ROLE OF SERUM IgA

Hepatobiliary Transport of Circulating Antigen*

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Recent investigations indicate that the liver is involved in the selective transport of serum IgA into the bile. In rats, ligation of the bile duct results in a rapid increase in the concentration of circulating IgA, which is reversed upon removal of the ligature (1, 2). Radiolabeled IgA infused intravenously in rats and mice is rapidly and selectively cleared from the circulation and transported to the bile (2–4). This process appears to be selective for oligomeric IgA, to involve secretory component (SC)[1] as an IgA receptor on the hepatocytes, and to result in the appearance of secretory IgA (SIgA) in the bile (5, 6).

The Kupffer cells of the liver are known to be involved in the removal from the circulation of opsonized particles such as bacteria and soluble immune complexes (7). These processes, however, are thought to apply mainly to particles or complexes containing IgG, because mononuclear phagocytes have receptors for IgG, but not, it appears, for IgM or IgA (8). Although conflicting claims have been made, it is generally held that IgA does not fix complement and is poorly opsonic. Thus, whereas the protective functions of IgG and IgM antibodies in complement- and phagocytosis-mediated processes are well understood, the function of serum IgA remains largely obscure. It has been suggested that the hepatobiliary transport of serum IgA serves to reinforce the intestinal supply of SIgA, which protects the mucosal surface against infection and prevents the absorption of antigens from the gut lumen.

We have considered the possibility that the hepatobiliary transport of IgA might also function as a mechanism for the removal of macromolecular antigens from the circulation. The experiments reported here were designed to investigate this, and the results indicate that serum IgA antibody mediates the transport of a foreign protein antigen from the circulation into the bile.

Materials and Methods

Antigen. Crystallized human serum albumin (HSA) was substituted with dinitrophenyl (DNP) hapten using DNP-ε-aminocaproic acid N-hydroxysuccinimide ester (Biosearch, San Rafael, Calif.) (9). The degree of substitution, determined spectrophotometrically, was ~8 mol DNP/1 mol HSA. DNP-HSA was radiolabeled with ^{125}I by the lactoperoxidase method (10) to achieve a specific activity of ~8 × 10^8 cpm/0.5 mg of protein. The labeled protein was separated from free label on a column of Sephadex G-25.

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[1] *Abbreviations used in this paper:* DNP, dinitrophenyl; HSA, human serum albumin; SC, secretory component; SIgA, secretory IgA.

Antibodies. IgA proteins with DNP- or levan-binding activity were obtained from the MOPC315 and UPC61 myelomas, respectively, which were grown as ascites tumors in CB6/F$_1$ mice (The Jackson Laboratory, Bar Harbor, Maine).

Monoclonal anti-DNP antibodies of IgG1 and IgM classes were prepared from hybridomas also grown as ascites tumors in CB6 mice. The hybridomas were prepared by the fusion of 10^8 P3-x63-Ag8.653 myeloma cells with 10^8 immune spleen cells using 41.3% polyethylene glycol 4000 (11). The immune spleen cells were obtained from BALB/c mice immunized 1 wk earlier with 100 μg of DNP-keyhole limpet hemocyanin in complete Freund's adjuvant. Hybrids were selected in hypoxanthine, aminopterin, thymidine medium, screened for secretion of anti-DNP antibody by enzyme-linked immunosorbent assay, and cloned by limiting dilution.

Immunoglobulin concentrations were estimated by radial immunodiffusion using goat antisera and a mouse immunoglobulin reference standard. The presence of anti-DNP antibody was determined by enzyme-linked immunosorbent assay (12) on microtiter plates coated with DNP$_{10}$-HSA (0.1 mg/ml). Alkaline phosphatase conjugates were prepared from goat anti-mouse IgG, IgM, or IgA antisera (Bionetics Laboratory Products, Litton Bionetics, Inc., Kensington, Md.). For controls, normal mouse serum and UPC61 ascitic fluid were used. Immunoglobulin class specificity of the conjugates was demonstrated by comparing ascitic fluids containing monoclonal anti-DNP antibodies of IgG and IgM classes with MOPC315 ascitic fluid.

Purification of IgA. 5 ml MOPC315 ascitic fluid was passed over a 15-ml column of DNP-Sepharose, prepared by reacting CNBr-activated Sepharose 4B with ethylene diamine and dinitrobenzene sulfonic acid. The nonretained material was designated as depleted ascitic fluid. The adsorbed anti-DNP IgA was eluted with 3 M KI, and the remaining albumin was removed on a 1.5-ml column of Affi-Gel Blue (Bio-Rad Laboratories, Richmond, Calif.). Immunoelectrophoresis and immunodiffusion against goat antisera to mouse serum and to mouse IgA, IgG, and IgM showed this to be essentially pure IgA, free of other immunoglobulins but with a trace of albumin. Purity was also confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Molecular Characterization of IgA. To determine the proportions of polymeric and monomeric IgA, MOPC315 ascitic fluid and purified IgA were chromatographed on Sephadex G-200 (88 × 1.5 cm) in 0.01 M Tris-HCl/0.14 M NaCl, pH 7.4. The distribution of IgA anti-DNP antibody was determined by radial immunodiffusion and enzyme-linked immunosorbent assay. The results indicated that MOPC315 IgA was ~75% polymeric.

Determination of Antibody Affinities. Anti-DNP IgG and IgM were purified from ascitic fluids as described above for MOPC315 IgA. Hapten dialysis (13) was performed using ^{14}C-DNP-ϵ aminocaproic acid, which was prepared by reacting 50 μCi (2.4 μmol) of 1-fluoro-2,4-dinitro[U-^{14}C]benzene (Amersham Corp., Arlington Heights, Ill.) with 25 μmol of ϵ-aminocaproic acid in 0.1 ml of 0.1 M NaHCO$_3$ for 40 h at room temperature. Purified antibody proteins (50 μg/ml in 0.15% gelatin) were dialyzed against 10^{-5}-10^{-8} M hapten in 0.01 M Tris-HCl/0.14 M NaCl/0.01% NaN$_3$ for 40 h at 4°C. Control dialyses included buffer only, and normal mouse IgG (N. L. Cappel Laboratories, Inc., Cochranville, Pa.) to assess nonspecific binding of hapten. After attainment of equilibrium, hapten concentrations inside and outside the dialysis bags were determined in a Beckman LS 8000 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). K$_A$ was determined for each immunoglobulin by plotting the reciprocal of bound hapten concentration against the reciprocal of free hapten concentration.

IgA Immune Complexes. 2 ml MOPC315 ascitic fluid was incubated with 0.2 ml of ^{125}I-DNP-HSA (~0.1 mg of protein containing 1.6 × 10^8 cpm) for 1 h at room temperature, and then chromatographed on a column (84 × 1.6 cm) of Sephadex G-200 in 0.15 M NaCl. The three fractions (~1.5 ml each) having the highest radioactivity in the void volume peak were pooled and used directly.

In Vivo Experiments. Injection mixtures were generally prepared from 0.1 ml of ^{125}I-DNP-HSA (~50 μg of protein and 5-9 × 10^7 cpm) and 1 ml of ascitic fluid or serum; aliquots of 0.1 ml were injected. With purified MOPC315 IgA, 2 ml of antibody solution (1.2 mg/ml IgA) was mixed with 0.1 ml of antigen, and 0.2-ml aliquots were injected. Similarly, 0.2-ml aliquots of isolated IgA immune complex fraction were injected.

Locally bred BALB/c mice of both sexes, 6-8 wk of age, were starved for ~3 h before the

start of the experiment to permit the accumulation of bile. Eight mice were injected in the lateral tail vein with each mixture. After 1 and 3 h, four mice from each group were anesthetized with ether and bled from the retro-orbital sinus. The mice were then killed and up to 10 μl of bile was collected from the gall bladder with a Hamilton syringe (Hamilton Co., Inc., Whittier, Calif.).

Examination of Samples. The radioactivity of serum and bile samples and of injection mixtures was measured in a Beckman Gamma 4000 counter (Beckman Instruments, Inc.).

To determine the molecular weight range of the radioactivity, samples of injection mixtures and of pooled bile containing 20–100 × 10³ cpm were mixed with 0.4 ml of 1:8 diluted normal mouse serum and chromatographed on a column (88 × 1.5 cm) of Sephadex G-200 eluted with 0.01 M Tris–HCl/0.14 M NaCl, pH 7.4. The distribution of the radioactivity in fractions of ~1.5 ml was related to the protein elution profile of mouse serum determined by absorbance at 280 nm.

Staphylococcal Protein A Immunosorption. Samples were also analyzed for the association of radioactivity with HSA and with immunoglobulins. Duplicate 1-μl aliquots of pooled bile samples and of 1:10 diluted injection mixtures were mixed with 10 μl of rabbit anti-HSA or goat anti-mouse IgA, IgG, or IgM antisera, or normal serum. After 16 h at 4°C, 0.1 ml of a 10% suspension of *Staphylococcus aureus* was added (14). After 1 h, the percentage of the total radioactivity that was associated with the twice-washed staphylococci was determined.

Statistical Analysis. Student's t test for independent means was applied to logarithmically transformed data.

Results

Requirement for Specific IgA Antibody in the Hepatobiliary Transport of Antigen. The levels of radioactivity in the serum and bile of mice injected with ^{125}I-DNP-HSA and various antibody-containing fluids are shown in Table I. At 1 and 3 h, the bile of mice receiving MOPC315 and ^{125}I-DNP-HSA contained high levels of radioactivity, which at 3 h exceeded the radioactivity present in the serum. In contrast, the bile of mice injected with normal serum and ^{125}I-DNP-HSA contained low levels of radioactivity. The difference in bile radioactivity between the two groups was highly significant ($P < 0.001$) at both sampling times. The levels of radioactivity in the bile of mice given ascitic fluid containing anti-levan IgA (UPC61) were similar to those of the serum controls. The injection of unhaptenated ^{125}I-HSA with MOPC315 ascitic fluid resulted in the recovery of only low levels of radioactivity in the bile. These results indicated that the transport of antigen into bile required specific antibody.

When MOPC315 ascitic fluid depleted of anti-DNP IgA was injected with ^{125}I-DNP-HSA, the recovery of radioactivity in bile remained low and was significantly different from that obtained with the original MOPC315 ascitic fluid ($P < 0.001$) at both sampling times. However, with the use of purified MOPC315 IgA, large amounts of radioactivity were recovered in the bile. Thus, it was concluded that the transport of antigen into bile was mediated by IgA antibody.

To determine whether hepatobiliary transport of antigen is a unique property of IgA, ^{125}I-DNP-HSA was injected together with the ascitic fluids of anti-DNP hybridomas containing IgG or IgM antibody. With the IgM antibody, there was no significant transport of radioactivity into the bile, in comparison with the normal serum controls. However, with IgG antibody, there was a significant level of radioactivity in the bile ($P < 0.001$, compared with IgM or normal serum control), but this was substantially less than the bile radioactivity obtained when IgA antibody was used. In comparison with IgA or IgM antibody, the serum levels of radioactivity were lower in the mice given IgG, although similar amounts of radioactivity were injected

TABLE I

IgA Antibody-dependent Specific Transport of Antigen into Bile

Antibody fluid injected	^{125}I-DNP-HSA injected	Time	Radioactivity recovered (mean ± SD)	
			Serum	Bile
	cpm	*h*	*cpm/μl*	
MOPC315 ascitic fluid*	6.9 × 10^6	1	2,723 ± 491	2,247 ± 590
		3	1,886 ± 110	3,840 ± 739
Normal serum	7.5 × 10^6	1	3,485 ± 474	286 ± 33
		3	2,852 ± 171	227 ± 31
UPC61 ascitic fluid	7.6 × 10^6	1	3,792 ± 481	324 ± 51
		3	2,992 ± 393	263 ± 21‡
Depleted MOPC315 ascitic fluid§	6.2 × 10^6	1	3,640 ± 486	293 ± 30
		3	2,715 ± 195	285 ± 71
Purified MOPC315 IgA‖	5.3 × 10^6	1	2,465 ± 120	2,939 ± 534
		3	1,761 ± 82	1,465 ± 182
Anti-DNP IgM ascitic fluid¶	8.4 × 10^6	1	4,800 ± 163	244 ± 20
		3	4,104 ± 579	256 ± 27
Anti-DNP IgG ascitic fluid**	8.9 × 10^6	1	1,912 ± 187	786 ± 204
		3	1,482 ± 182	605 ± 26
MOPC315 ascitic fluid	^{125}I-HSA	1	18,317 ± 749	886 ± 210
	24 × 10^6	3	14,239 ± 1,910	658 ± 139

*Contained 27 mg/ml IgA. Anti-DNP titer (IgA) = 3 × 10^6. K$_A$ = 1.2 × 10^7 M^{-1}.
‡ Three animals.
§ Contained 0.13 mg/ml IgA. Anti-DNP titer (IgA) < 1 × 10^2.
‖ Contained 1.2 mg/ml IgA. Anti-DNP titer (IgA) = 2.4 × 10^5.
¶ Contained 4.0 mg/ml IgM. Anti-DNP titer (IgM) = 1.3 × 10^6. K$_A$ = 3.0 × 10^6 M^{-1}.
** Contained 14.5 mg/ml IgG. Anti-DNP titer (IgG) = 6.4 × 10^5. K$_A$ = 1.6 × 10^7 M^{-1}.

into each group of mice. It was conjectured that this was due to the clearance of immune complexes containing IgG and ^{125}I-DNP-HSA by the mononuclear phagocytic system, which includes the Kupffer cells of the liver. As a result, radioactive breakdown fragments might be released into the bile. On the other hand, IgA-mediated transport of radiolabeled antigen into the bile through the hepatocytes should not necessarily involve breakdown of the antigen. Bile samples were therefore analyzed to determine the molecular weight range of the radioactivity.

Analysis of Radiolabeled Antigen Transported to Bile. When the bile of mice injected with anti-DNP IgG and ^{125}I-DNP-HSA was chromatographed on Sephadex G200, the radioactivity was confined to the low molecular weight fractions (Fig. 1 A). This was in sharp contrast to the elution profiles of bile samples from mice injected with immune complexes of MOPC315 IgA and ^{125}I-DNP-HSA (see below; Fig. 1 C). In the latter, ~70% of the recovered radioactivity was present in the void volume and albumin fractions together. These results therefore indicated that IgA antibody could mediate the hepatobiliary transport of antigen that was largely intact, whereas IgG antibody brought about the appearance in bile of only low molecular weight fragments.

To confirm these findings, attempts were made to determine the binding of radioactivity in bile by antisera to HSA and to immunoglobulin, using a staphylococcal immunosorption assay. For comparison, injection mixtures were also analyzed.

In the bile taken from mice after injection with MOPC315 ascitic fluid and ^{125}I-
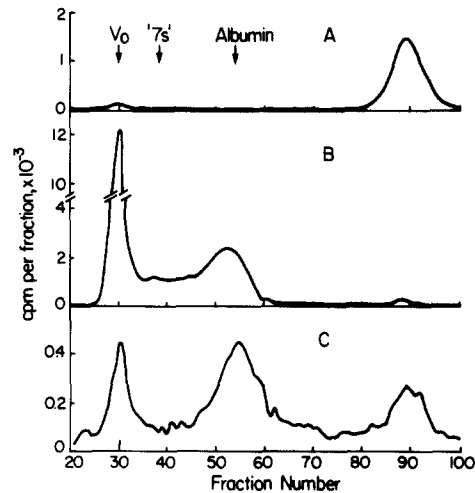
FIG. 1.   Sephadex G-200 chromatography of: A, bile 1 h after injection of IgG anti-DNP hybridoma ascitic fluid and ^{125}I-DNP-HSA; B, separated immune complex fraction prepared from MOPC315 ascitic fluid and ^{125}I-DNP-HSA; C, bile 1 h after injection of separated immune complex of MOPC315 and ^{125}I-DNP-HSA.

DNP-HSA, ~60% of the radioactivity was bound by anti-HSA serum, and 50% was bound by anti-IgA serum. However, in the controls, 28% of the bile radioactivity was bound to staphylococci in the absence of specific antibody. Similar analysis of the injection mixture of MOPC315 ascitic fluid and ^{125}I-DNP-HSA showed that 78% of the radioactivity was bound by anti-HSA, 50% was bound by anti-IgA, and 7% was bound directly by the staphylococci. It therefore appeared that components of the bile itself influenced the direct binding of either the labeled antigen or the putative IgA immune complexes to the staphylococci. In the bile of mice injected with anti-DNP IgG and ^{125}I-DNP-HSA, the radioactivity was not appreciably bound by antisera to HSA (4.3%) or IgG (3.5%), or directly by staphylococci (2.1%). These results were compatible with those obtained by the chromatographic analysis of bile samples, and it was therefore concluded that at least part of the antigen transported into bile by IgA antibody remained intact and also bound to IgA. In contrast, the much lower amount of radioactivity present in the bile of mice injected with IgG was associated with fragments of degraded antigen.

Biliary Transport of Preformed Complexes of MOPC315 IgA and ^{125}I-DNP-HSA.   When separated, preformed immune complexes of MOPC315 IgA and ^{125}I-DNP-HSA were injected into mice, high levels of radioactivity, relative to serum, were observed in the bile (Table II). In the injected complex, 93% of the radioactivity was bound by anti-HSA and 63% was bound by anti-IgA antiserum. Sephadex G-200 chromatography showed that most of the radioactivity was in the void volume, but that some had dissociated and was present in the albumin fraction (Fig. 1 B). The Sephadex G-200 elution profile of the bile showed radioactivity in the void volume, albumin, and low molecular weight fractions (Fig. 1 C). In the control mice, which received a similar amount of ^{125}I-DNP-HSA in 10% normal serum, bile radioactivity remained low. Thus, it was concluded that immune complexes of IgA and antigen were efficiently transported into bile.

Table II

Transport of Preformed IgA Immune Complexes into Bile

Material injected	Percentage of injected radioactivity bound by antiserum to					Time	Radioactivity (mean ± SD)	
	HSA	IgA	IgG	IgM	Control		Serum	Bile
						h	*cpm/μl*	
Immune complex	93	63	12	7.1	5.0	1	329 ± 5.3	1,103 ± 233
(1.1 × 10⁶ cpm)						3	208 ± 50	650 ± 200
Antigen control	84	0.3	0.8	0.5	0.1	1	797 ± 108	47 ± 5.0
(1.5 × 10⁶ cpm)						3	718 ± 152	61 ± 15

Although much of the transported antigen remained intact and partly complexed to IgA antibody, it was apparent that there was also a certain amount of breakdown. This breakdown may have occurred in transit through the liver, or in the bile. To test the latter possibility, preformed immune complexes of ^{125}I-DNP-HSA and MOPC315 IgA were incubated with normal bile in vitro for 1 h at 37°C. Subsequent chromatography on Sephadex G-200 did not reveal any change in the elution profile of the radioactivity in the complex. Thus, it appeared that breakdown of antigen may have occurred within the liver.

Discussion

The results presented here demonstrate that specific IgA antibody mediated the transport of a haptenated protein from the circulation to the bile. At least part of the antigen was present in the bile in intact form and was bound to IgA. This finding implies that degradation of the antigen was not a prerequisite of its transport into bile.

In contrast, transfer of the haptenated protein was not mediated by IgM antibody, and IgG brought about the appearance in bile of only small amounts of low molecular weight fragments. It seems most likely that this was due to phagocytosis and digestion of IgG-antigen complexes by the Kupffer cells, which possess receptors for IgG (15). The failure to observe biliary transport of antigen by IgM or IgG antibody was not attributable to lack of affinity for DNP. Both IgM and IgG antibodies had high association constants that were comparable with that of MOPC315 IgA (Table I). Furthermore, we have found that another IgA protein, MOPC460, which binds DNP with lower affinity ($K_A = 3 \times 10^5$ M^{-1}) (16) than the anti-DNP IgG and IgM used in these studies, will also mediate the transport of ^{125}I-DNP-HSA to bile (M. W. Russell, T. A. Brown, and J. Mestecky, unpublished observations).

In all of the present experiments, anti-DNP antibody was used in considerable excess over the amount of antigen. Lack of hepatobiliary transport by IgG and IgM antibody cannot therefore be attributed to insufficient antibody. It remains to be investigated whether the IgA:antigen ratio and the size and composition of IgA-antigen complexes will affect the efficiency of the transport.

The mechanism responsible for the appearance of IgA in bile has not been conclusively established. In rats, the transport of IgA from the circulation to the bile is mediated by SC, which functions as a receptor for IgA on the hepatocyte membrane, and is selective for polymeric forms (5, 6). The MOPC315 protein used in our

experiments was 75% polymeric. It is likely that the transport of IgA immune complexes through the hepatocytes is analogous to the transport of free IgA. Although human hepatocytes selectively bind polymeric IgA, SC has not been detected on their surfaces (17, 18). It remains to be investigated whether other secretory glands, which transport polymeric IgA across the epithelium to generate SIgA, might also be capable of transporting antigen complexed with IgA.

There are other mechanisms of uptake of immune complexes by the liver. In addition to the clearance of IgG immune complexes by Kupffer cells (19), mannose receptors on nonparenchymal cells have been implicated in the binding of immune complexes that contained IgM (20). However, these mechanisms do not explain our observations that hepatobiliary transport of immune complexes was selective for IgA rather than for IgG or IgM. In this connection, HSA, which is not a glycoprotein, would not bind directly to carbohydrate receptors.

The results presented here suggest a beneficial role for serum IgA, in contrast to potentially deleterious effects such as the inhibition of complement-mediated bacteriolysis, neutrophil chemotaxis, and phagocytosis (21–23). The rapid clearance of IgA immune complexes from the circulation has recently been described (24), but the site or mechanism of removal was not investigated. This observation is consistent with our results, which demonstrate the specific IgA-mediated transport of antigen into bile.

In the intestine, antigens that penetrate the mucosal barrier and become absorbed would encounter an abundant supply of IgA produced by the submucosal plasma cells (25). Much of this IgA is secreted through the epithelium as SIgA, and might conceivably re-export antigens immediately in the form of immune complexes. Some of this IgA, however, is carried to the liver and could thereby mediate the excretion of absorbed antigens into the bile. This process could help explain the apparent role of the liver in limiting the systemic immune response to antigens absorbed in the gut (26). Antigens absorbed through other mucosal surfaces, e.g., the lungs and the bronchial tree, or generated by infectious agents, might also be eliminated by the liver in this way.

Although the role of SIgA in protecting mucosal surfaces is generally accepted, the function of serum IgA has been inadequately investigated. We propose that an important and hitherto undisclosed function of serum IgA is to mediate the removal of antigens from the circulation to the bile by a mechanism that may be found to circumvent the inflammatory consequences of the activation of complement and phagocytosis.

Summary

The IgA-mediated hepatobiliary excretion of antigen from the circulation was studied using a radiolabeled haptenated protein (dinitrophenyl-human serum albumin) injected intravenously in mice together with monoclonal anti-dinitrophenyl antibodies of different immunoglobulin classes. Antibodies were obtained from ascitic fluids of mice bearing the MOPC315 myeloma (IgA), or immune spleen cell hybridomas (IgG and IgM). IgA antibody brought about the transport of large amounts of antigen from the circulation to the bile during 1–3 h. Analysis of bile by gel filtration showed that a large part of the transported antigen remained intact and complexed with IgA. Neither IgA of different specificity nor anti-dinitrophenyl IgM mediated

biliary transport of antigen. With anti-dinitrophenyl IgG, only small amounts of low molecular weight fragments of labeled antigen were found in the bile. Preformed immune complexes of radiolabeled antigen and IgA antibody were rapidly transported from the circulation to the bile, resulting in threefold-higher levels of radioactivity in bile than in serum. It is proposed that an important function of serum IgA is to mediate the hepatobiliary excretion of corresponding circulating antigens.

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