# Structural Composition of Canine Secretory Component and Immunoglobulin A†

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ABSTRACT: Dog serum and colostral immunoglobulin A (IgA) and free secretory component from colostrum were isolated using affinity chromatography. Both serum and colostral IgA showed similar susceptibility to reduction with dithiothreitol, but only colostral IgA released the additional subunit, bound secretory component. This released secretory component was identical with free secretory component with respect to electrophoretic migration, isoelectric focusing point, and molecular weight, but lacked some antigenic determinants. The amino acid composition and the N-terminal sequence of canine free secretory component was similar to that reported for the human and cow.

Secretory immunoglobulin A (sIgA)[1] is the predominant immunoglobulin (Ig) in the external secretions of most mammalian species and man (Tomasi and Grey, 1972; Vaerman and Heremans, 1970). This immunoglobulin, which plays an essential role in the first line defense of mucosal surfaces, is an interesting product of two distinct cellular lines. Sub-mucosal plasma cells synthesize the dimeric form of the IgA molecule which contains four heavy chains ($\alpha$ chain), four light chains, and one J chain. Secretory component (SC), a glycoprotein produced by mucosal epithelial cells, is attached to this dimeric immunoglobulin prior to its release into extracellular spaces (Tomasi et al., 1965; Poger and Lamm, 1974; Brandtzaeg, 1974).

Secretory component is found attached to sIgA (BSC), and in addition is isolated unattached to other proteins (Tomasi and Grey, 1972). The role of this free secretory component (FSC), if any, has not yet been determined. In contrast, BSC is thought to be a transport protein (Tourville et al., 1969; Brandtzaeg, 1974) which may also serve to protect sIgA from proteolytic digestion (Steward, 1971; Ghetie and Mota, 1973; Underdown and Dorrington, 1974).

sIgA and secretory component have been well described in the human, rabbit, cow, and to a lesser extent in the dog. Johnson and Vaughan (1967) reported the existence of an immunoglobulin in canine secretions which was later verified to be sIgA by Vaerman and Heremans (1968). Ricks et al. (1970) reported the presence of BSC and FCS in canine colostrum. Reynolds and Johnson (1971) noted differences in the sedimentation coefficients and molecular weights of sIgA and serum IgA plus an immunological difference between these two species but failed to conclusively identify secretory component.

In this report, we describe the isolation of sIgA and FSC from canine colostrum, and have obtained BSC from sIgA. The structure and composition of FSC were determined and comparisons made between FSC and BSC.

## Materials and Methods

*Materials and Reagents.* Chemicals used were of reagent or analytical grade if possible and their sources have been previously cited (Reynolds and Johnson, 1970, 1971). Cytochrome *c* and dithiothreitol were obtained from Calbiochem. [$^{14}$C]Iodoacetamide ($ICH_3{}^{14}CONH_2$) was obtained from New England Nuclear (11.8 Ci/mol). Cyanogen bromide and dimethylformamide were obtained from Fisher Scientific Co. Ampholines, pH 3–10, pH 5–7, and pH 7–9, were purchased from LKB. Sodium dodecyl sulfate was obtained from Matheson.

Human IgG (150,000), bovine albumin (67,000) and ovalbumin (43,000) were purchased from Schwarz/Mann. Human transferrin (90,000) and rabbit phosphorylase *a* (94,000) were obtained from Sigma. Aldolase (40,000) was provided by Pharmacia. These proteins were used in sodium dodecyl sulfate disc gel electrophoresis as molecular weight markers.

Sephadex and Sepharose filtration gels were obtained from Pharmacia. Whatman preswollen microgranular diethylaminoethyl (DEAE)-cellulose was obtained from Reeve Angel. Electrophoresis paper 2043-3, used for isoelectric focusing, was obtained from Schleicher and Schuell, Inc. Ultrafiltration chambers and membranes were obtained from Amicon. A Desaga thin layer isoelectric focusing chamber was obtained from Brinkmann Instruments. A Fisher Accumet (Model 320) expanded scale pH meter was employed for pH determinations.

*Chromatography.* The method of gel filtration through Sephadex and Sepharose in borate-saline buffer (0.16 *M* NaCl–0.2 *M* borate, pH 8.0) has previously been described (Reynolds and Johnson, 1970). Ion exchange chromatography on DEAE-cellulose in phosphate buffer, employing linear gradient salt elution, was a modification of the method of O'Daly and Cebra (1971a). Pooled protein fractions were concentrated by ultrafiltration using Amicon UM 10 membranes.

*Analytical Polyacrylamide Gel Electrophoresis.* Disc gel electrophoresis in 7 *M* urea was performed at 25° according to the method of Reisfeld and Small (1966). Bromophenol Blue was used to mark the solvent front; gels were electrophoresed at 1.5 mA/gel. Sodium dodecyl sulfate disc gel electrophoresis was done using the method described by Weber and Osborn (1969) at 25° with a constant current of 10 mA/gel. Cytochrome *c*, previously purified by gel filtra-

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[1] Abbreviations used are: sIgA, secretory immunoglobulin A; Ig, immunoglobulin; IgA, immunoglobulin A; SC, secretory component; BSC, bound secretory component; FSC, free secretory component; L chain, light chain; Gdn-HCl, guanidine hydrochloride; DEAE, diethylaminoethyl.

tion, was used as a marker for relative mobility ($R_f$) calculations. Molecular weight determinations of unknown proteins were obtained from linear regression plots of $R_f$ vs. log molecular weight using known calibration proteins. Both samples and standards were reduced with 2-mercaptoethanol prior to electrophoresis in sodium dodecyl sulfate gels with a 11% acrylamide concentration. After staining with Coomassie Brilliant Blue, the gels were scanned with a Beckman Acta III spectrophotometer at 550 nm.

*Preparative Isoelectric Focusing.* Focusing in Sephadex G-75 superfine gel was performed with modifications of the method described by Radola (1973). The protein preparation (1–100 mg) was dialyzed against 5 *M* urea, 20 ml of dialyzed protein was added to 1 g of Sephadex, and sufficient ampholine was added to give a final 1% dry weight composition. The slurry was applied to a 10 × 20 cm glass plate and allowed to air dry until the edges began to recede. Approximately 30% of the water was evaporated leaving a final urea concentration of 7 *M*. Contact with the electrode solutions (0.5% $H_2SO_4$ and 0.75% NaOH) was made with 2043-3 electrophoresis paper. Proteins were focused for 12 hr at 400 V on a Desaga thin layer isoelectric focusing chamber cooled at 4°. At the completion of focusing, gel sections 8 mm wide were removed and mixed in 0.5 ml of distilled water. Ouchterlony analysis, pH measurements, and radioactivity determinations were performed on the supernatant fluid from each gel section.

*Carbohydrate Analysis.* Free secretory component was hydrolyzed in 4 *N* HCl in vacuo for 4 hr at 110°. Analysis for glucosamine and galactosamine was performed using a Beckman Model 119 amino acid analyzer. Neutral hexose was measured by the phenol-sulfuric acid method (Dubois et al., 1956) with galactose standards.

*Amino Acid Analysis.* Proteins were hydrolyzed in vacuo at 105° for 24, 48, and 72 hr in 6 *N* HCl. Analyses were performed by the single column method with a Beckman Model 119 amino acid analyzer. Extrapolation to zero hydrolysis time was made for the labile amino acids, serine and threonine. The 72-hr values were used for the slow released amino acids valine and isoleucine. Cys/2 and methionine were determined as cysteic acid and methionine sulfone after performic acid oxidation (Moore, 1963).

*Amino Acid Sequence.* The N-terminal amino acid of native FSC was determined by dansylation (Gray, 1967) and thin layer chromatography on polyamide sheets (Gottlieb et al., 1970). Amino acid sequences were determined with a Beckman sequencer (Model 890), using both the dimethylbenzylamine (Hermodson et al., 1973) and Quadrol double cleavage programs; 5 mg of protein was used in each determination. Thiazolinone derivatives were converted to phenylthiohydantoin derivatives with 1 *N* HCl at 80° for 10 min and were subsequently identified by mass spectroscopy (Finnigan Model 1015 mass spectrometer) and amino acid analysis after back hydrolysis in vacuo with 4 *N* methanesulfonic acid at 150° for 18 hr (Lin and Chang, 1971).

*Source of Canine Sera and Colostrum.* Arterial blood from mongrel dogs was allowed to clot overnight at 4°. Following centrifugation, the serum was removed and sodium azide added to give a final concentration of 0.02%. Sera were pooled and frozen (−20°) until subsequent immunoglobulin isolation.

Colostrum was obtained by manual expression from lactating mongrels within 3 days of parturition. Sodium azide was added as a preservative and the samples were frozen until used. The colostrum was clarified by ultracentrifugation at 70,000*g* and by dialysis against borate-saline buffer. This clarified colostrum was applied to specific immunoadsorbents for the removal of sIgA and FSC.

*Antisera.* The preparation of rabbit antisera to canine IgG Fab fragments and of goat antisera to canine colostral IgA has been previously described (Reynolds and Johnson, 1971). Other antisera were raised in New Zealand white rabbits given primary immunizations with 2 mg of protein emulsified in 1 ml of complete Freund's adjuvant. Rabbits were boosted at 2 weeks using 2 mg of protein/ml of incomplete Freund's adjuvant and exsanguinated at 4–5 weeks. Goat anti-rabbit T-Piece antiserum was kindly supplied by Dr. Alexander R. Lawton (Lawton et al. 1970). Antisera to free human secretory component were obtained from Behring (batch 2535-B) (Reynolds and Newball, 1974), and from Dr. David S. Rowe[2] (M33A). Antisera to human sIgA were obtained from Hyland (lot 8212E001A1), Meloy (lot A10318631), and Behring (batch 2454 B). Antiserum to human light chains was obtained from Kallestad (lot E012).

*Preparation and Utilization of Immunoadsorbents.* Immunoadsorbents were prepared according to the method of Rejnek et al. (1969) with minor modifications; 20 ml of settled Sepharose 2B, in an equal volume of 0.1 *M* $NaHCO_3$ (pH 11.0 ± 0.2) was activated with 1 g of cyanogen bromide (previously dissolved in 3 ml dimethylformamide) for 1 hr at 4°. The activated gel was washed thoroughly with a buffer that was 0.1 *M* in $NaHCO_3$ and 0.15 *M* NaCl (pH 8.0) and the protein was coupled at a ratio of 2 mg of protein/ml of settled gel. Protein coupling was found to be 90% or greater by spectroscopic measurements.

Immunoadsorbents were utilized for the isolation of rabbit and goat antibodies using the canine antigens, IgG, IgM, and sIgA (antigen-immunoadsorbents). The isolation of desired proteins from canine colostrum and serum was accomplished using antibodies specific for light chain, $\alpha$ chain, and SC coupled to Sepharose (antibody-immunoadsorbents). In general, the protein mixtures were applied to the immunoadsorbent column and nonreacting components were removed by copious washing of the column with borate-saline buffer. Subsequent elution of the specifically bound protein was accomplished with 0.1 *M* citrate buffer (pH 2.2) followed by immediate neutralization with 2 *M* Tris (pH 10.5).

*Antibody Isolation.* Anti-canine light (L) chain antibody was isolated from rabbit anti-canine IgG Fab antisera by elution of the cross-reacting L chain antibodies from a sIgA antigen-immunoadsorbent (infra).

Antibodies to canine IgA $\alpha$ chain and SC were obtained from rabbit antisera made against peak I proteins ($\alpha$ chain and BSC, see Figure 1). These antibodies were eluted from the sIgA antigen-immunoadsorbent and the L chain cross reacting components were removed by repetitive passage of the antibody through canine IgG and IgM antigen-immunoadsorbents.

In addition, antibodies specific for $\alpha$ chain were isolated from goat antiserum to colostral IgA. This antiserum was previously shown to have no secretory component reactivity (Reynolds and Johnson, 1971). L chain cross-reactivity was removed using IgG and IgM antigen-immunoadsorbents.

*Isolation of IgG and IgM.* Canine IgG and IgM were isolated from pooled sera after ammonium sulfate precipi-

[2]World Health Organization, Lausanne, Switzerland.

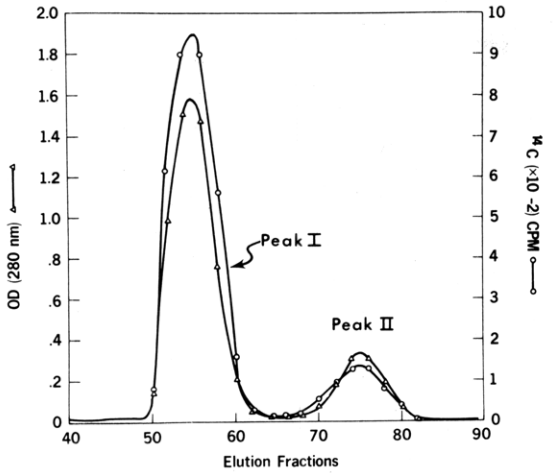


FIGURE 1: The elution profile is shown for 30 mg of lightly reduced (5 m*M* dithiothreitol) and radioalkylated sIgA filtered through a Sephadex G-100 column (2.5 × 90 cm) which was equilibrated with 6 *M* Gdn-HCl. Peak I was shown to consist of $\alpha$ chain and BSC while L chain and J chain were found within peak II (see Figure 5).

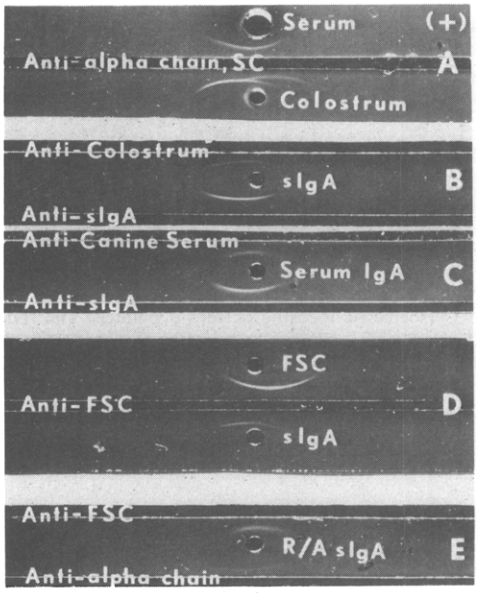


FIGURE 2: Immunoelectrophoresis was performed at 18 mA constant current for 1 hr in 1.2% agar. (A) Canine serum and clarified colostrum were reacted with rabbit anti-$\alpha$ chain, anti-SC antibody (4 mg/ml). The anodal migrating protein in colostrum was subsequently shown to be FSC. (B) sIgA (5 mg/ml) was precipitated with rabbit anti-colostrum antisera and rabbit anti-sIgA unabsorbed antiserum. (C) Rabbit anti-canine serum antiserum and rabbit anti-sIgA antiserum reacted against serum IgA (5 mg/ml). (D) Unabsorbed rabbit antiserum to FSC reacted with FSC (2 mg/ml) and also recognized the BSC in intact purified sIgA (5 mg/ml). (E) Reduced (5 m*M* dithiothreitol) and alkylated sIgA (5 mg/ml) reacted with rabbit anti-FSC antiserum to show the release and change in migration of BSC. Goat anticolostral sIgA antibody (2 mg/ml) identified the migration of $\alpha$ chain.

tation, DEAE ion exchange chromatography, and repeated gel filtration as previously described (Reynolds and Johnson, 1970).

*Isolation of sIgA and FSC.* Previously, canine sIgA has been isolated by repeated gel filtration through Sephadex G-200 and Sepharose 4B (Reynolds and Johnson, 1971). Although these procedures were adequate, a method based on affinity chromatography was desired for the preparative isolation of sIgA and FSC.

sIgA, purified by repeated gel filtration, was coupled to Sepharose 2B and used as an antigen–immunoadsorbent in order to isolate the specific antibodies to sIgA. Rabbit antisera to reduced and alkylated sIgA (peak I in Figure I) were applied to this sIgA immunoadsorbent column. The eluted antibody was shown to react with one protein in serum and two proteins in colostrum (Figure 2A). The second component in colostrum was later identified to be FSC. This antibody preparation, hereafter designated as anti-$\alpha$ chain, anti-SC, was in turn coupled to Sepharose 2B and used as an antibody–immunoadsorbent for the preparative isolation of sIgA and FSC from colostrum.

Clarified colostrum was applied to the anti-$\alpha$ chain, anti-SC immunoadsorbent column. Following the removal of unreactive proteins, FSC and sIgA were eluted and then were separated from each other by gel filtration through Sephadex G-100. sIgA was purified by additional gel filtration using Sephadex G-200 and Sepharose 6B. FSC was further purified by gel filtration in Sephadex G-100 and ion exchange chromatography using DEAE-cellulose equilibrated with 0.01 *M* phosphate buffer (pH 7.5).

As an alternate method for separating sIgA from FSC, clarified colostrum was initially applied to the anti-L chain immunoadsorbent column. The colostral Ig concentrate eluted from this column was in turn applied to the anti-$\alpha$ chain, anti-SC immunoadsorbent column for sIgA removal.

From 1 l. of colostrum, approximately 1000 mg of sIgA and 100 mg of FSC were recovered.

*Isolation of Serum IgA.* Pooled mongrel serum was applied to the anti-L chain immunoadsorbent. The eluted Ig-rich concentrate was then applied to the anti-$\alpha$ chain, anti-SC immunoadsorbent for serum IgA removal. The dimeric, 10S form of serum IgA (Reynolds and Johnson, 1971) was separated from smaller IgA monomers and aggregates by gel filtration in Sephadex G-150 and Sepharose 6B.

*Reduction of IgA Disulfide Bonds.* Disulfide bond reduction studies were performed on sIgA and serum IgA in order to determine the minimal concentration of reducing agent, dithiothreitol, required to cause maximal subunit dissociation. Studies were performed at a protein concentration of 5 mg/ml in 0.2 *M* Tris-HCl (pH 8.6). Native, unreduced samples of IgA were used as controls. Varying amounts of dithiothreitol (0.1–10 m*M*) were added to individual samples and the IgA preparations were incubated for 1 hr at 37°. After alkylation with a twofold excess of iodoacetamide, the IgA samples were analyzed using sodium dodecyl sulfate disc gel electrophoresis and by immunoelectrophoresis. The polyacrylamide gels were stained and scanned at 550 nm using a Beckman spectrophotometer.

Dissociation of the molecule was followed by measuring the increase in L chain absorbancy which corresponded with the decrease in high molecular weight protein that would not penetrate the 11% gel. Comparisons were based upon the amount of L chain released because at low concentrations of dithiothreitol the large subunits ($\alpha$ chain and SC) were not well resolved.

*Recovery of Secretory Component Bound to $\alpha$ Chain.* BSC was isolated from $\alpha$ chain using two methods. Peak I material (Figure 1) from reduced and alkylated sIgA was dialyzed against 5 *M* urea and subjected to isoelectric focusing in Sephadex (Figure 3). Utilizing a pH gradient of 5–7, BSC was partially separated from $\alpha$ chain. BSC under these conditions focused between pH 5.25 and pH 6.40 while $\alpha$ chain focused between pH 5.65 and pH 6.92. The area where no overlap occurred was pooled and the BSC was eluted from the gel. BSC was precipitated twice with

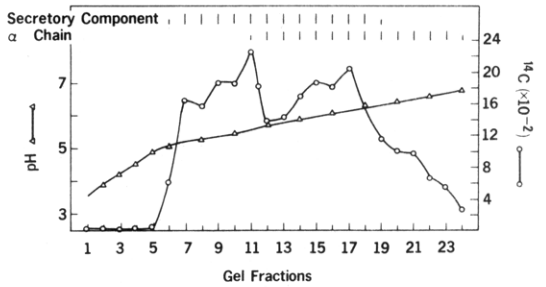


FIGURE 3: Isoelectric focusing pattern of reduced and radioalkylated sIgA; 10 mg of peak I (Figure 1) proteins were focused at 4° for 13 hr at 400 V, utilizing Sephadex G-75 as the solid support. Ampholines, pH 5–7 (1.4%) and pH 7–9 (0.14%), were used to establish the gradient in 7 *M* urea. Ouchterlony analysis (vertical bars), radioactivity measurements (○), and pH determinations (△) were performed on eluted gel fractions.

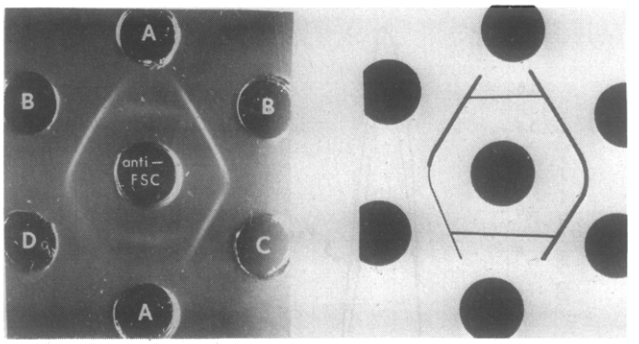


FIGURE 4: Immunodiffusion of anti-FSC antiserum against: (A) BSC isolated from the goat anti-α chain immunoadsorbent; (B) FSC; (C) sIgA; and (D) lightly reduced and alkylated FSC following denaturating and renaturing.

50% ammonium sulfate and after dialysis against 0.01 *M* $NH_4HCO_3$ was freeze dried. Although Ouchterlony analysis showed the isolated BSC to be pure, a small amount of an unknown contaminating protein was found by disc electrophoresis in sodium dodecyl sulfate.

As an alternative approach to the isolation of BSC, an antibody–immunoadsorbent was prepared using goat antibodies specific for α chain (designated goat anti-α chain immunoadsorbent). Radiolabeled peak I proteins (Figure 1) were applied to this goat immunoadsorbent column, and upon washing with borate–saline buffer, approximately 10% of the radioactivity passed through the column. The protein in the effluent buffer was concentrated and had the same mobility as FSC in disc gel electrophoresis. Ouchterlony analysis of this protein showed cross-reactivity with FSC (Figure 4). The immunoadsorbent was further eluted with 2 *M* urea and analysis of the eluted protein showed both α chain and BSC to be present. Attempts to selectively remove more of the BSC by using a 2 *M* urea gradient or by heating the sample to 60° (to dissociate noncovalent interactions) prior to application were unsuccessful.

*Reduction and Alkylation of FSC.* To assay for free sulfhydryl groups, native FSC was dissolved in 6 *M* guanidine-HCl (Gdn-HCl), 1 *M* Tris-HCl, and 1.2% EDTA (pH 8.5). After thoroughly flushing with nitrogen, a fourfold molar excess of [$^{14}$C]iodoacetamide was added and the protein was incubated at 25° for 2 hr. Following extensive dialysis, the protein was counted for radioactivity using a Packard (Model 3375) TriCarb scintillation spectrometer.

In order to compare immunological determinants, a sample of FSC was subjected to the same reducing and denaturing conditions that were used to release BSC from sIgA (Figure 4). FSC was dialyzed against 0.2 *M* Tris-HCl (pH 8.6) and was reduced with 5 m*M* dithiothreitol for 1 hr at 37°, and alkylated with 10 m*M* iodoacetamide. This FSC was dialyzed against 6 *M* Gdn-HCl for 24 hr to promote unfolding and was further dialyzed against borate–saline buffer in order to renature the protein.

## Results

*Isolation of sIgA.* By the use of specific immunoadsorbents, sIgA and FSC were isolated directly from clarified colostrum and further separated from each other by gel filtration through Sephadex G-100. Protein which eluted at the void volume of the column (37%) contained sIgA while FSC was recovered at 44% of the total gel bed volume. To purify sIgA from aggregates and smaller molecular weight monomers, sIgA was gel filtered through Sephadex G-200 and Sepharose 6B. In several experiments an additional isolation procedure with antibody–immunoadsorbents to L chain and to α chain and SC was used to isolate sIgA from colostrum. Both procedures resulted in identical preparations of sIgA. Purity of sIgA was established by immunoelectrophoresis at a wide range of protein concentrations against rabbit antisera to canine colostrum and whole serum. In each case a single precipitin line resulted (Figure 2B).

*Isolation of Free Secretory Component.* Free SC was subjected to additional gel filtration in Sephadex G-100 followed by ion exchange chromatography on DEAE-cellulose at pH 7.5 in order to remove possible sIgA contamination. The FSC preparation was eluted from the DEAE-cellulose with a 0.3 *M* NaCl linear gradient at a conductivity between 7 and 14 mmhos. FSC appeared pure by immunoelectrophoresis using rabbit antisera prepared against colostrum, whole serum, and isolated FSC. Disc electrophoresis in urea and in sodium dodecyl sulfate of 10 μg of FSC revealed a single band (Figure 5B, e and f), but electrophoresis of 50 μg of FSC in sodium dodecyl sulfate revealed a faint band corresponding to a smaller molecular weight protein (less than 10% contamination). This contaminating protein could not be identified immunologically and may possibly represent fragments of FSC or sIgA.

*Isolation of Serum IgA.* IgA was isolated from pooled mongrel sera using two immunoadsorbents. The Ig-rich concentrate which eluted from the anti-L chain immunoadsorbent was in turn applied to the anti-α chain, anti-SC immunoadsorbent. This eluted IgA was gel filtered in order to isolate the dimeric molecule. This IgA preparation produced a single line on immunoelectrophoresis at various protein concentrations against rabbit antisera to whole serum and colostrum (Figure 2C).

*Reduction Studies Using sIgA and Serum IgA.* In order to determine the optimal amount of dithiothreitol necessary to reduce interchain disulfide bonds, samples of sIgA and serum IgA were incubated with varying amounts of reducing agent (Table I). The alkylated samples were subjected to disc electrophoresis in sodium dodecyl sulfate and the release of light chains was monitored along with the disappearance of high molecular weight protein. In addition, immunoelectrophoresis was used to monitor the release of SC, since released BSC assumes a migration similar to that of FSC (Figure 2D and E).

The results (Table I) indicate that 5 m*M* dithiothreitol was optimal for the dissociation of both sIgA and serum IgA, since there was maximum release of L chain and all

Table I: Release of L Chain from IgA with Increasing Amounts of the Reducing Agent Dithiothreitol.[a]

| [Dithiothreitol] (m*M*) | Serum IgA % L Chain Release | sIgA % L Chain Release |
|---|---|---|
| 0 (control) | 10 | 11 |
| 0.1 | 7 | 10 |
| 0.5 | 15 | 22 |
| 1.0 | 55 | 78 |
| 5.0 | 100 | 105 |
| 10.0 | 100 | 100 |

[a] Representative study showing the release of L chains from native IgA and reduced and alkylated IgA following reduction with increasing amounts of dithiothreitol. The proteins were subjected to disc electrophoresis in sodium dodecyl sulfate and the gels were scanned at 550 nm after staining. Percentage L chain release was determined by comparing the area of the L chain band for each sample to the area obtained by reduction with 10 m*M* dithiothreitol.

the protein migrated into the polyacrylamide gel. At 10 m*M* no increase in dissociation was observed while at 1 m*M* the dissociation was significantly less. As an indication of maximum L chain release, IgA was totally reduced with a large excess of 2-mercaptoethanol (20% v/v) (Weber and Osborn, 1969). The amount of L chain released under these conditions was similar to that obtained with the higher dithiothreitol concentrations.

The unreduced controls of both sIgA and serum IgA showed a similar release of trace amounts of small molecular weight proteins (5–10%),[3] while the majority of the sample was not able to penetrate the gel. These small molecular weight proteins are believed to be light chain and light chain dimers. The unreduced sIgA released a small amount of BSC (less than 10%) while a corresponding band was not observed for the serum IgA control.

The disc electrophoresis data suggested that reduced and alkylated IgA could be partially separated into subunits by gel filtration. sIgA was lightly reduced with 5 m*M* dithiothreitol and alkylated with [$^{14}$C]iodoacetamide in an excess of cold iodoacetamide. When subjected to gel filtration through Sephadex G-100, equilibrated with 6 *M* guanidine-HCl, two peaks were observed (Figure 1). Sodium dodecyl sulfate disc electrophoresis of concentrated peak I material was shown to consist of two major protein bands (Figure 5A, b). Further analysis of the peak by double immunodiffusion showed that these proteins corresponded to reduced $\alpha$ chain and BSC. Analysis of peak II (Figure 1) by disc electrophoresis in sodium dodecyl sulfate (Figure 5A, c) revealed a single band, while in urea gels, the typical banding of L chains and what appeared to be the fast migrating J chain bands (Halpern and Koshland, 1970) were observed (Figure 5A, d).

In contrast, lightly reduced and radioalkylated serum IgA gave a similar chromatographic separation in Sephadex G-100 but analysis of peak I revealed the absence of BSC by immunoprecipitation. Urea disc electrophoresis of peak II showed the L chain and J chain pattern observed for sIgA.

*Isolation of Bound Secretory Component.* sIgA was lightly reduced and separated into its major subunits by gel filtration in 6 *M* Gdn-HCl. Peak I (Figure 1) was shown immunologically to contain $\alpha$ chain and BSC. To isolate

[3] Percentages are based on the total amount of protein observed by scanning the stained gel.

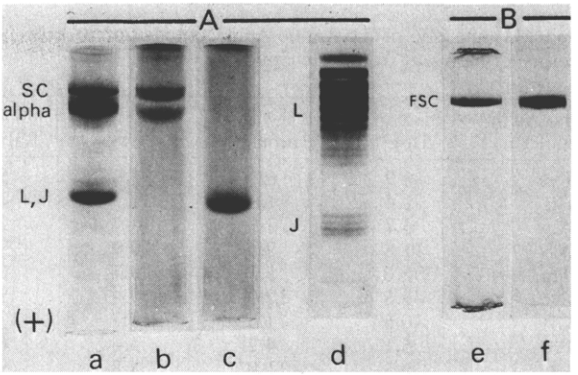


FIGURE 5: (A) Sodium dodecyl sulfate disc gel electrophoresis with a 11% acrylamide concentration. Lightly reduced (5 m*M* dithiothreitol) and alkylated sIgA (50 $\mu$g) (a) was gel filtered through Sephadex G-100 and resolved into (b) peak I (30 $\mu$g) and (c) peak II (10 $\mu$g) subunits. Disc electrophoresis of peak II (100 $\mu$g) in alkaline urea (pH 9.4) and 4% acrylamide (d) revealed the multiple banding pattern of L chain and the fast migrating anodal J chain. (B) Following gel filtration through Sephadex G-100 and ion exchange chromatography, FSC (10 $\mu$g) was subjected to disc electrophoresis in sodium dodecyl sulfate (e) with an acrylamide gel concentration of 11% and in alkaline urea (f) with a 4% acrylamide concentration.

BSC, peak I proteins were subjected to isoelectric focusing in 7 *M* urea (Figure 3). Under these conditions BSC was shown to have an average focusing pH of 5.8 while $\alpha$ chain focused at an average pH of 6.3. Due to the close spacing only a small amount of BSC could be isolated free of $\alpha$ chain.

As an alternative approach, the mixture of BSC and $\alpha$ chain was applied to an immunoadsorbent specific for $\alpha$ chain and approximately 10% of the radioactivity passed through the column. The borate–saline protein effluent was shown to contain released BSC by immunological cross-reactivity with FSC antiserum. The two isolation procedures resulted in low yields of BSC and were unsuccessful in isolating BSC free of unknown trace contaminants.

*Characterization of Secretory Component.* Ouchterlony analysis of canine FSC against two antisera to human secretory component and antiserum to rabbit T-Piece showed no cross-reactivity. Anti-canine FSC antisera showed no cross reactivity against partially purified human secretory component (Reynolds and Newball, 1974). Double immunodiffusion of FSC and serum IgA against antiserum to purified FSC showed no cross-reactivity, whereas a line of complete identity was seen with FSC and sIgA (Figure 4). The isolated BSC was shown to give a line of partial identity with native FSC, with reduced and alkylated FSC following denaturing and renaturing, and with sIgA.

Totally reduced FSC and BSC showed identical mobilities in dodecyl sulfate disc electrophoresis corresponding to a molecular weight of 77,500 ± 6500 ($n$ = 6). Isoelectric pH values of 5.6 and 5.8 were obtained for FSC and isolated BSC by thin layer isoelectric focusing in 7 *M* urea.

The carbohydrate content of FSC is 18.9% by weight (5.9% neutral hexose, 6.4% glucosamine, 6.6% galactosamine). An extinction coefficient ($E_{280}$(1%)) of 12.7, determined for human FSC in water (Kobayashi, 1971), was used in these determinations.

The amino acid analysis of FSC was performed on timed hydrolysates and the corrected data are reported in Table II as mole percent in order to allow for comparison with the data accumulated from other species.

Following identification of the N-terminal amino acid of

Table II: Comparison of the Amino Acid Composition of Free Secretory Component from Four Species.

| Amino Acids (mol %) | Dog[a] | Human[b] | Cow[c] | Rabbit[d] |
|---|---|---|---|---|
| Lys | 6.9 | 6.2 | 5.6 | 5.6 |
| His | 1.4 | 1.0 | 1.3 | 2.1 |
| Arg | 3.7 | 3.8 | 4.7 | 3.7 |
| Asx | 11.8 | 9.4 | 10.9 | 9.5 |
| Thr | 6.0 | 5.3 | 6.7 | 6.1 |
| Ser | 8.5 | 12.0 | 9.4 | 9.2 |
| Glx | 10.5 | 11.3 | 10.7 | 12.0 |
| Pro | 5.1 | 4.2 | 4.5 | 6.7 |
| Gly | 9.3 | 11.0 | 8.8 | 9.8 |
| Ala | 4.2 | 5.7 | 6.2 | 5.2 |
| Cys/2 | 4.9 | 2.4 | 2.2 | 3.7 |
| Val | 8.4 | 8.1 | 9.3 | 8.4 |
| Met | 1.2 | 0 | 0.4 | 0.4 |
| Ile | 4.0 | 2.9 | 0.3 | 3.0 |
| Leu | 7.5 | 8.0 | 7.2 | 8.1 |
| Tyr | 4.0 | 3.7 | 3.9 | 2.8 |
| Phe | 4.3 | 3.1 | 3.4 | 3.8 |

[a] Canine values were based on 18-hr hydrolysates with corrections for the labile amino acids. The 72-hr values were used for the slow releasing amino acids. Cys/2 was determined as cysteic acid and methionine was determined as methionine sulfone following performic acid oxidation. No free cysteine residues were detected by radioalkylation with [$^{14}$C] iodoacetamide in the presence of 6 *M* Gdn-HCl. [b] Obtained from Lamm and Greenberg (1972). Values for labile amino acids were extrapolated to zero hydrolysis time and for slow releasing amino acids the 70-hr values were used. [c] Data obtained from Tomasi et al. (1974). Values for threonine, serine, and Cys/2 were corrected for loss due to 20-hr hydrolysis. [d] Obtained from O'Daly and Cebra (1971b). Hydrolysis time was 20 hr. The Cys/2 value was corrected for degradation.

Table III: N-Terminal Sequence of Free Secretory Component.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Canine | Lys-Ser-Pro-Ile-Phe-Gly-Pro-Glu-Glu-Val-Asn-Ile | | | | | | | | | | | |
| Human[a] | Lys-Ser-Pro-Ile-Phe-Gly-Pro-Glu-Glu-Val-Asp-Ser | | | | | | | | | | | |
| Bovine[b] | Lys-Ser-Pro-Ile-Phe-Gly-Pro-Glu-Glu-Val-Asp-Ser | | | | | | | | | | | |

[a] Sequence reported by Cunningham-Rundles et al. (1974). [b] Sequence reported by Tomasi et al., in preparation.

Table IV: Composite Amino Acid Sequence for Canine FSC.[a]

| Step | Residues Identified by Amino Acid Analysis | Residues Identified by Mass Spectroscopy | Composite Sequence | Recoveries[c] (%) |
|---|---|---|---|---|
| 1 | Lys | ND | Lys | 97 |
| 2 | NF | Ser | Ser | ND |
| 3 | Pro | ND | Pro | 54 |
| 4 | Ile | ND | Ile | 78 |
| 5 | Phe | Phe | Phe | 74 |
| 6 | Gly | Gly | Gly | 76 |
| 7 | Pro | Pro | Pro | 34 |
| 8 | Glu[b] | Glu | Glu | ND |
| 9 | Glu | Glu | Glu | 55 |
| 10 | Val[b] | Val | Val | 54 |
| 11 | Asx | Asn | Asn | 64 |
| 12 | Ile | Leu | Ile | 56 |

[a] ND, not determined; NF, not found. [b] These residues contained a higher than background amount of Gly. [c] Recoveries were based on the total amount of protein used in the sequence studies as determined from the amino acid composition of an aliquot.

canine FSC as lysine, the sequence of the N-terminal end was examined. The sequence of the first 12 amino acids, based on three determinations, proved to be similar to the sequence reported for human and bovine free secretory component (Table III). Recoveries beyond residue 12 were low and since this segment of the sequence is questionable, the next three amino acids are only tenatively reported as: Val-Glu-Gly. Repetitive recoveries, based on Ile in steps 4 and 12, proved to be 94%. The recoveries at each residue (Table IV) were calculated from amino acid analyses of a Quadrol double cleavage sequence determination.

## Discussion

In initial studies of canine sIgA (Reynolds and Johnson, 1971), it was difficult to immunologically identify secretory component; in this study, using antiserum to reduced and alkylated sIgA, we were able to detect secretory component (SC) in its various forms. This in turn led to an isolation procedure for IgA and secretory component based on affinity chromatography. The two-step isolation, using columns with immunological affinity for L chain and $\alpha$ chain, eliminated extraneous protein contamination of the IgA preparations. Purified sIgA was used to raise antibody to SC, which subsequently resulted in FSC preparations free of contaminants which have traditionally presented difficulties. The yields of FSC isolated by our procedure were approximately five times greater than the recoveries reported for human FSC (Lamm and Greenberg, 1972); however, a difference in colostral FSC concentration between species cannot be discounted. The isolation procedures for BSC, following the reduction of sIgA, were unsatisfactory because the recoveries were low and contaminating protein (believed to be $\alpha$ chain) was found.

Complete immunological cross-reactivity was observed for FSC and sIgA using antiserum to FSC, whereas the isolated BSC showed only partial cross-reactivity with native FSC, with reduced and alkylated, denatured and renatured FSC, and with sIgA. These findings indicate that a loss of antigenic determinates on isolated BSC may occur during its release from the reduced sIgA. Since the isolated BSC and FSC were shown to have similar physical properties (electrophoretic migration, isoelectric focusing point, and molecular weight), this antigenic dissimilarity suggests a possible conformational difference between the two forms of secretory component. This structural difference or rearrangement may occur at the cellular level as SC becomes covalently attached to the dimeric IgA, but it can only be demonstrated when sIgA is in reduced form.

BSC has been shown to be covalently bound to the $\alpha$ chain (Mestecky et al., 1974) and to attach only to the dimeric form of IgA (Mach, 1970; Brandtzaeg, 1974). Since secretory component has been found attached to exocrine IgM (Brandtzaeg, 1974), it has been suggested that J chain, in addition to promoting polymerization, may be responsible for the binding of SC to immunoglobulins via disulfide bond rearrangement (Mestecky et al., 1973; Wilde and Koshland, 1973). If disulfide rearrangement does occur, a conformation change in SC may also result, which possibly explains the partial immunological reactivity between the released BSC and FSC found in these studies.

sIgA and serum IgA both dissociated into their subunits when reduced with 5 m*M* dithiothreitol, but with a concentration of 1 m*M*, only partial release of constituents occurred. However, unreduced sIgA and serum IgA released small molecular weight proteins believed to be analogous to the L chain and L chain dimers previously reported for

human sIgA (Mestecky et al., 1972) and for the human $IgA_2$ subclass (Grey et al., 1968). A small amount of SC (less than 10%) appeared to be noncovalently bound to the native canine sIgA. This noncovalently attached SC seems to occur in vivo but may also represent an artifact of the isolation procedure even though a similar finding was reported for human sIgA (Mestecky et al., 1972). The appearance of non-covalently bound subunits of canine colostral and serum IgA suggests that two subclasses of IgA exist. These subclasses may prove to be similar to the $A_1$ and $A_2$ subclasses found in human IgA (Grey et al., 1968).

J chain was released from lightly reduced and alkylated serum and colostral IgA. The presence of J chain has been previously described in canine myeloma IgA (Kehoe et al., 1972) serum IgA (Kobayashi et al., 1973), and in colostral IgA (Meinke and Spiegelberg, 1974).

Molecular weight determination of SC by disc electrophoresis revealed an identical value of 77,500 for both BSC and FSC. This value should be used cautiously. Earlier studies (Fletcher and Woolfolk, 1972) showed aberrant behavior of glycoproteins due to a lower free mobility during electrophoresis in sodium dodecyl sulfate, suggesting that the estimate of molecular weight for SC may be high.

The amino acid sequence of the N-terminal region of canine FSC was similar to the sequence reported for human (Cunningham-Rundles et al., 1974) and bovine FSC (T. B. Tomasi and J. D. Capra, in preparation). The first ten residues were found to be identical (Table III) while differences occur at residues 11 and 12 for the human and cow. These differences can be explained by a single base change in the gene coding for these amino acids.

Although these initial sequence data and the amino acid compositions suggest homology among human, bovine, and canine FSC, immunological cross-reactivity could not be detected between human and canine FSC. In addition, no cross-reactivity was observed for human sIgA and L chain antisera against canine sIgA or serum IgA. Canine FSC was shown to contain more methionine residues than the human (Lamm and Greenberg, 1972), cow (T. B. Tomasi, J. D. Capra, M. Kehoe, R. Labib, and N. Calvanico, in preparation; Tomasi and Capra, 1974), or rabbit (O'Daly and Cebra, 1971b). The presence of these methionine residues has provided a feasible approach (CNBr cleavage) for our current studies on the structure of SC and its attachment to the $\alpha$ chain in sIgA.

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# The Complete Amino Acid Sequence of a Cardiotoxin from the Venom of *Naja naja* (Cambodian Cobra)†

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ABSTRACT: The complete amino acid sequence of a small, basic protein with cardiotoxic activity is described. This toxin, designated *Naja naja* F8, was isolated from the venom of *Naja naja,* of Cambodian origin, by gel filtration on Sephadex G-75 followed by gradient ion exchange chromatography on Bio-Rex 70. The cardiotoxin F8, molecular weight 6727 from amino acid composition, consists of 60 amino acids in a single peptide chain cross-linked by four disulfide bridges and is devoid of histidine, tryptophan, and glutamic acid. The chymotryptic and tryptic peptides from the performic acid oxidized toxin were separated by gel filtration on Sephadex G-25 and zone electrophoresis in columns of cellulose powder. The sequence was established by Edman degradation, using the direct phenylthiohydantoin method, and with the aid of carboxypeptidase A, and is similar to the sequences reported for other cardiotoxins, cytotoxins, and/or lytic factors from cobra venoms, all of which show considerable homology with the functionally distinct neurotoxins.

Cobra venoms contain many small basic proteins representing several different pharmacological activities (Lee, 1971) and immunological classes (Boquet et al., 1972). The *Naja naja* cardiotoxin F8 described below is serologically related to the *Naja nigricollis* toxin $\gamma$ and is distinct from all the curariform neurotoxins tested (Boquet et al., 1972). Cardiotoxin F8 is similar in sequence to the cardiotoxin from *Naja naja atra* venom (Narita and Lee, 1970), *Naja nigricollis* toxin $\gamma$ (Fryklund and Eaker, 1975), the *Naja naja* cytotoxins I (Hayashi et al., 1971) and II (Takechi and Hayashi, 1972), the lytic protein 12B from *Haemachatus haemachates* venom (Fryklund and Eaker, 1973), the two cytotoxins from *Naje naje annulifera* (Weise et al., 1973), the major cytotoxin from *Naja melanoleuca* (Carlsson and Joubert, 1974), and *Naja mossambica mossambica* (Louw, 1974).

## Materials and Methods

*Isolation of the Cardiotoxin.* The *Naja naja* venom of Cambodian origin was a gift from Dr. Paul Boquet, Pasteur Institute, Garches, France, and had been desiccated over silica gel; 1.0 g of dried crude venom was dissolved in 10.0 ml of 0.2 *M* ammonium acetate and the solution was centrifuged for 10 min at 20,000*g*. The clarified solution was separated on a 3.2 × 70 cm column of Sephadex G-75 in the same medium. The fraction containing the cardiotoxic and neurotoxic activities was further separated by ion exchange chromatography on Bio-Rex 70, minus 400 mesh, equilibrated with 0.20 *M* ammonium acetate at pH 7.3, using a 2-l. concave gradient of 0.11 vs. 1.5 *M* ammonium acetate. Details regarding the preparation and equilibration of the resin and the chromatographic technique have been described elsewhere (Karlsson et al., 1971).

*Characterization and Sequence Analysis.* Toxicity assays were done in triplicate at each dose level using female albino mice weighing ca. 20 g. The injections were done intravenously or intraperitoneally in 0.1 or 0.5 ml of 0.9% saline, respectively.

For sequence analysis the cardiotoxin was oxidized with preformed performic acid and recovered by lyophilization as described by Hirs (1956).

Amino acid analyses of the native and oxidized toxin were done with a Bio-Cal BC-200 analyzer equipped with an Infotronics CRS-110A integrator following hydrolysis at 110° in thoroughly evacuated tubes in 6 *N* HCl containing 10 mg/ml of reagent grade phenol. The molar absorptivity of the native cardiotoxin was determined in conjunction with the amino acid analyses as described by Karlsson et al. (1972).

Digestions with trypsin and chymotrypsin and the separation of peptides by gel filtration and column electrophoresis were done as described by Fryklund et al. (1972). Digestions with carboxypeptidases A and B and manual Edman degradation by the direct phenylthiohydantoin procedure were performed as described by Fryklund and Eaker (1973).

## Results

*Isolation and Characterization.* Gel filtration on Sephadex G-75 resolved the crude venom into five protein fractions (Figure 1) which, together with the nonprotein fraction VI, accounted for all of the material applied to the column. Fraction IV containing the cardiotoxic and neurotoxic activity was further separated by ion exchange chromatography as illustrated in Figure 2. Peak 8 exhibited cardiotoxic activity and eluted in a single symmetrical peak upon rechromatography under the same conditions. Further evi-

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