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Report

Intestinal Transport of Sulfanilic Acid in Rats Immunized with Protein–Sulfanilic Acid Conjugate

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Intestinal transport of sulfanilic acid was examined by means of an *in vitro* everted sac technique in rats immunized with a bovine γ-globulin–sulfanilic acid conjugate. At a low concentration of sulfanilic acid, the intestinal transport of sulfanilic acid was decreased in rats immunized with bovine γ-globulin–sulfanilic acid conjugate. This phenomenon was dose dependent and antigen specific, since there was no difference in the transport of sulfanilic acid at a high concentration and of an unrelated hapten. These results suggested that parenteral immunization impaired not only the intestinal transport of macromolecular antigens, as previously shown, but also the transport of the low molecular weight hapten, sulfanilic acid.

KEY WORDS: intestinal transport; sulfanilic acid; hapten; immunization; protein-hapten conjugate; everted sac.

INTRODUCTION

Walker *et al.* previously reported that oral and parenteral immunization interfered with the *in vitro* uptake of macromolecular antigens (bovine serum albumin, horseradish peroxidase) by the small intestine of the rat (1,2). In subsequent studies, they investigated the mechanisms whereby immunization interfered with the intestinal absorption of macromolecules. It was shown that antigen was found to bind rapidly with antibodies present on the surface of the intestine, and formation of antigen–antibody complexes seemed to prevent binding of antigen to intestinal epithelial cells and its subsequent pinocytosis (3). In addition, antigen–antibody complexes retained on the surface of the intestine were in turn more rapidly degraded by local proteases than the antigen alone (3,4). This proteolysis was considered to contribute the decreased availability of antigen for uptake. Further, antigen–antibody complexes appeared to stimulate secretion of mucus, thereby preventing contact with the surface of the enterocyte from whence uptake of pinocytosis is initiated (5).

While the effect of immunization on the intestinal uptake of macromolecules has been thoroughly investigated, few studies have examined the intestinal uptake and transport of low molecular weight compounds, such as haptens, in immunized animals. Recently, Shimura *et al.* reported that the absorption of dinitrophenylated lysine was inhibited in rats immunized with dinitrophenylated bovine serum albumin conjugate (6). In our previous report, we showed that a dose-dependent and an antigen-specific decrease in intestinal transport of *p*-aminobenzoic acid was observed in rats immunized with protein–*p*-aminobenzoic acid conjugate (7). This result was due to the antihapten antibodies present on the surface of the intestinal mucosa and the increased metabolic transformation of hapten caused by the enhancement of mucosal *N*-acetyltransferase activity of the gut. However, we could not distinguish the contribution of these two factors regulating the intestinal transport of *p*-aminobenzoic acid (hapten) in immunized rats. In the present study, we have therefore selected sulfanilic acid as a hapten which is hardly metabolized in the intestinal mucosa (8), and the intestinal transport of sulfanilic acid was examined in rats immunized with a protein–sulfanilic acid conjugate.

MATERIALS AND METHODS

Materials

Bovine γ-globulin and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, MO. Freund's incomplete adjuvant was obtained from Difco Laboratories, Detroit, Mich. [^{14}C]Sulfanilic acid (3.91 mCi/mmol) was kindly supplied by Daiichi Radioisotope Co., Japan. *p*-[^{14}C]Aminobenzoic acid (56 mCi/mmol) was purchased from Japan Radioisotope Association. All other reagents used in these experiments were of reagent grade obtained from Nacalai Tesque, Inc., Japan.

Synthesis of the Immunizing Antigen

The immunizing antigen used in this experiment was synthesized by diazotizing 50 ml of sulfanilic acid and coupling at 0°C to 1 g of bovine γ-globulin or bovine serum albumin dissolved in 20 ml of borate buffer. The pH was maintained between 9.0 and 9.5 during the coupling reaction

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with the addition of 0.5 *M* NaOH. After the coupling reaction, the pH was adjusted to between 7.0 and 7.5 with 0.1 *M* HCl. The antigen was dialyzed against 5 liters of isotonic saline solution at 4°C over a period of 3 days and diluted with isotonic saline to a final concentration of 1 g/100 ml. The antigen solution was purified by size-exclusion chromatography with a Sephadex G-25 column (2.2 × 66 cm) to separate the free sulfanilic acid at 4°C. Elution was performed with 0.5 *M* acetate buffer at 15 ml/hr and 5-ml fractions were collected automatically. Sulfanilic acid and its protein conjugate were determined spectrophotometrically.

Animals and Immunization

Male Wistar albino rats ranging from 150 to 200 g were used. The rats, which had been fed on a diet free of bovine γ-globulin, were immunized according to the following schedule. Bovine γ-globulin–sulfanilic acid solution (2.5 mg/0.25 ml) was emulsified with an equal volume of Freund's incomplete adjuvant and was injected intraperitoneally to rats under light ether anesthesia. Animals were immunized two, four, and six times at 10-day intervals and absorption studies were carried out 10 days after the final immunization. Since there was no significant difference in the intestinal uptake and transport of sulfanilic acid between bovine γ-globulin and saline-treated animals, control rats were administered with 0.5 ml of Freund's incomplete adjuvant emulsified with saline on the same schedule as for bovine γ-globulin–sulfanilic acid-treated rats.

Radial Immunodiffusion Reaction Technique

Antihapten antibodies in immune sera were determined by a radial immunodiffusion technique. Agar (1.5 g) was dissolved in pH 7.4 heated phosphate buffer at a concentration of about 1%. A petri dish was filled with 10 ml of semisolid agar solution. After the agar hardened, five wells were bored as indicated in Fig. 3. The diameter of the wells was 5 mm and the distance between the antigen- and the antiserum-containing wells was 5 mm. The center well was filled with undiluted antiserum obtained from rats immunized with bovine γ-globulin–sulfanilic acid conjugate. Antigens (bovine γ-globulin–sulfanilic acid, bovine γ-globulin, bovine serum albumin–sulfanilic acid conjugate, bovine serum albumin) were placed in wells surrounding the central well. After 24–72 hr, the antigen–antibody reaction proceeded and we have observed and taken pictures of precipitin lines between these wells.

Absorption Studies

Intestinal transport of [^{14}C]sulfanilic acid and *p*-[^{14}C]-aminobenzoic acid was studied by means of an *in vitro* everted sac experiment (7). Under pentobarbital anesthesia, the small intestine was washed with pH 6.5 isotonic phosphate buffer solution and quickly removed from the rat. The removed intestine was everted by a wire inserted through the lumen. The upper 20-cm segment (jejunum) and the lower 20-cm segment (ileum) were everted and ligated at one end. As serosal fluid, 3 ml of pH 7.4 phosphate buffer solution was introduced from the opposite end. The everted sac was ligated and placed in the mucosal fluid (10 ml of pH 6.5 isotonic phosphate buffer solution containing radiolabeled sulfanilic acid or *p*-aminobenzoic acid) bubbled with 5% CO_2 in O_2 at 37°C. At the end of the incubation period, the sac was cut and opened at one end, and the serosal fluid was collected. The radioactivity in the serosal fluid was determined in a liquid scintillation system. The viability of gut sacs during the test period was monitored by measuring the transport of trypan blue dye. There was minimal transport of dye during the incubation.

Protein and Mucus Determination in the Mucosal Fluid

The procedure was similar to that used for the absorption studies. The everted sacs from immunized and control rats were placed in the mucosal fluid (10 ml of pH 6.5 phosphate buffer solution containing 5.1 nmol of sulfanilic acid). After incubation of the everted sac for 15 min with sulfanilic acid, the mucosal solution was centrifuged for 10 min at 400*g* and protein and mucus concentrations were assayed.

Analytical Method

The determination of ^{14}C radioactivity was performed as follows. One milliliter of sample solution was put into the counting vial, 5 ml of scintillation medium (Univer-gel, Nacalai Tesque, Inc., Japan) was added, and the radioactivity was determined in a liquid scintillation system. The counts obtained were corrected using external standards. The protein concentration was determined by the method of Lowry (9) with bovine serum albumin as the standard and the mucus was determined by an anthrone method using D-glucose as the standard (10).

Statistical Analyses

Results are expressed as the mean ± standard deviation (SD). Statistical analyses were performed using Student's *t* test.

RESULTS

The Synthesis of Protein–Hapten Conjugate

Bovine γ-globulin–sulfanilic acid conjugate was purified by size-exclusion chromatography. Figure 1 shows chromatographic profiles of sulfanilic acid and bovine γ-globulin–sulfanilic acid conjugate. The conjugate was eluted at a different position from free sulfanilic acid, and fractions of the conjugate were collected and used for the following experiments. The synthesis of bovine γ-globulin–sulfanilic acid conjugate was confirmed by absorption spectra. Figure 2 shows the absorption spectra of bovine γ-globulin–sulfanilic acid conjugate, bovine γ-globulin, and sulfanilic acid. The spectrum of the conjugate had a different pattern from that of the protein and hapten only, suggesting the synthesis of bovine γ-globulin–sulfanilic acid conjugate. One mole of protein bound approximately 27 mol of sulfanilic acid.

The Determination of Anti-Sulfanilic Acid Antibody

Figure 3 shows the radial immunodiffusion reaction between antiserum and various antigens. There existed precipitin lines between antiserum and antigens (bovine γ-glob-

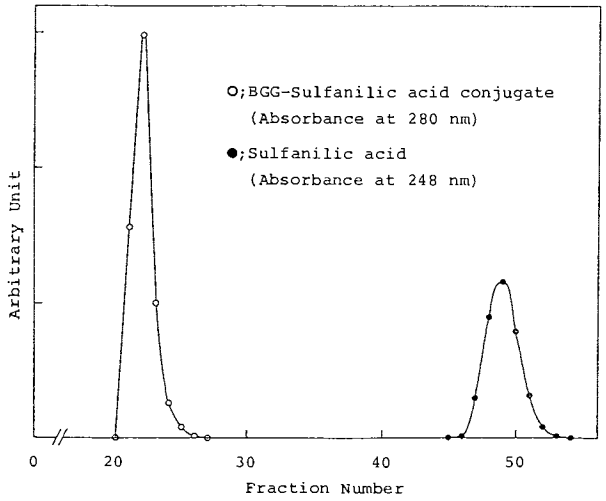

Fig. 1. Size exclusion chromatographic patterns of bovine γ-globulin–sulfanilic acid conjugate and sulfanilic acid. Chromatography was performed on Sephadex G-25.

ulin–sulfanilic acid conjugate, bovine γ-globulin, bovine serum albumin–sulfanilic acid conjugate), while we found no precipitin line between antiserum and bovine serum albumin. These findings indicated the presence of antibodies for sulfanilic acid and bovine γ-globulin in immunized animals.

Intestinal Transport of Sulfanilic Acid in Rats Immunized with Bovine γ-Globulin Sulfanilic Acid Conjugate

There was no significant change in the body weight and wet weight of the small intestine between normal and immunized animals. Tables I and II show the jejunal and ileal transport of sulfanilic acid in rats immunized twice, four times, and six times, with bovine γ-globulin–sulfanilic acid

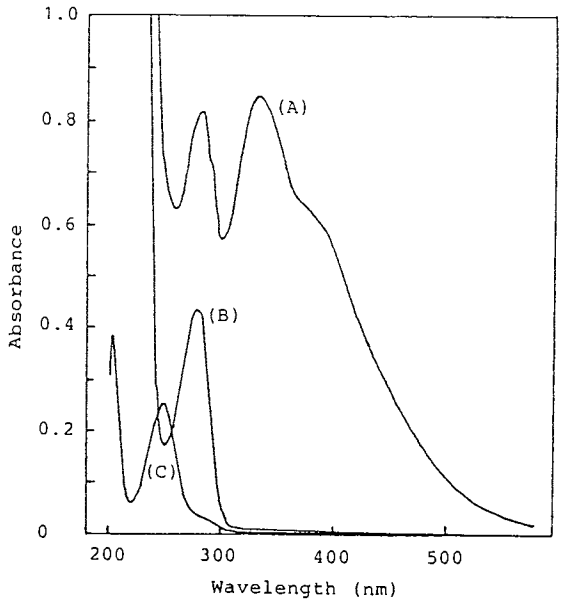

Fig. 2. Absorption spectra of bovine γ-globulin–sulfanilic acid conjugate (A), bovine γ-globulin (B), and sulfanilic acid (C). The concentration of solution A was 0.2 mg (of protein)/ml, whereas the concentrations of solutions B and C were 1 mg/ml.

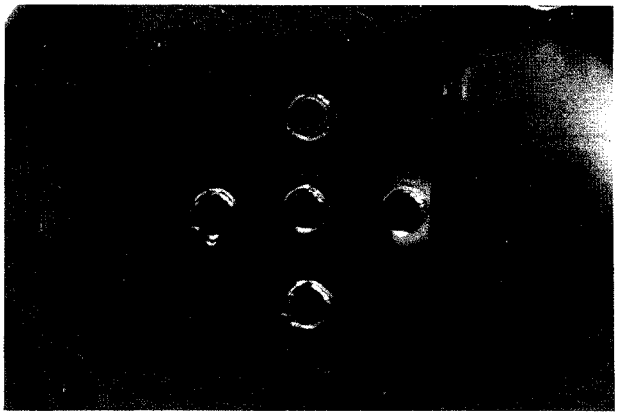

Fig. 3. Radial immunodiffusion reaction between antiserum and various antigens. BGG-S, bovine γ-globulin–sulfanilic acid conjugate; BGG, bovine γ-globulin; BSA-S, bovine serum albumin–sulfanilic acid; BSA, bovine serum albumin.

conjugate. A significant decrease in jejunal and ileal transport of sulfanilic acid was noted after 15 min in immunized rats compared with the control but not in the transport of sulfanilic acid after 30 min. In addition, there was no difference in the intestinal uptake of sulfanilic acid at high concentration. Further, the transport of *p*-aminobenzoic acid, an unrelated hapten, was unchanged in jejunum or ileum of immunized rats vs controls, suggesting an antigen-specific decrease in transport of sulfanilic acid in these animals.

Protein and Mucus Release After Intraluminal Exposure to Sulfanilic Acid in Control and Immunized Rats

As summarized in Table III, we found no significant difference between these two groups of rats in protein and mucus release in jejunal and ileal mucosal solutions.

DISCUSSION

We have reported that local and systemic immune responses affect the intestinal absorption of low molecular weight drugs. In particular, the intestinal transport was influenced by these immune responses (11–16) and the immunomodulator (17–19). Further, the transport of *p*-aminobenzoic acid was decreased from the small intestine of rats immunized with ovalbumin–*p*-aminobenzoic acid conjugate (7). These results suggested that immunization regulates the intestinal transport of low molecular weight drugs as well as macromolecular antigens.

In this study, we have shown that parenteral immunization leads to specific inhibition of sulfanilic acid transport by gut sacs prepared from jejunum and ileum of treated animals. This inhibition may be due to the presence of anti-sulfanilic acid antibodies on the mucosal surface of the gut, since such antibodies were detectable by the radial immunodiffusion technique (Fig. 3) and the decrease in transport of sulfanilic acid was dose dependent and antigen specific (Tables I and II). These findings were consistent with the results of Shimura *et al.* (6) and Walker *et al.*, who demonstrated that the intestinal uptake of dinitrophenol–bovine serum albumin was considerably less than that of controls in rats immunized with dinitrophenol–bovine γ-globulin be-

Table I. Transport of [^{14}C]Sulfanilic Acid in Jejunum in Rats Immunized with Bovine γ-Globulin–Sulfanilic Acid Conjugate[a]

Drug	Immunization	Incubation time (min)	Transport (% of dose)		Statistics
			Control	Immunization	
Sulfanilic acid (low conc.)	× 2	15	0.99 ± 0.21	0.65 ± 0.15	$P < 0.05$
		30	2.04 ± 0.49	2.27 ± 0.52	NS
	× 4	15	0.86 ± 0.11	0.44 ± 0.11	$P < 0.01$
	× 6	15	0.73 ± 0.15	0.53 ± 0.14	$P < 0.01$
		30	2.23 ± 0.25	2.11 ± 0.32	NS
Sulfanilic acid (high conc.)	× 2	15	1.45 ± 0.32	1.20 ± 0.17	NS
p-Aminobenzoic acid	× 2	15	2.43 ± 0.23	2.37 ± 0.17	NS

[a] Results are expressed as the mean ± SD of at least three animals. The concentrations of sulfanilic acid were 0.51 and 2.56 μ*M*, respectively, while that of *p*-aminobenzoic acid was 0.51 μ*M*. NS, not significantly different compared with the control.

cause of the presence of antidinitrophenol antibodies (3). Increased metabolic transformation of the hapten, which plays a major role in the decreased absorption of *p*-aminobenzoic acid in immunized rats (7), could not account for transport inhibition of the very slowly metabolizing hapten, sulfanilic acid.

Inhibition of the intestinal transport of sulfanilic acid was rather independent of the number of immunizations. This result was inconsistent with the previous finding that a significant and specific decrease in uptake of antigen (bovine serum albumin) was noted in rats injected five times with the antigen but not in rats injected only twice (2). This discrepancy may be due to the differences of antibodies present on the gut surface between bovine serum albumin and sulfanilic acid and may be related to the type or pattern of local antibody distribution.

The inhibition in transport of sulfanilic acid disappeared within 30 min. One possible mechanism is that the antibodies present on the mucosal surface of the gut may be saturated with sulfanilic acid during the test period. Further, the antibodies for sulfanilic acid may be detached from the mucosal surface of everted sac in 30 min, since antigen–antibody complexes were shown to be loosely bound on the intestinal mucosal surfaces (5). However, if the lack of inhibition for sulfanilic acid transport was due to these mechanisms, the differences between immunized and control animals in transport of sulfanilic acid should continue after 30 min of incubation. Consequently, it is more plausible that the antigen–antibody complexes stimulate the release of various chemical mediators affecting the transport of sulfanilic acid during the test period. Indeed, Turnberg reported that chemical mediators such as histamine and serotonin can influence intestinal transport under certain experimental conditions (20). These chemical mediators may decrease not only the intestinal membrane permeability of drugs because of the increase in water secretion but also the surface area of the intestine as a result of induced smooth muscle contraction. The action of these substances may be reversible, since the decrease in sulfanilic acid absorption was seen only for the first 15 min.

We have also examined the protein and mucus release in the mucosal solution after intraluminal exposure to sulfanilic acid to address additional mechanisms decreasing intestinal transport of sulfanilic acid in immunized rats. We found no significant difference in the release of the macromolecular substances between immunized and control rats (Table III), suggesting that the protein and mucus release might not play an important role, although the decreased transport of macromolecular antigens was due partly to the mucus release from goblet cells in the small intestine (5,21,22).

In conclusion, the transport of hapten by gut sacs obtained from rats parenterally immunized with protein–hapten

Table II. Transport of [^{14}C]Sulfanilic Acid in Ileum in Rats Immunized with Bovine γ-Globulin–Sulfanilic Acid Conjugate[a]

Drug	Immunization	Incubation time (min)	Transport (% of dose)		Statistics
			Control	Immunization	
Sulfanilic acid (low conc.)	× 2	15	1.06 ± 0.06	0.61 ± 0.07	$P < 0.001$
		30	1.64 ± 0.32	1.98 ± 0.61	NS
	× 4	15	1.01 ± 0.27	0.52 ± 0.08	$P < 0.02$
	× 6	15	0.73 ± 0.11	0.36 ± 0.11	$P < 0.001$
		30	1.86 ± 0.51	1.85 ± 0.48	NS
Sulfanilic acid (high conc.)	× 2	15	1.11 ± 0.27	1.01 ± 0.43	NS
p-Aminobenzoic acid	× 2	15	1.78 ± 0.33	1.59 ± 0.14	NS

[a] Results are expressed as the mean ± SD of at least three animals. The concentrations of sulfanilic acid were 0.51 and 2.56 μ*M*, respectively, while that of *p*-aminobenzoic acid was 0.51 μ*M*. NS, not significantly different compared with the control.

Table III. Protein and Mucus Release in the Mucosal Fluid After Intraluminal Exposure to Sulfanilic Acid in Rats Immunized Six Times with Bovine γ-Globulin–Sulfanilic Acid Conjugate[a]

Region	Control	Immunization[b]
Protein (μg/ml)		
Jejunum	143.6 ± 29.2	114.4 ± 26.8
Ileum	96.1 ± 5.7	105.2 ± 26.4
Mucus (μg/ml)		
Jejunum	3.61 ± 0.64	4.97 ± 2.30
Ileum	4.83 ± 0.66	5.73 ± 1.46

[a] Results are expressed as the mean ± SD of at least four animals.
[b] No results were significantly different compared with the control.

conjugate was significantly less than the transport of gut sacs obtained from control animals. However, the mechanism for the inhibition of hapten transport depends on the type of hapten. In the case of *p*-aminobenzoic acid, this result was due to the anti-*p*-aminobenzoic acid antibodies present on the mucosal surfaces of the gut and to the increase in the metabolic transformation of hapten caused by the enhancement of intestinal mucosal *N*-acetyltransferase activity, whereas antibodies play the main role in the decreased transport of sulfanilic acid in immunized rats.

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