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The Effect of Immunization with Protein-sulphanilic Acid Conjugate on Sulphanilic Acid Disposition in the Rat

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Abstract—Rats were immunized with bovine γ-globulin-sulphanilic acid conjugate and the plasma concentration of sulphanilic acid examined after an intravenous injection of this drug. There was a significant increase in plasma half-life and AUC and a significant decrease in clearance of sulphanilic acid in the immunized rats compared with the control. In the immunized rats, binding of sulphanilic acid to macromolecules in serum, determined by ultrafiltration, decreased with increase of sulphanilic acid concentration. At low concentrations, there was a significant increase in % binding of the drug in the serum of immunized rats compared with controls. There was also a significant reduction in urinary excretion of total drug in the immunized rats compared with controls. These findings suggest that sulphanilic acid-specific antibodies in the serum of immunized animals bind [^{14}C]sulphanilic acid, giving rise to higher serum levels thereby making it unavailable for normal excretory processes.

We have previously reported that immunization of rats against *p*-aminobenzoic acid or sulphanilic acid interfered with the intestinal transport of these drugs (Yamamoto et al 1989a, 1990). The result with sulphanilic acid was mainly due to the existence of antibodies on the mucosal surface of the small intestine (Yamamoto et al 1990). However, an increase in the metabolic transformation of *p*-aminobenzoic acid and a decrease in the unchanged drug caused by the enhancement of mucosal *N*-acetyltransferase activity was observed in animals immunized with protein-*p*-aminobenzoic acid conjugate (Yamamoto et al 1989a). Therefore, not only the antibodies present on the mucosal surface but also an increase in the metabolite contributed to the decreased transport of *p*-aminobenzoic acid in the immunized rats. Those results suggested that the intestinal immune system might regulate the transport of these low molecular weight drugs.

Berkowitz et al (1974) found that intravenous administration of [^{3}H]dihydromorphine to actively immunized mice resulted in higher serum levels and lower brain levels of the drug compared with control animals. These actively immunized animals also had a decreased responsiveness to subsequent administration of morphine (Berkowitz & Spector 1972). Similarly, Schmidt et al (1974) demonstrated that, after a dose of [^{3}H]digoxin, serum levels of digoxin were higher in actively immunized rabbits compared with control animals and there was an increase in the biological half-life of the drug. They also demonstrated that anti-digoxin antibodies could block or reverse the pharmacological response to digoxin (Schmidt & Butler 1971). Cerreta et al (1977) and Flynn & Cerreta (1978) have shown that animals actively immunized against barbiturates have higher serum levels of drug following administration of [^{3}H]phenobarbitone. These higher serum levels of phenobarbitone were also accompanied by a prolongation of the half-life of the drug, and mice actively immunized against barbiturates had a shift to the right in their dose-response curve for pentobarbitone-induced ataxia (Flynn et al 1977; Cerreta et al 1979). These findings suggested that immunization of rats with hapten-protein conjugates alters not only the intestinal transport of haptens but also the distribution, excretion and pharmacological actions of these drugs.

In the present study, sulphanilic acid was chosen as a model compound and the effect of immunization with protein-sulphanilic acid conjugate on disposition of this compound was examined.

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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, MI. [^{14}C]Sulphanilic acid (3·91 mCi mmol^{-1}) was kindly supplied from Daiichi Radioisotope Co., Japan. *N*-Acetylsulphanilic acid was synthesized in our laboratory. All other reagents used in these experiments were of reagent grade obtained from Nacarai Tesque, Inc. Japan.

Synthesis of the immunizing antigen

The immunizing antigen used was made by diazotizing 50 mg of sulphanilic acid (2 mL) 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 with the addition of 0·05 M NaOH. After the coupling reaction, the pH was adjusted to between 7·0–7·5 with 0·1 M HCl. The antigen was dialysed against 5 L of 0·9% NaCl (saline) at 4°C over 3 days and diluted with 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 sulphanilic acid at 4°C. Elution was performed with 0·5 M acetate buffer at 15 mL h^{-1} and 5 mL fractions were collected automatically. Sulphanilic acid and its protein conjugate were determined spectrophotometrically. In this experiment, the conjugate was eluted at a different position from free sulphanilic acid, indicating the separation and purification of the conjugate. To confirm the synthesis of protein-hapten conjugate, we also examined the absorption spectra of bovine γ-globulin-sulphanilic acid conjugate, bovine γ-globulin and sulphanilic 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-sulphanilic acid conjugate. One mol of protein bound approximately 27 mol of sulphanilic acid.

Animals and immunization

Male Wistar albino rats, 150–200 g fed on a diet free of bovine γ-globulin, were immunized according to the following schedule. Bovine γ-globulin-sulphanilic 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 anaesthesia. Animals were immunized six times at 10-day intervals and experiments were carried out 10 days after the final immunization. Since there was no significant difference in the disposition of sulphanilic acid between bovine γ-globulin and saline-treated animals in our preliminary studies, control rats received 0·5 mL Freund's incomplete adjuvant emulsified with saline on the same schedule as for bovine γ-globulin-sulphanilic acid-treated rats.

Radial immunodiffusion technique

Antihapten antibodies in immune sera were detected by a radial immunodiffusion technique as described previously (Yamamoto et al 1990). Agar (1·5 g) was dissolved in pH 7·4 warmed phosphate buffer at a concentration of about 1%. A petri dish was filled with 10 mL of semi-solid agar solution. After the agar had hardened, 5 wells were bored. The diameter of the wells was 5 mm and the distance between antigen- and antiserum-containing well was 5 mm. The centre well was filled with undiluted antiserum obtained from rats immunized with bovine γ-globulin-sulphanilic acid conjugate. Antigens (bovine γ-globulin-sulphanilic acid, bovine γ-globulin, bovine serum albumin-sulphanilic acid conjugate, bovine serum albumin) were placed in wells surrounding the central wells. After 24–72 h, antigen-antibody reaction proceeded with precipitin lines observed between these wells.

Plasma elimination of sulphanilic acid

Rats anaesthetized by intraperitoneal injection of sodium pentobarbitone (60 mg kg^{-1}), received [^{14}C]sulphanilic acid (27·5 nmol kg^{-1}) dissolved in 0·2 mL saline into the femoral vein, and 0·25 mL of blood was collected from the jugular vein, at 5, 10, 20, 40, 60 and 90 min. The radioactivity in plasma was determined in a liquid scintillation system. In a pilot study, no metabolites of sulphanilic acid were detected in plasma over the time course of this experiment.

Binding of sulphanilic acid with macromolecules in serum

Ultrafiltration was adopted to estimate the binding of sulphanilic acid to macromolecules in serum. Blood obtained from control and immunized rats by cardiac puncture was allowed to clot at room temperature, stored overnight at 4°C and centrifuged twice for 10 min at 1000 *g*. The supernatant (0·5 mL) was collected and added to [^{14}C]sulphanilic acid (10 μL, 0·01–1·5 μCi mL^{-1}). The mixture was incubated at 37°C and ultrafiltration was carried out (Ultracent-30, Toyo Soda., Co., Ltd) with a filter membrane (mol. wt cut off 30 000). A sample was applied to the reservoir cup and centrifuged at 3000 *g* for 10 min at 4°C. Ultrafiltrate was assayed to determine the free drug concentration.

Urinary excretion of sulphanilic acid

Rats were anaesthetized by intraperitoneal injection of sodium pentobarbitone (60 mg kg^{-1}) and the abdomen was opened. The bile duct was cannulated with polyethylene tubing (PE-10) and bile was collected at intervals for 90 min. The bladder was also cannulated with polyethylene tubing (i.d. 0·28 mm, o.d. 0·61 mm, Dural Plastics, Dural, Australia) and urine was collected for 90 min after intravenous injection. The radioactivity in bile and urine was determined in a liquid scintillation system.

Analytical method

Total ^{14}C radioactivity of 1 mL samples was determined by liquid scintillation. The ^{14}C radioactivity of *N*-acetylsulphanilic acid, a major metabolite of sulphanilic acid, was also determined after separation from unchanged drug by thin layer chromatography (chloroform–methanol, 6:4; R_F 0·23 and 0·3 for unchanged drug and metabolite, respectively). All counts were corrected using external standards.

Pharmacokinetic and statistical analyses

The pharmacokinetic parameters after intravenous administration were calculated by MULTI (Yamaoka et al 1981). Results were expressed as the mean ± standard deviation (s.d.). Statistical analyses were performed using Student's *t*-test.

Results

The detection of anti-sulphanilic acid antibody

In the radial immunodiffusion reaction, there were precipitin lines between antiserum and antigens (bovine γ-globulin-sulphanilic acid conjugate, bovine γ-globulin, bovine serum albumin-sulphanilic acid conjugate) while no precipitin lines between antiserum and bovine serum albumin were observed. These findings indicate the presence of antibodies for sulphanilic acid and bovine γ-globulin in immunized animals.

Plasma elimination of sulphanilic acid

Fig. 1 shows plasma concentration-time curves of unchanged [^{14}C]sulphanilic acid after intravenous administration in control and immunized rats. There were significantly higher amounts of radioactivity present in the plasma of actively immunized rats compared with the control rats at all times up to 90 min. However, radiolabelled metabolites could not be detected over the time of this experiment. Table 1 summarizes the pharmacokinetic parameters after the intravenous administration of sulphanilic acid. There was a

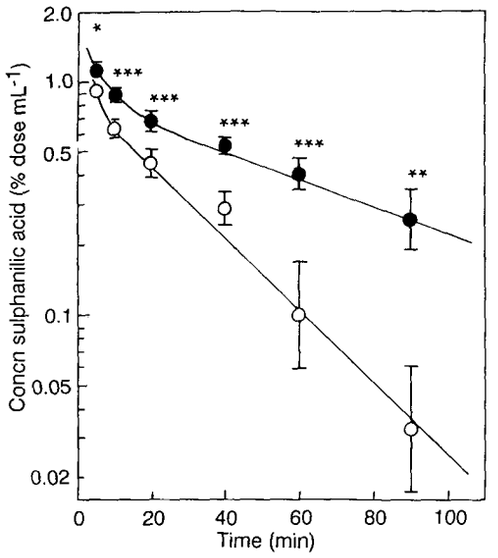

FIG. 1. Plasma concentration-time curves of [^{14}C]sulphanilic acid in control (○) and immunized (●) rats. Results are expressed as the mean ± s.d. of 4 animals. *$P<0.05$, **$P<0.02$, ***$P<0.01$, compared with the control.

Table 1. Pharmacokinetic parameters after intravenous administration of [^{14}C]sulphanilic acid to control and immunized rats.

	Control	Immunized
AUC (% of dose min mL^{-1})[a]	36·3 ± 4·7	79·4 ± 10·1**
$t_{\frac{1}{2}}$ (min)[b]	21·9 ± 3·4	50·9 ± 5·9**
CL (mL min^{-1} kg^{-1})[c]	6·9 ± 1·5	2·8 ± 0·3**
Vd (mL kg^{-1})[d]	213·0 ± 18·9	202·1 ± 6·4

Results are expressed as the mean ± s.d. of 4 animals. [a] area under the plasma concentration time-curve, [b] plasma half-life of terminal phase, [c] plasma clearance, [d] distribution volume of terminal phase, **$P<0.01$.

Table 2. Binding of [^{14}C]sulphanilic acid to macromolecules in plasma of control and immunized rats.

Concn (μM)	Binding (%)	
	Control	Immunized
2·56	58·9 ± 1·9	74·8 ± 10·1
7·67	58·6 ± 0·7	60·3 ± 6·5
76·7	54·3 ± 0·4	59·7 ± 10·6
384	54·3 ± 3·9	57·6 ± 6·9

Results are expressed as the mean ± s.e. of 3 experiments.

significant increase in plasma half-life and AUC and a significant decrease in clearance of sulphanilic acid in immunized rats compared with control. However, no significant change was observed between control and immunized animals in the apparent volume of distributon.

Binding of [^{14}C]sulphanilic acid to macromolecules in serum

Binding of the drug in serum was examined by ultrafiltration. As shown in Table 2, a significant increase in % binding of sulphanilic acid was noted in immunized rats compared with the control at a very low concentration of the drug and there was higher % binding of sulphanilic acid in serum of immunized rats than in control rats. Further, binding of sulphanilic acid was decreased with an increase in total sulphanilic acid concentration in the immunized rats.

Biliary and urinary excretion of sulphanilic acid

The urinary excretion of total radioactivity was significantly reduced in immunized rats compared with the control at every time up to 90 min (Fig. 2). In contrast, the biliary excretion of sulphanilic acid was low (< 4% of dose in 90 min) in both groups of animals and there was no significant difference between these groups (data not shown). Fig. 3 shows the cumulative urinary excretion of [^{14}C]sulphanilic acid and its main metabolite in 90 min. There was a significant decrease in cumulative urinary excretion of unchanged sulphanilic acid in 90 min, whereas we found no significant difference in the cumulative urinary excretion of the metabolite.

Discussion

When injected into immunized animals, proteins and other macromolecular antigens form complexes with their corresponding antibodies and the resulting antigen-antibody complexes are rapidly removed from the circulation (Dixon & Talmage 1951). In contrast, some small antigens and haptens which also formed complexes with their corresponding antibodies, have been shown to be retained longer in the circulation of specifically immunized animals than in non-immunized animals (Schmidt et al 1974). However, few studies have been carried out on the disposition of the protein-sulphanilic acid complex.

The present study has demonstrated that immunization of rats with protein-sulphanilic acid conjugate results in an increased plasma half-life and a decreased urinary excretion of sulphanilic acid. The result is in agreement with previous findings that immunization of rats with protein-hapten conjugates affected the disposition of various drugs such as morphine (Berkowitz & Spector 1972; Berkowitz et al 1974), digoxin (Schmidt & Butler 1971; Schmidt et al 1974) and phenobarbitone (Cerreta et al 1977; Flynn & Cerreta 1978). We have previously shown that local and systemic immune responses and immunomodulators altered the intestinal transport of drugs (Yamamoto et al 1989 a, b). These results suggested that immunization might play an important role in both intestinal transport and disposition of low molecular weight compounds.

The prolongation of higher plasma concentration of sulphanilic acid was noted in immunized rats, indicating the binding of this drug to plasma circulating antibodies.

The binding of sulphanilic acid in serum was decreased with increased concentration of sulphanilic acid in the immunized rats and there was no significant difference in the serum binding of sulphanilic acid to macromolecules between control and immunized animals at higher concentrations. This result may be explained by saturation binding of sulphanilic acid to the antibodies at high concentrations so the observed binding is dependent only on drug binding to plasma proteins.

We have found no significant difference between control and immunized rats in the biliary excretion of sulphanilic acid. In contrast, there was a significant reduction in the total urinary excretion of sulphanilic acid (Fig. 3), in keeping with

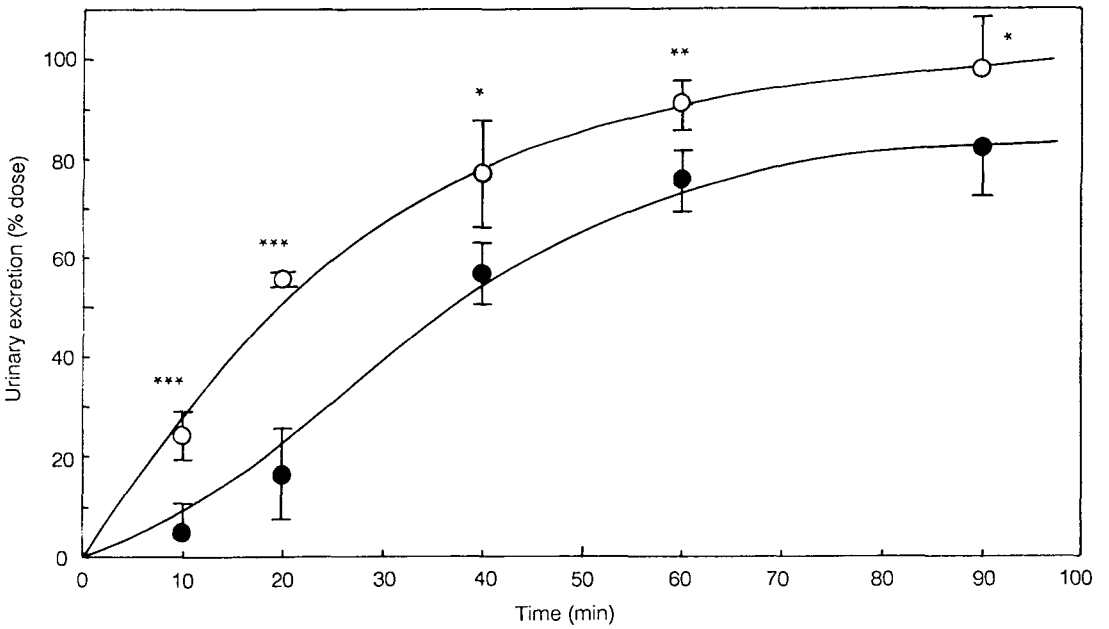

FIG. 2. Time course of urinary excretion of total ^{14}C in control (○) and immunized (●) rats. Results are expressed as the mean ± s.d. of 3 animals. $*P < 0.05$, $**P < 0.02$, $***P < 0.01$, compared with the controls.

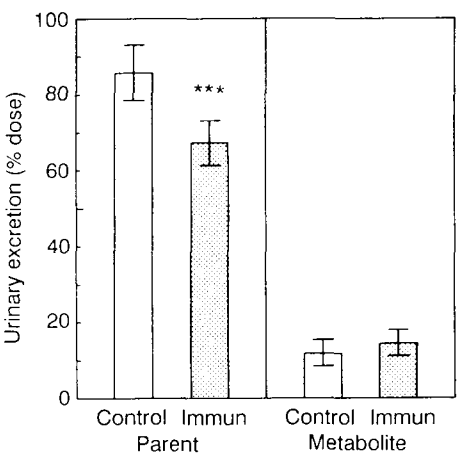

FIG. 3. Cumulative urinary excretion of [^{14}C]sulphanilic acid and its metabolite during 90 min in control and immunized rats. Results are expressed as the mean ± s.d. of 3 animals. Keys: $***P < 0.01$.

the prolonged plasma half-life of the hapten. There was no significant difference in the urinary excretion of sulphanilic acid metabolite between control and immunized animals, consistent with the previous finding that sulphanilic acid is insignificantly metabolized in plasma (Yasuhara et al 1984).

In conclusion, we have demonstrated that immunizaton against sulphanilic acid could alter the plasma elimination and urinary excretion of sulphanilic acid by binding of drug to circulating antibodies. Further studies are necessary to clarify the effect of immunization with sulphanilic acid on tissue distribution of the hapten.

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