mechanism, which prevents it from leaking to the surrounding anococcygeal smooth muscle in sufficient concentration to cause relaxation.

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The effect of digoxin-specific active immunization on digoxin toxicity and distribution in the guinea-pig

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In guinea-pigs intravenously infused with digoxin, prior immunization using a digoxin-human serum albumin conjugate increased by 3- and 2·4-fold, respectively, the digoxin doses causing the first signs of cardiotoxicity and death. At death, serum digoxin concentration was four times higher in immunized than in control animals. In the immunized guinea-pigs 50% of the serum digoxin was protein bound, presumably mainly to digoxin-specific antibodies, since in the controls the bound fraction was only 1–2%. Generally, tissue digoxin concentrations were not increased to the same extent as the lethal dose, and in the heart and lungs the increase was not significant. With cardiac (ventricle) subcellular fractions, there was no difference between control and immunized animals in the digoxin concentration of the 'microsomal' pellet. This subfraction contains the plasma membrane and the associated sodium pumps which are considered to be the sites at which the pharmacologically active digoxin binds. It seems likely, therefore, that the greater digoxin resistance in the immunized animals can be explained on the basis of reduced drug access to the site of action within the heart.

Schmidt & Butler (1971) showed that rabbits actively immunized using a digoxin-human serum albumin conjugate could tolerate intravenous digoxin doses of 0·6–0·9 mg kg^{-1}, while in control animals (immunized with albumin) a dose of 0·6 mg kg^{-1} was uniformly lethal. The present study, using guinea-pigs as a further digoxin-sensitive species, investigates more fully the protective effect of digoxin-specific immunization. The study involving continuous intravenous infusion of digoxin, determines more precisely the effect of immunization on the lethal digoxin dose, as well as measuring the digoxin concentrations in the heart and other tissues at the end of the infusion.

Materials and methods

Preparation of immunogen. A human serum albumin-digoxin conjugate was prepared by the method of Smith et al (1970) in which the terminal digitoxose residue of digoxin 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.

Digoxin-specific active immunization. Female Dunkin-Hartley guinea-pigs between 6 and 9 months old (mean weight 0·95 kg) were injected (i.m. in the hind legs) with 0·8 ml of an emulsion containing equal volumes of Freund's complete adjuvant and 2 mg ml^{-1} (in saline) of human serum albumin (control) or human serum albumin-digoxin conjugate ('immunized'). The animals were used 9 weeks after immunization, and were allowed free access to food and water up to this time.

Digoxin infusion. Seven immunized and seven control guinea-pigs were anaesthetized with urethane (25%, 1·5 g kg^{-1} i.p.) and infused via a jugular vein with [^{3}H]digoxin (500 μg ml^{-1}, 10 μCi ml^{-1} in an aqueous vehicle containing 10% ethanol, 40% propylene glycol, 0·069% citric acid and 0·45% Na_2HPO_4) at a rate of 1·9 ml h^{-1} until death (no cardiac activity for more than 60 s). Cardiac activity was monitored by lead II electrocardiographic recording.

Treatment of tissue and blood samples. After infusion, tissues were removed and weighed, and blood samples (cardiac puncture) were allowed to clot to obtain serum. Protein (antibody)-bound serum digoxin was separated by precipitation in '50% saturated' ammonium sulphate solution (Hudson & Hay 1980).

Following the removal of small samples for the determination of tissue radioactivity, the ventricles

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from each heart were homogenized (2–3 g tissue in sucrose–ethylenediaminetetraacetic acid, 0·33 M : 0·001 M, to a total volume of 10 ml) for 30 s in an Ultra Turrax (Janke & Kunkel, IKA-Werk, Stauffen, FRG) homogenizer and subfractions prepared essentially according to Dutta et al (1968). The homogenate was spun at 110 000*g* for 1 h and the supernatant ('soluble fraction') removed. The pellet was resuspended in the previously described vehicle and consecutively spun at 450*g* for 10 min (this spin was repeated), 12 000*g* for 15 min and 110 000*g* for 1 h to obtain 'nuclear', 'mitochondrial' and 'microsomal' pellets, respectively. All operations were carried out at 4 °C, with the pellets being resuspended in 1·5–3 ml of 0·1 M Tris (pH 7·5) buffer. Protein concentrations of the ventricular homogenate and subfractions were determined by the method of Lowry et al (1951).

Radioactivity in serum, tissues, heart homogenate and subcellular fractions was determined by standard liquid scintillation counting methods (Griffiths et al 1985a). Radioactivity thus determined was divided by the specific activity of the [^{3}H]digoxin injected, to give digoxin concentration. Digoxin concentration calculated in this way is strictly 'digoxin equivalent' since it may include digoxin metabolite concentration as well as that of unchanged digoxin.

Statistics. Comparisons were made using Student's *t*-test with $P < 0·05$ being taken as significant. Results are given as means ± s.e.

Results

Digoxin-induced cardiotoxicity and death. Immunization caused statistically significant elevations in both the toxic and lethal doses of digoxin. The dose at which the first cardiotoxic effects (ventricular ectopic beats, atrioventricular block or missed beats) were seen was increased by about 3-fold (1·49 ± 0·14 versus 0·47 ± 0·04 mg kg^{-1}). The subsequent lethal dose was increased 2·4 times (2·0 ± 0·13 versus 0·83 ± 0·05 mg kg^{-1}).

Serum and tissue concentrations of digoxin at death. Serum digoxin concentrations were four times higher in immunized than in control animals (Fig. 1). Whereas in controls only 1–2% of the total serum digoxin was protein bound, the corresponding value in experimental guinea-pigs was about 50% (Fig. 1).

In immunized animals, digoxin concentrations in the liver, kidney, spleen, skeletal muscle (abdominal), fat (peritoneal) and brain were increased by some 70, 60, 10, 160, 500 and 150%, respectively (Fig. 1). Although the mean values for digoxin concentrations in the heart (ventricles) and lung tissue of immunized animals were greater, these increases were not significant.

With the ventricular homogenates there was no significant difference between the digoxin concentrations in immunized and control animals (Table 1). The order of decreasing digoxin concentration in the various ventricular subfractions was soluble fraction > microsomes > mitchondria = nuclei (Table 1). There were no differences between control and experimental animals in digoxin concentration in the soluble fraction and microsomes, although in the nuclei and mitochondria the digoxin concentrations were greater for the immunized guinea-pigs.

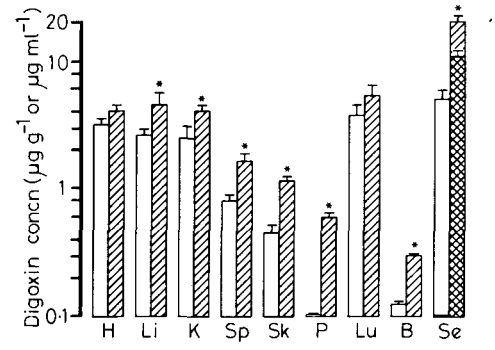

FIG. 1. The influence of digoxin-specific active immunization on digoxin disposition. The guinea-pigs had been intravenously infused with [^{3}H]digoxin (500 μg ml^{-1}, 10 μCi ml^{-1}) until death. The mean lethal doses were 2·0 and 0·83 mg kg^{-1} digoxin in immunized and control animals, respectively. The hatched columns indicate the immunized animals (for details see Materials & methods). The additional cross hatching indicates protein-bound serum digoxin. Digoxin concentration was determined directly from the concentration of radioactivity and is strictly 'digoxin equivalent'. Means ± s.e. are given (n = 7). Asterisks indicate differences ($P < 0·05$) between control and immunized animals. Key: H, heart (ventricle); Li, liver; K, kidney; Sp, spleen; Sk, skeletal muscle; P, peritoneal fat; Lu, lung; B, brain; Se, serum.

Table 1. The effect of digoxin-specific active immunization on the cardiac subcellular digoxin distribution in guinea-pigs receiving a lethal digoxin infusion.

	Digoxin concentration at death (ng mg^{-1}) protein	
Fraction	Control	Immunized
Whole homogenate (ventricle)	21·3 ± 2·5	22·5 ± 1·5
Soluble fraction (110 000*g* supernatant)	129·0 ± 18·5	117·5 ± 11·5
Nuclei (450*g* pellet)	4·5 ± 0·5	8·5 ± 0·5*
Mitochondria (12 000*g* pellet)	5·5 ± 0·3	8·2 ± 0·7*
Microsomes (110 000*g* pellet)	22·5 ± 3·6	22·0 ± 3·5

The data are from the experiment described in the legend to Fig. 1, the immunized and control animals receiving mean doses of 2·0 and 0·3 mg kg^{-1} digoxin, respectively. Digoxin concentration was determined directly from the concentration of radioactivity and is strictly 'digoxin equivalent'. Means ± s.e. are given (n = 7). Asterisks indicate differences ($P < 0·05$) between control and immunized animals.

Discussion

As reported by Schmidt & Butler (1971) digoxin-specific active immunization was associated with a tolerance to the cardiac effects of digoxin. There was a 3-fold increase in the digoxin dose causing cardiotoxicity and the lethal dose was increased 2·4 times. In general, tissue digoxin concentrations at death were not increased to the same extent as the lethal dose. This was presumably due to the digoxin being partly sequestered in the serum, where the drug concentration was elevated 4-fold. Even more marked increases in serum digoxin concentration after active immunization have been reported in the rabbit (Schmidt et al 1974; Griffiths et al 1985b). In the present work, half of the increased serum digoxin concentration was protein-bound, probably to digoxin-specific antibody. The data therefore suggest that, during infusion, digoxin-specific antibodies in the blood reduced access of digoxin to the tissue of immunized animals. It appears that in these animals cardiotoxic symptoms became apparent only when the digoxin binding capacity of these antibodies was exceeded, since the dose required to cause cardiotoxicity was much greater in immunized guinea-pigs, whereas the drug dosage increment between the first manifestations of cardiotoxicity and death was similar in control and experimental animals.

In control animals, apart from the somewhat high concentration in the lungs, the tissue distribution of digoxin agrees with that of earlier findings (Dutta & Marks 1966; Griffiths et al 1985a) with the digoxin concentrating in the liver, kidney and heart. The tissue digoxin concentrations in the immunized animals will tend to be overestimated due to the presence of blood containing high concentrations of digoxin, the extent of the error being dependent on the amount of blood normally contained within each particular tissue.

Such 'contamination' with blood/serum however, is negligible with the pellets obtained after differential centrifugation of ventricular homogenate. The centrifugation procedure we used was essentially that of Dutta et al (1968) who, with subsequent workers (Kim et al 1972; Stephen et al 1976), used this method to obtain subfractions from guinea-pig isolated hearts that had been perfused with digoxin. Although in the present study the final serum digoxin concentration was 10–100 times that in the perfusion fluid used by the earlier workers, there was a general similarity in the two sets of results in that, of the subfractions that could be sedimented, the highest digoxin concentration was in the microsomes, while lower and rather similar drug concentrations were in the nuclei and mitochondria.

The microsomal subfraction as prepared in 'Materials and methods' also includes the plasma membrane (Dutta et al 1968) which contains the sodium pumps that are generally accepted to be the sites at which the pharmacologically active cardiac glycoside binds (Hansen 1984). It is of interest to note, therefore, that although toxic rather than pharmacological effects were measured in this subfraction containing 'active' digoxin, the drug concentrations were not significantly different at death in control and immunized guinea-pigs, despite the latter receiving a 2·4 times greater dose.

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