution progression. Absorbancy values were read at 280 nm. (B) Western blot analysis using anti-IgA (α-chain-specific) antibody of a selection of fractions applied onto 6% polyacrylamide gels in SDS. (C) Silver staining analysis of the pool of the $IgA_{p/d}$-containing fractions (11–29) after migration in a 6% polyacrylamide gel in SDS.

and free ra-hSC occurs, yielding more than 96% of the raSIgA present in the loaded material.

Fractions containing the raSIgA were pooled and concentrated to 2.5 mg/ml. The presence of every component of interest was checked by immunodetection using anti-IgA (α-chain), anti-hSC, anti-GroES and anti-FLAG antisera under non-reducing or reducing conditions. This confirmed that every component is present in molecules migrating at a high relative mass after SDS–PAGE under non-reducing conditions (Fig. 4C, lanes marked −). The variation in relative intensity of the signals was due to the use of antisera with different antigen binding capacity, and also to the different availability of epitopes within the raSIgA complex. Such a discrepancy in signal intensity was attenuated when immunodetection was carried out using reduced material (Fig. 4C, lanes marked +). The silver staining analysis convincingly showed that the preparation contains highly purified raSIgA molecules, with very few non-covalently complexed ra-hSC molecules (Fig. 4D, lane marked −). Together, our data indicate that the driving force contributed by $IgA_{p/d}$ in binding ra-hSC allows to associate raSIgA complexes even though the ra-hSC partner is part of a crude culture SN. Furthermore, this does not prevent subsequent purification and proper assembly similar to native SIgA present in mucosal secretion.

4. Conclusion

We reported here a method to recover highly purified raSIgA by taking advantage of optimized

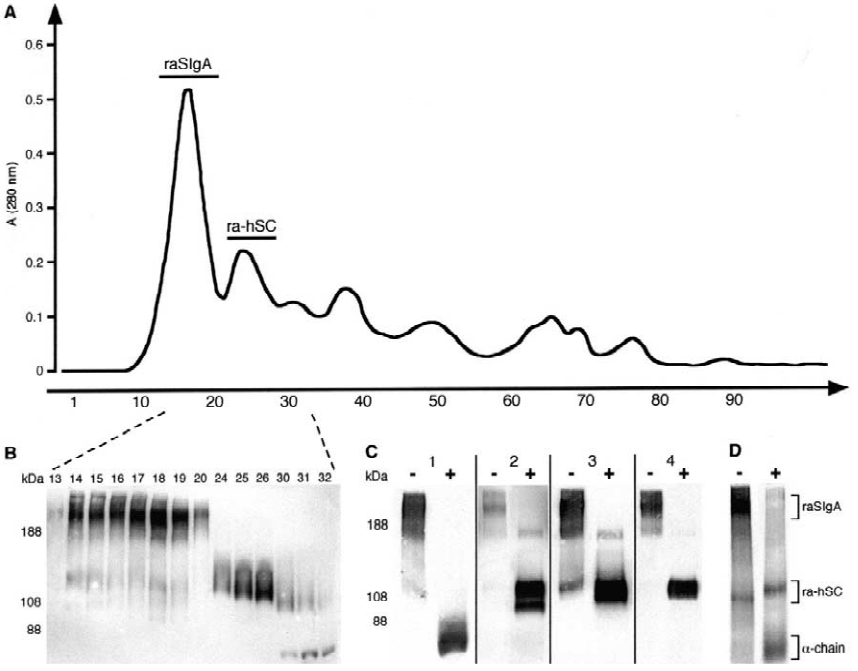

Fig. 4. Purification of raSIgA following association between purified $IgA_{p/d}$ and crude ra-hSC in culture SN. (A) Elution profile recovered after chromatography on a 1-m long Superdex 200 column. Fraction numbers are indicated under the arrow showing the elution progression. Absorbancy values were read at 280 nm. (B) Analysis by immunodetection with anti-hSC antiserum of a selection of fractions run onto a 6% polyacrylamide gel in SDS. High molecular mass bands correspond to covalent raSIgA complexes. (C) Fractions 14–19 were pooled and analyzed by Western blot (SDS–PAGE on 6% polyacrylamide gel). Antisera against IgA α-chain (lane 1), hSC (lane 2), GroES (lane 3) and FLAG (lane 4) confirmed the presence of all constituents in the complex. Samples were loaded under non-reducing (−) or reducing conditions (+). (D) Silver staining analysis of the concentrated pool (14–19) comprising raSIgA following migration in a 6% polyacrylamide gel in SDS under non-reducing conditions.

cell culture conditions and a simple two-step purification procedure based on size exclusion chromatography separations. Sufficient yields of recombinant IgA immunoglobulin and ra-hSC proteins could be obtained after adapting hybridoma and CHO cells to suspension culture using CELLine 350 bioreactors. The diverse IgA populations present in culture SN were resolved on two 1-m long size exclusion column (Sephacryl S-300 beads). $IgA_{p/d}$ were then associated with an excess of the ra-hSC present in the CHO cells crude SN. Finally, highly purified raSIgA were recovered after resolving the associated product by passage over a 1-m long size exclusion column (Superdex 200 beads).

This novel protocol allows, once the cell culture conditions are established, to obtain rapidly large amounts of highly purified raSIgA (or derivative) suitable for diverse in vitro or in vivo applications. For instance, the material prepared in this way proves successful for in vitro or in vivo experiments, such as SIgA–cell interaction or mouse oral or nasal immunization (ongoing experiments in our laboratory).

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