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The Effect of Drug-specific Active Immunization on Digoxin and Benzylpenicillin Disposition in the Bile Duct-cannulated Rat

P. C. JOHNSTON, I. H. STEVENSON AND D. S. HEWICK

Department of Pharmacology & Clinical Pharmacology, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, UK

Abstract—Rats were immunized with a digoxin-human serum albumin conjugate i.m. This resulted in a several hundred-fold increase in plasma radioactivity and a 90% reduction in biliary drug elimination when [^{3}H]digoxin (10 μg kg^{-1}, i.v.) was subsequently injected into anaesthetized bile duct-cannulated rats. It was calculated that about 90% of the drug dose remained antibody-bound within the plasma compartment, with essentially no drug distributing into organs such as the heart and liver. Digoxin-specific antibody levels, determined by equilibrium dialysis, were high in the plasma but at least an order of magnitude lower in the bile. Immunization via Peyer's patches did not increase antibody levels in the bile. Immunization (i.m.) with a benzylpenicillin-human serum albumin conjugate gave specific antibody plasma titres with values less than 10% of those obtained after immunization with a digoxin-protein conjugate. However, although subsequent injection of the hapten (40 μg kg^{-1}, [^{14}C]benzylpenicillin, i.v.) was associated with much lower increases and decreases in plasma and biliary radioactivity, respectively, they were still statistically significant. It appears that endogenously-formed drug-specific antibodies, when present in the blood, will inhibit drug distribution and elimination. It is unlikely that their secretion in the bile plays a significant role in mediating biliary drug hapten elimination.

Previous studies in rabbits (Schmidt et al 1974) and guinea-pigs (Andrews et al 1986) have shown that immunization, using a digoxin-human serum albumin conjugate, produces digoxin-specific antibodies which sequester the hapten in the plasma with a consequential reduction in elimination both in the urine (Schmidt et al 1974; Hewick et al 1986) and faeces (Schmidt et al 1974). In line with reduced faecal elimination, preliminary experiments in guinea-pigs suggested that excretion of digoxin in the bile was also reduced (Hewick et al 1986). It is proposed, using protein conjugates of digoxin and benzylpenicillin as immunogens, to examine the effect of immunization on drug hapten disposition. A bile duct-cannulated rat model has been chosen to allow the biliary elimination of the hapten to be studied, so that a more precise indication of the likely effects on subsequent faecal elimination can be obtained.

This experimental model also provides the opportunity to investigate if drug-specific antibodies secreted in the bile are capable of facilitating the biliary elimination of drugs. Immunoglobulin A (IgA), the class of antibody which is present in the bile in by far the greatest amount, is synthesized by the mucosal-associated lymphoid tissue (e.g. Peyer's patches). In the rat, such IgA can enter the blood and be subsequently secreted in the bile via the so-called IgA pump (Hodgson 1985). This pump has been shown (Peppard et al 1982) to facilitate the biliary elimination of blood-borne protein antigen bound to specifically induced IgA. Whether drug-specific IgA would have a similar role in the biliary elimination of low molecular weight lipid-soluble drug haptens remains to be established. In the present study, hapten elimination in the bile has been compared after injection of a drug-protein immunogen i.m. and via the Peyer's patches. The use of the latter route was suggested by the work of Mullock et al (1983) who found that immunization via Peyer's patches, using a chlorpromazine-protein conjugate, resulted in chlorpromazine-specific antibody secretion in the bile.

Correspondence to: D. S. Hewick, Department of Pharmacology & Clinical Pharmacology, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, UK.

Materials and Methods

Materials

[^{3}H]Benzylpenicillin (15 Ci mmol^{-1}) and [^{14}C]benzylpenicillin (58·9 Ci mmol^{-1}) were purchased from Amersham International, Amersham, Bucks, UK, and 12α[^{3}H]digoxin (15·4 Ci mmol^{-1}) from New England Nuclear, Boston, MA. Tissue solubiliser (TS-1) was brought from Koch-Light Ltd., Haverhill, Suffolk and liquid scintillant (NE 260) from Nuclear Enterprises (Edinburgh, UK). Digoxin, benzylpenicillin and Freund's complete adjuvant (FCA) were obtained from Sigma Chemical Co. (Poole, Dorset, UK). All other chemicals were purchased from British Drug Houses, Poole, Dorset, UK.

Preparation of immunogens

A human serum albumin-digoxin conjugate was prepared by the method of Smith et al (1970) in which the terminal digoxin residue is oxidized with sodium metaperiodate so that the resulting dialdehyde reacts with a primary amino group of the albumin to form a Schiff base linkage. The bond is then stabilized by reduction with sodium borohydride. Using the spectrophotometric method of Smith et al (1970) it was estimated that the conjugate possessed 6–7 digoxin molecules per molecule of albumin.

A human serum albumin-benzylpenicillin conjugate was prepared by mixing human serum albumin (200 mg) with benzylpenicillin (100 mg) in 0·5 M carbonate buffer at pH 10 according to Lee et al (1985). Using the penamaldate assay

(Parker et al 1962) it was estimated that the conjugate possessed 22 penicilloyl groups per molecule of protein.

Immunization

Female Sprague-Dawley rats (200–270 g) with free access to food and water were used. Drug-albumin conjugates were administered in FCA as an emulsion containing equal volumes of the FCA and a 2 mg mL^{-1} solution (in saline) of human serum albumin drug conjugate. When immunization was via the intramuscular route, 0·25 mL of the emulsion was injected into each of the back legs. When immunization involved Peyer's patches (method after Hall et al 1979) the emulsion (0·1 mL) was first injected i.p. Two weeks later, the animals were anaesthetised with pentobarbitone (55 mg kg^{-1} i.p.) and a small opening made in the abdomen. After carefully lifting out the small intestine and placing it on sterile saline-soaked gauze, 0·1 mL of the emulsion was injected into each discernible Peyer's patch along the length of the intestine. The intestine was then replaced, the abdominal opening closed by sutures and the rats allowed to recover. In control animals human serum albumin was used in place of the drug-albumin conjugate.

Disposition of radioactivity in anaesthetized bile duct-cannulated rats

The rats were anaesthetized with pentobarbitone (60 mg mL^{-1}, 55 mg kg^{-1} i.p.) 16 weeks after immunization via the intramuscular route, or one week after immunization via Peyer's patches. Then after cannulating a carotid artery and jugular vein, the bile duct was cannulated essentially as described by Klaassen & Strom (1978). Rectal temperature was maintained at 37°C by means of a heat-lamp regulator device (Yellow Springs Instrument Company, Yellow Springs, OH).

Before injection of drug hapten, bile and plasma samples were taken for the determination of antibody titre. The penicillin-immunized rats were given [^{14}C]benzylpenicillin i.v. (40 μg kg^{-1}, 6·25 μCi kg^{-1}). The digoxin-immunized rats were given [^{3}H]digoxin i.v. at doses of either 10 μg kg^{-1}, 12·5 μCi kg^{-1} (immunization via intramuscular route) or 0·725 μg kg^{-1}, 12·5 μCi kg^{-1} (immunization via Peyer's patches). For experiments involving "intramuscular immunization" the molar dose of penicillin was about ten times that used for digoxin in an attempt to reflect the higher clinically-used doses of the former drug. The lower of the two digoxin doses was given after immunization via Peyer's patches, on the basis that any changes in biliary elimination due to the presence of digoxin-specific antibodies in the bile would be more readily detectable in the presence of lower concentrations of hapten.

Bile, and blood (0·25 mL), samples were collected at the times indicated in Results. Saline was given i.v. to replace fluid lost as bile or blood. Blood was centrifuged (3000 *g* for 10 min) to obtain plasma. Two hours after drug injection the animals were killed and heart, liver and kidneys removed and weighed. The intestines (small intestine, caecum and colon) were weighed before and after removing the contents, which were then homogenised before assay. Both urine voided during 2 h and that remaining in the bladder was collected.

Radioactivity present in plasma, bile, urine, gut contents and tissues was determined by standard liquid scintillation counting methods as detailed previously (Griffiths et al 1984). Total radioactivity (which may have included metabolites along with unchanged drug) was expressed as digoxin or benzylpenicillin equivalents.

Determination of antibody titre

Antibody titre, which is a function of both antibody concentration and affinity, was determined by equilibrium dialysis, basically as described by Smith et al (1970). Diluted plasma and bile samples in buffer solution were dialysed, using the Dianorm apparatus (Diachema A. G. Zurich, Switzerland), against buffered [^{3}H]digoxin or [^{3}H]benzylpenicillin solution (4 pmol mL^{-1}, 15·4 or 15 Ci $mmol^{-1}$). The buffer solution (0·15 mM NaCl, 0·01M phosphate pH 7·4) contained 1 mg mL^{-1} bovine serum albumin to inhibit any non-specific binding. After incubating for 18 h at 25°C, samples were taken from each side of the dialysis membrane so that the concentrations of free and bound tritiated hapten could be determined after liquid scintillation counting. Antibody titre was defined as the dilution of plasma or bile which bound 50% of the added tritiated hapten.

Statistics

Means $\pm$ s.e. are given. Data were analysed using a non-paired Student's t-test with a probability of $P < 0.05$ being taken as significant.

Results

Hapten disposition after digoxin-specific immunization

Fig. 1a shows that digoxin-specific immunization (via the intramuscular route) increased plasma digoxin-derived radioactivity several hundred-fold, this increase being largely maintained for the 2 h experimental period. The control values, despite being very low in relative terms, were still well within the limits of detection. Associated with the high concentration of radioactivity in the plasma of immunized rats, there was a 90% reduction of drug elimination in the bile (Fig. 1b). In control and immunized rats, respectively, there was no significant difference in the percentage of the dose eliminated in the collected urine during 2 h (0·88 $\pm$ 0·67 vs 0·16 $\pm$ 0·07). The corresponding data for secretion into the intestine were: small intestine, 4·1 $\pm$ 0·4 and 0·8 $\pm$ 0·1; large intestine, 1·0 $\pm$ 0·2 and 0·05 $\pm$ 0·02; caecum, 1·3 $\pm$ 0·1 and 0·14 $\pm$ 0·04. The values for the immunized rats were significantly lower.

Heart and kidney digoxin-derived radioactivity concentrations at 2 h, uncorrected for plasma radioactivity content, were apparently significantly increased by immunization (heart, 8·0 $\pm$ 0·6 vs 23·0 $\pm$ 3·0 ng g^{-1}; kidney, 5·6 $\pm$ 0·4 vs 37·0 $\pm$ 6·0 ng g^{-1}), while in the liver, concentrations were reduced (44·0 $\pm$ 6 vs 25·0 $\pm$ 3·0 ng g^{-1}).

After digoxin-specific immunization via Peyer's patches and injecting a digoxin dose (0·725 μg kg^{-1}) about one-fourteenth that previously used, similar effects on plasma radioactivity concentrations and cumulative biliary excretion were obtained. For instance, at 4 min the plasma concentrations for control and immunized rats were 0·33 $\pm$ 0·04 and 19·4 $\pm$ 1·6 ng mL^{-1}, respectively, and the corresponding values at 2 h were 0·08 $\pm$ 0·01 and 11·9 $\pm$ 1·3 ng mL^{-1}. Cumulative biliary excretion (% of dose) after 2 h

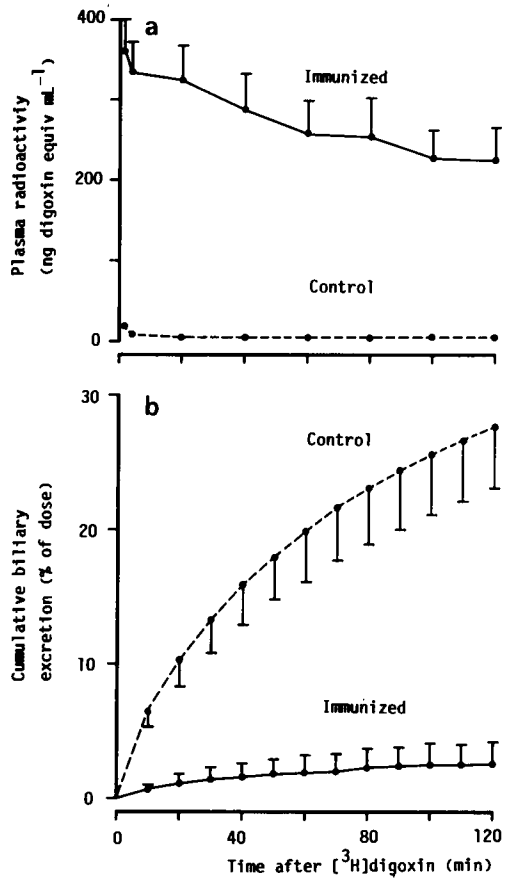

FIG. 1. The effect of digoxin-specific active immunization on hapten plasma concentrations (a) and cumulative biliary excretion (b) in rats. Sixteen weeks after immunization with a digoxin-human serum albumin conjugate (i.m.) the rats were anaesthetized (pentobarbitone 55 mg kg^{-1} i.p.), bile duct-cannulated and injected with [^{3}H]digoxin i.v. (10 $\mu g\ kg^{-1}$, 12·5 $\mu Ci\ kg^{-1}$). Hapten concentrations ([^{3}H]digoxin-derived radioactivity) are expressed as digoxin equivalents. Means are given ±s.e. (n = 6). Control and experimental data were significantly different ($P < 0.05$) at each time point.

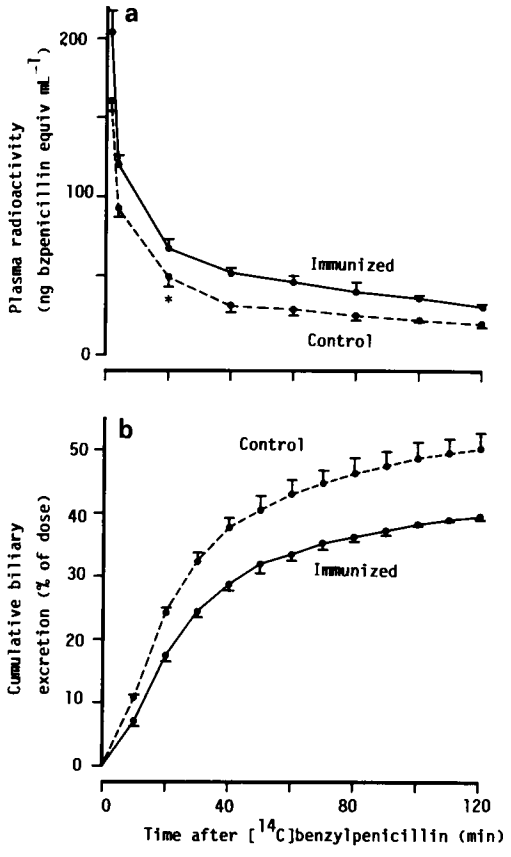

FIG. 2. The effect of benzylpenicillin-specific active immunization on hapten plasma concentrations (a) and cumulative biliary excretion (b) in rats. Sixteen weeks after immunization with a benzylpenicillin human serum albumin conjugate (i.m.) the rats were anaesthetized (pentobarbitone 55 mg kg^{-1} i.p.), bile duct-cannulated and injected with [^{14}C]benzylpenicillin i.v. (40 $\mu g\ kg^{-1}$, 6·25 $\mu Ci\ kg^{-1}$). Hapten concentrations ([^{14}C]benzylpenicillin-derived radioactivity) are expressed as benzylpenicillin equivalents. Means are given ±s.e. (n = 4). Control and experimental data were significantly different ($P < 0.05$) at each time point, except where indicated by an asterisk.

for control and immunized rats respectively, was 18·7 ± 2·03 and 1·77 ± 0·26.

Hapten disposition after penicillin-specific immunization

Benzylpenicillin-specific immunization had a less-marked effect on the disposition of the hapten; immunization increased penicillin-derived radioactivity by only 25–50% in the plasma (Fig. 2a) and reduced the biliary elimination over 2 h by 24% (Fig. 2b). For control and immunized rats, respectively, the percentage of the benzylpenicillin dose excreted in the urine during 2 h was 36 ± 2·4 and 42 ± 5·5 (no significant difference). There was also no significant difference between control and immunized rats in the amount of drug secreted into the intestines or in the tissue radioactivity concentration of the heart, liver and kidney.

Hapten-specific antibodies in plasma and bile

Although in digoxin-immunized rats digoxin-specific binding was detected in the bile, it was of a relatively low order and insufficent volumes of bile were available to enable titres to be determined. Therefore, digoxin-specific binding in a 1 in 10 dilution of bile was compared (Table 1). If it is assumed that rats 1 and 2 had titres of about 10, it is seen that the lowest titre in the plasma (rat 4) was some 8 times higher than the highest titres obtained in the bile.

Digoxin-specific immunization via Peyer's patches produced more consistent plasma titres than when the immunogen was given intramuscularly, but did not enhance digoxin-specific binding in the bile (Table 1).

After penicillin-specific immunization via the intramuscular route, hapten-specific binding in the plasma was relatively low and determination of percentage binding for a 1 in 10 plasma dilution for the 4 immunized rats used, gave values of 4, 25, 15 and 35%. If it is assumed that the highest value represents a titre of about 5, it is seen that this value is one-sixteenth of the lowest plasma titre obtained in the digoxin experiment (rat 4, Table 1).

Discussion

The high plasma digoxin-derived radioactivity levels found after digoxin-specific immunization in rats, agree with

Table 1. Digoxin-specific binding in plasma and bile after immunization with a digoxin-human serum albumin conjugate (see Materials and Methods). No detectable digoxin binding was observed in samples from control rats which had been "immunized" with human serum albumin alone.

Route of Immunization	Rat	Plasma titre[a]	Percent digoxin-specific binding in 1 in 10 dilution of bile
	1	2000	47
	2	524	57
Intramuscular	3	188	4
	4	80	14
	5	761	34
	6	780	21
	7	810	0
	8	880	23
Via Peyer's	9	870	18
patches	10	480	5
	11	280	61

[a] The dilution which binds 50% of [^{3}H]digoxin added (see Materials & Methods).

similar findings in rabbits (Schmidt et al 1974; Griffiths et al 1985) and guinea-pigs (Hewick et al 1986). In the present study, after digoxin-specific immunization via the intramuscular route and a subsequent digoxin dose of 10 μg kg^{-1}, it can be calculated that at 2 h after hapten injection about 90% of the digoxin dose remains in the plasma (using the 2 h plasma concentration of 222 ng mL, and assuming a plasma volume of 40·4 mL kg^{-1}, Baker et al 1980). The drastic reduction in biliary elimination is therefore not unexpected. This along with the detectable decrease in direct secretion of drug into the intestine, would result in reduced faecal excretion. In the experimental model used, the amount of hapten eliminated by the urinary route was small and highly variable, and it was not surprising that the decrease in urinary excretion associated with immunization was not statistically significant.

Measurement of tissue radioactivity in immunized rats is complicated by the presence of plasma which contains very high concentrations of hapten. However, the amounts of such plasma-associated hapten can be approximately determined by multiplying published values for the appropriate tissue content of plasma in female rats (Everett et al 1956) by the respective control or immunized plasma radioactivity concentrations at 2 h. If such calculated values for plasma-associated hapten are subtracted, the respective net tissue concentrations (ng g^{-1}) for [^{3}H]digoxin-derived radioactivity in control versus immunized rats receiving 10 μg kg^{-1} digoxin are 7·7 and −12·5 (heart), 5·4 and 9·5 (kidney), 43·6 and −16·5 (liver). Taking into account the impreciseness of the correction procedure used, the negative values obviously indicate that essentially no hapten is distributed in the cardiac or hepatic tissue. This is in line with the calculation that some 90% of the drug is contained within the plasma compartment.

The low plasma titres of benzylpenicillin-specific antibodies were unexpected. Especially when compared with the corresponding values obtained for digoxin-specific antibodies after immunization using an identical carrier protein, route of immunization and injection vehicle (containing FCA). The relatively low titres are also surprising in view of the fact that the procedure used to produce the benzylpenicillin-protein conjugate has been reported to give a product with a high degree of immunogenicity (Lee et al 1985). However, despite this, it is clear that even relatively low amounts of benzylpenicillin-specific antibodies can still cause detectable changes in hapten disposition.

In the case of benzylpenicillin, the interpretation of antibody titres obtained by standard equilibrium dialysis procedures, may be complicated by degradation of benzylpenicillin and the possibility of it, or its degradation products, convalently binding to plasma protein (Kitteringham et al 1987). In the current studies, the negligible level of protein-bound radioactivity detected in control experiments, suggest that the amount of covalent binding was very small compared to the reversible binding occurring between hapten and antibody. Concerning degradation, although the conditions of our incubations in-vitro were somewhat different to those of Kitteringham et al (1987), it is possible that the hapten(s) binding to antibodies could have included degradation products (in particular benzylpenicilloic acid) as well as benzylpenicillin. To some extent this situation resembles that in-vivo, since Kitteringham et al (1987) reported that after dosing rats with [^{3}H]benzylpenicillin, much of the plasma radioactivity comprised benzylpenicilloic acid.

Regarding Peyer's patch immunization, our results after direct injection of the immunogen into the mucosal-associated lymphoid tissue were disappointing in that there was no increased digoxin-specific binding (to IgA) in the bile, compared with more "distal" immunization via the intramuscular route. This was unexpected since Hall et al (1979) had reported that after injecting a bacterial immunogen into Peyer's patches, antibody titres in the bile were increased to values somewhat greater than those found in the serum after injection of the immunogen subcutaneously.

Although our experiments involving injection of the protein conjugate into Peyer's patches did not selectively increase antibody titre in the bile compared with that after immunization via the intramuscular route, the former procedure clearly had a stimulatory effect on the Peyer's patches since it increased their size by about 50% (results not presented). The work of Mullock et al (1983) however, in which a chlorpromazine-haemocyanin conjugate was injected into the Peyer's patches, seems in greater agreement with our findings. These workers, although they only examined the single route of immunization, found that chlorpromazine-specific antibody levels were much higher in the serum than bile. The likely presence of high levels of antibodies in the blood after drug-specific immunization, means that it is improbable that the IgA pump would make an appreciable contribution to facilitating biliary drug elimination. As can be seen in the present work, the presence of drug specific antibodies in the body will more probably reduce drug elimination by this route.

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