Property and Physiological Role of Biliary Secretory IgA in Rats

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(Received July 29, 1982)

Summary Secretory IgA (sIgA) and monomeric IgA (mIgA) were purified from normal rat bile, and their role in the gastrointestinal tract was investigated. Biliary sIgA has a molecular weight of approximately 400,000 daltons and a sedimentation constant of 11.7S. Thus, sIgA obtained from rat bile had physicochemical properties similar to those reported for sIgA in other secretions, and probably consists of L-chains, α -chains and secretory component.

After *in vitro* incubation with trypsin or intestinal fluid, sIgA remained intact, whereas mIgA was hydrolyzed. Rats challenged repeatedly with dinitrophenylated bovine serum albumin (DNP-BSA) had specific IgA antibody against DNP in the bile and feces as measured by a radioimmunoassay using [³H]-DNP-lysine. Our results demonstrate a possibility that biliary sIgA, not mIgA, has an important role which inhibits the absorption of foreign antigen from intestine.

Key Words secretory IgA, biliary, antitryptic, IgA, secretory component

Secretory IgA (sIgA) protects infants against food allergies, and certain infectious diseases and toxins to which they are particularly vulnerable, *e.g.* neonatal meningitis (1–3). In the intestine and respiratory tract, sIgA is produced by plasma cells located close to the epithelial surface (3–5). External secretions such as tears, saliva and milk contain sIgA (6), and colostrum contains particularly high concentrations. For young infants with only trace amounts of sIgA in the gut lumen, sIgA in breast milk is an important biologically active substance capable of providing passive host defense (7).

Recently, it has been demonstrated that sIgA is also secreted into the bile, across a strong concentration gradient. Several observations suggest that the liver plays a major role in the selective transport of circulating sIgA into the upper gastrointestinal tract (8, 9). In particular, biliary sIgA seems to play the principal role in protecting weanling infants against foreign antigens. It is not clear, however,

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whether the function of biliary sIgA in the intestine is the same as that of colostrum sIgA. In the present paper, we characterized sIgA purified from rat bile and studied the physiological role of biliary sIgA in the gastrointestinal tract.

MATERIALS AND METHODS

Animals. Male Wistar rats, weighing about 300 g, were anesthetized (2.5 mg nembutal/100 g body weight) and cannulae were inserted for bile collection. During the few days of bile collection, rats were fed a commercial chow (Oriental Yeast Co., Tokyo) and water containing 5% glucose and 0.9% NaCl *ad libitum*.

Rats weighing 100 g were raised on the same commercial chow for 6 weeks and injected intraperitoneally at weekly intervals with 0.2 ml of dinitrophenylated bovine serum albumin (DNP-BSA) (20 mg/ml) in an equal volume of Freund's incomplete adjuvant. Bile was obtained by cannulation of the common bile duct for a few days prior to decapitation. Also, feces collection was carried out for 2 days before the bile collection.

Purification of biliary sIgA and monomeric IgA. sIgA and monomeric IgA (mIgA) were purified from 410 ml (total IgA 279 mg) of bile by the procedure shown in Fig. 1. After centrifugation at $15,000 \times g$ for 15 min, the clarified bile was precipitated with 50% saturated ammonium sulphate. The precipitate was dialyzed against 0.01 M Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl for 18 hr at 2°C. The sample was concentrated using a Diaflo membrane UM-10 and applied to a Sepharose 6B column. All columns for gel filtration were equilibrated and eluted with 0.01 M Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl. For ion exchange

Fig. 1. Outline of purification of sIgA and mIgA from rat bile.

column chromatography, DEAE-cellulose DE-52 was equilibrated with 0.01 M Tris-HCl buffer (pH 8.0) containing 0.02 M NaCl, and samples were eluted with a linear gradient of 0.02–0.4 M NaCl in 0.01 M Tris-HCl buffer (pH 8.0). During the purification, IgA was detected by single radial immunodiffusion using antiserum against rat α -chain (10).

Immunochemical methods. Biliary IgA was detected by conventional immunoelectrophoresis as described by Grabor and Williams (11) and Ouchterlony immunodiffusion using 1.2% agarose gel (12). Quantitative estimation of IgA was performed by single radial immunodiffusion according to the procedure of Mancini *et al.* (10).

The anti-DNP antibody binding activity in bile, feces and serum was determined by the modified radioimmunoassay of Hanson *et al.* (13) using [3 H]-DNP-lysine. Samples of wet feces, from rats immunized with DNP-BSA, were homogenized in 5 volumes of 0.1 M borate buffer (pH 8.4) containing 0.9% NaCl in a Waring blender. After centrifuging at $41,000 \times g$ for 30 min, the supernatant was pretreated with DEAE-Sephadex A-25 to remove the color and mucus.

Samples (20 μ l) were incubated with 30 pmol of [3 H]-DNP-lysine (10,000 cpm in 10 μ l) in the presence or absence of unlabeled DNP-lysine (3 nmol) for 60 min at 2°C. To separate free from bound DNP-lysine, 200 μ l of 40% polyethylene glycol 6,000 was added and the incubation continued for 16 hr at 2°C. The mixture was centrifuged at 3,000 rpm for 60 min and the precipitate was dissolved in 1 ml of redistilled water. The sample was added to 10 ml of toluene-Triton X-100 (2:1) scintillation cocktail containing 0.4% (w/v) of Omunifluor and the radioactivity was determined in a liquid scintillation counter (Beckman LS-7500). The specific binding is expressed as total binding minus non-specific binding.

Treatment of biliary sIgA with trypsin or intestinal fluid. Resistance to proteolytic enzymes was tested by incubating 2 mg of purified sIgA, mIgA and bovine γ -globulin in 1 ml of 0.01 M Tris-HCl buffered saline (pH 8.0) with 0.1 mg of trypsin or 0.1 ml of intestinal fluid obtained from normal rats. The mixtures were incubated for 16 hr at 22°C or for 3 hr at 37°C. Trypsin digestion was stopped by the addition of a 10-fold excess of soybean trypsin inhibitor. The substrate digests were fractionated on a Sephadex G-150 column (1.7 $\times$ 14 cm) with 0.01 M Tris-HCl buffer saline (pH 8.0) and by polyacrylamide gel disc electrophoresis.

Other determinations. Polyacrylamide gel disc electrophoresis was performed by the method of Davis using a separating gel containing 7.5% (w/v) acrylamide, and with a running buffer of pH 9.5 (14). SDS-gel disc electrophoresis was carried out by the method of Hokin *et al.* (15). Sucrose density gradient analysis (5 to 20% sucrose) was carried out at $368,400 \times g$ for 5 hr at 2°C in a preparative ultracentrifuge (Hitachi 65P with RPS-65-TA rotor). The sedimentation constant was estimated by the method of Martin and Ames using bovine serum γ -globulin (7 S) as the standard (16). Protein was determined by the method of Lowry *et al.* using bovine serum albumin as the standard (17).

Chemicals. ϵ -[Phenyl-3,5- 3 H]-DNP-L-lysine (5.3 Ci/mmol) and Omunifluor

were purchased from New England Nuclear Co. (Boston, U.S.A.). Bovine pancreatic trypsin (9,000 units/mg) and soybean trypsin inhibitor were obtained from Sigma Chemical Co. (St. Louis, U.S.A.). Antisera against rat α -chain and L-chain

Fig. 2. Molecular weight estimation of biliary sIgA and its subunits. Molecular weights of purified biliary sIgA and mIgA were estimated by gel filtration of Sepharose 6B column (2.6×57 cm) equilibrated with 0.01 M Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl. Molecular weight of the subunits of purified biliary sIgA were estimated by SDS-gel disc electrophoresis using 0.1% SDS and 7.5% polyacrylamide gel. Sample was treated with 1% SDS in adequate buffer for 90 min at 37°C . Standard proteins employed were cytochrome *c* (M.W. 12,400), chymotrypsinogen (M.W. 25,000), ovalbumin (M.W. 45,000), bovine serum albumin (M.W. 67,000), bovine γ -globulin (M.W. 160,000) and horse spleen apo-ferritin (M.W. 480,000).

were purchased from Milea/Mann Co. (U.S.A.). DNP-BSA was prepared by the method of Iverson (18).

RESULTS

Properties of rat biliary sIgA

Purification of sIgA and mIgA from rat bile yielded 57 and 9 mg, respectively. Bile collected in one day contained approximately 10 mg of sIgA. The purified sIgA had a molecular weight of about 400,000 daltons as estimated by SDS-gel disc electrophoresis and gel filtration through Sepharose 6B column (Fig. 2-A). Biliary sIgA was composed of α -chains, L-chains and secretory component, as demonstrated by SDS-gel disc electrophoresis (Fig. 2-B). By this method, however, J-chain could not be clearly detected. The sedimentation constant was about 11.7S according to sucrose density gradient analysis. When the specificity and reactivity of the purified biliary sIgA antiserum were tested in Ouchterlony immunodiffusion, fusion of the adjacent precipitin lines indicated that the sIgA obtained from bile was serologically pure (Fig. 3).

Resistance of biliary sIgA to proteolytic enzymes

If biliary sIgA functions to block the intestinal absorption of foreign macromolecules which could otherwise induced both local and systemic immune response, it needs to be resistant to proteolytic enzymes. Accordingly, purified biliary sIgA was

Fig. 3. Ouchterlony immunodiffusion of purified biliary sIgA against its antiserum. Rabbits (2.8 kg) were immunized with 1.7 mg of purified biliary sIgA in 2.6 ml of redistilled water in an equal volume of Freund's incomplete adjuvant to obtain biliary sIgA antiserum. After 4 weeks, 0.4 ml of the same solution was injected additionally. The antiserum with 0.1% (w/v) of sodium azide was frozen at -20°C until used. A sample (6 μl) was placed in each well. A, anti sIgA serum; 1, purified biliary sIgA; 2, crude bile.

Fig. 4. Elution profiles of biliary sIgA digests by trypsin. Two mg of purified biliary sIgA, mIgA or bovine γ -globulin in 1 ml of 0.01 M Tris-HCl buffered saline (pH 8.0) was incubated with 0.1 ml of trypsin (1 mg/ml) for 0 hr, 3 hr at 37°C or 16 hr at 22°C. After adding 0.1 ml of soybean trypsin inhibitor (2 mg/ml) to stop the reaction, the substrate digests were fractionated on a Sephadex G-150 column (1.7 $\times$ 14 cm) with 0.01 M Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl. The flow rate was 30 ml/hr.

incubated with trypsin, and the incubated mixture was fractionated on a Sephadex G-150 column and by disc electrophoresis. The resulting elution profile and the electrophoretic patterns were not detectably different from those of untreated sIgA (Figs. 4, 5). In contrast, after biliary mIgA and bovine serum γ -globulin were incubated with trypsin, the elution profile and electrophoretic patterns were greatly altered. Similarly, while the biliary sIgA was not digested by incubation with intestinal fluid obtained from normal rats, both biliary mIgA and bovine γ -globulin

Fig. 5. Electrophoretic patterns of biliary sIgA digests by trypsin. A portion of the incubated mixtures described in Fig. 4 was used for 7.5% polyacrylamide gel disc electrophoresis. The density of disc gel was read by densitometry.

Fig. 6. Elution profiles of biliary sIgA digests by intestinal fluid. The incubation conditions and others were the same as Fig. 4, except that 0.1 ml of intestinal fluid was substituted for trypsin. The proteolytic activity of intestinal fluid was adjusted to that of trypsin.

were degraded (Fig. 6). These results suggest that biliary sIgA, but not mIgA, is resistant to digestion by proteolytic enzymes.

Specific antibody against DNP-BSA in bile and feces

In order to examine whether sIgA antibody appears in bile after immunization and if biliary sIgA is hydrolyzed during passage through the intestinal tract, rats were given weekly injections of DNP-BSA intraperitoneally for 6 weeks. The concentration of specific antibody in bile and feces was determined by a radioimmunoassay using [^3H]-DNP-lysine. The specific binding activity in bile and feces

Table 1. Specific binding of [^3H]-DNP-L-lysine to bile, feces and serum of rats immunized with dinitrophenylated bovine serum albumin.

Rats weighing 100 g were raised on a commercial chow for 6 weeks and 2 mg of DNP-BSA with Freund's incomplete adjuvant were intraperitoneally injected at weekly intervals. The collection of feces was carried out for 3 days from the 5th day through the 2nd day before sacrifice, and bile was collected for 2 days before sacrifice. The binding activity of [^3H]-DNP-lysine to bile, feces and serum was determined by the modified method of Hanson *et al.* (13). The specific activity are expressed as total binding minus non-specific binding.

Groups	Number of rat	Bile	Feces	Serum
			(dpm/mg of protein)	
Control	5	N.D.	N.D.	N.D.
Immunized	6	5.07 $\pm$ 0.42	107.1 $\pm$ 27.0	5,069 $\pm$ 721

The values are mean $\pm$ SEM. N.D.: non-detectable.

extract to DNP-lysine was clearly detected in immunized rats, although there was a large variation in binding activities (Table 1). These results suggest that sIgA secreted to bile passes through the gastrointestinal tract, maintaining antigen binding ability.

DISCUSSION

The physicochemical properties and the physiological function of colostral sIgA have been well documented. Colostral sIgA, which is clearly different from serum IgA, has a molecular weight of 385,000 daltons and a sedimentation constant of 11 S (19, 20). It is comprised of 2 molecules of IgA (M.W. 159,000), a secretory component (M.W. 75,000) and a J-chain (M.W. 15,000), similar to sIgA in other secretions (19, 20). In our study, the molecular weight and sedimentation constant of biliary sIgA were about 400,000 daltons and 11.7 S respectively, and, thus were similar to those of colostral sIgA. Furthermore, when the components of biliary sIgA were identified by SDS-gel disc electrophoresis and gel filtration, 4 kinds of proteins were detected with molecular weights of 25,000, 55,000, 80,000 and 160,000 daltons, findings compatible with the presence of L-chain, α -chain, secretory component and IgA of immunoglobulin, respectively. However, a protein corresponding to J-chain, which is contained essentially in sIgA from other sources (21), could not be detected in biliary sIgA by our methods. It is possible that J-chain may be present but in amounts too small to be detected by the methods used. In all other respects, though sIgA obtained from rat bile was identical to classical secretory IgA found in other secretions.

Although bile contained sIgA and mIgA, sIgA was present in higher concentrations than mIgA. In addition, only sIgA had an antiproteolytic activity.

Furthermore, sIgA excreted in the feces maintained antigen binding ability. Therefore, these facts suggest a possibility that biliary sIgA is not digested and acts physiologically in the gastrointestinal tract.

Colostrum sIgA is a biologically active substance capable of providing passive host defense and is thought to perform an important role in the gastrointestinal tract of young infants, whose immune system is not fully developed (2, 7). sIgA is secreted into bile in relatively large amounts. Therefore, after infants are weaned, if exogenous sIgA is not supplemented, biliary sIgA may play an important function in the intestine in place of colostrum sIgA. The discharge of biliary sIgA into the upper gastrointestinal tract may prevent the absorption of macromolecular substances with antigenic activity as a result of the formation of antigen-antibody complex.

Two mechanisms for the function of biliary sIgA in the intestine have been proposed: one is to facilitate the excretion of antigen into feces, thereby inhibiting antigen absorption by the gut, and the other is to stimulate digestion of antigen by adhering the antigen-antibody complex to the glycocalyx of mucosal surfaces. As demonstrated in the present study, biliary sIgA was resistant to treatment with trypsin and intestinal fluid *in vitro* and was excreted into feces still capable of binding ability to antigen. However, our results could not demonstrate whether biliary sIgA facilitates the digestion of antigen by proteolytic enzymes or promotes their removal by the flow of secretions.

We would like to thank Dr. Pamela, J. Bier-Nakajima (Department of Immunology, University of Tokyo, Tokyo, Japan) for helpful comments on our manuscript.

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