Br. J. Pharmac. (1985), **84**, 157–163

The influence of digoxin antibodies on digoxin disposition and effect: studies in guinea-pigs and HeLa cells

N.M. Griffiths. D.S. Hewick[1]. J.F. Lamb* & I.H. Stevenson

Department of Pharmacology and Clinical Pharmacology, Ninewells Hospital and Medical School, University of Dundee, Dundee, DD1 9SY and Department of Physiology and Pharmacology*, Bute Medical Buildings, University of St. Andrews, St. Andrews, Fife, KY16 9TS

1 Pretreatment of guinea-pigs with digoxin-specific Fab (fragment antigen binding) fragments reduced the cardiotoxicity of intravenously infused digoxin (the lethal doses in Fab-treated and control animals were 1.0 and 0.6 mg kg^{-1}, respectively).
2 At death the serum digoxin concentration was elevated 2 fold in the Fab-treated animals, while the tissue concentrations were generally lower.
3 The 30–40% lower cardiac digoxin concentration (seen in whole homogenate and throughout the subcellular fractions examined) was surprising; presumably this reflects a difference from the controls in the proportion of pharmacologically active/inactive digoxin in this organ.
4 Adding digoxin-specific immunoglobulin G or the Fab fragments to HeLa cells before incubation with digoxin, reduced specific digoxin binding (Na pump-bound) slightly more than the non-specific binding.
5 Adding specific antibody after digoxin, however, did not reduce digoxin binding or effect a recovery in Na pump activity.
6 It seems that the protective effect of digoxin-specific antibodies seen in the guinea-pig can to some extent be simulated using HeLa cells. However, this is apparently not so regarding the widely-reported ability of these antibodies to reverse the action of digoxin.

Introduction

Digoxin-specific Fab (fragment antigen binding) fragments (prepared from sheep antiserum) have been used successfully in the treatment of severe and life-threatening digitalis toxicity (Smith *et al.*, 1976; Hess *et al.*, 1979; Aeberhard *et al.*, 1980; Smith *et al.*, 1982). The ability of digoxin-specific antibodies and their Fab fragments to prevent or reverse the effects of digoxin has also been demonstrated in experimental animals. For instance in dogs, both the positive inotropic (Ochs *et al.*, 1978) and cardiotoxic (Lloyd & Smith, 1978; Hougen *et al.*, 1979) effects of digoxin may be reversed by the administration of digoxin antibodies. In addition, these antibodies may cause profound changes in digoxin disposition, and other studies in dogs (Butler *et al.*, 1977; Ochs *et al.*, 1978) have shown that digoxin-specific IgG (immunoglobulin G) and Fab fragments effect very large increases in total serum digoxin concentration as a consequence of antibody binding in the vascular compartment. However, the free, and presumably pharmacologically-active digoxin concentration is markedly reduced. The urinary excretion of digoxin is delayed by the IgG but unaffected by treatment with Fab fragments (Butler *et al.*, 1977). However, despite these and other extensive animal studies (Smith *et al.*, 1979), little work has been reported on the influence of treatment with digoxin-specific antibodies on tissue digoxin concentration.

In parallel with the investigations *in vivo* there have been a number of studies *in vitro*. Digoxin-specific antibodies have been shown to reverse digoxin-induced electrophysiological abnormalities in canine cardiac muscle (Mandel *et al.*, 1972) and the positive inotropic effect in bovine ventricular trabeculae (Hess & Muller, 1982), as well as to remove digoxin from rat renal cortical slices and human erythrocytes that had been preincubated with the drug (Watson & Butler, 1972; Gardner *et al.*, 1973).

[1]Correspondence.

It is generally accepted that digoxin-induced cardiac effects are mediated by inhibition of the membrane-bound Na^+K^+-ATPase (the 'sodium pump') and it is thought (Gardner *et al.*, 1973: Hoeschen, 1976; Bosaller, 1981; Butler, 1982) that the digoxin antibodies exert their antidotal effect by combining with 'free' extracellular digoxin after its release from the membrane enzyme site and prevent the drug from rebinding to the site. The antibodies do not appear to combine with enzyme-bound digoxin so as to 'prise' it from the enzyme. However, it has been suggested in studies with HeLa cells (Cook & Brake, 1978; Lamb & Ogden, 1981) that recovery of sodium pump activity after inhibition by cardiac glycosides depends not only upon dissociation of drug from the membrane sodium pump sites, but also upon the manufacture and insertion of new pumps into the cell membrane. These workers postulate that a significant amount of sodium pump-bound cardiac glycoside does not dissociate from the membrane, but is 'internalized' as a glycoside-pump complex within the cell. It is therefore difficult to wholly predict the influence of digoxin-specific antibodies on the activity of digoxin-affected sodium pumps in such a situation.

The present paper examines further the pharmacokinetic and pharmacodynamic aspects of the protective/antidotal effects of drug-specific antibodies using a combination of *in vivo* and *in vitro* procedures. In the guinea-pig the influence of antibody on digoxin-induced cardiotoxicity and the resultant change in tissue (particularly cardiac) digoxin concentration was studied. In cultured human (HeLa) cells the effect of digoxin-specific antibodies on cellular digoxin retention/binding and the functioning of the sodium pump was examined.

Methods

Experiments in guinea-pigs

Two groups of five guinea-pigs (650–1120 g, female, Dunkin-Hartley) were anaesthetized with urethane ($1.0\,g\,kg^{-1}$, i.p.). The right jugular vein was cannulated and the cannula attached to an infusion pump. Electrocardiographic leads were connected to each limb and recordings made from Lead II. Thirty minutes before digoxin infusion, one group of animals received digoxin-specific Fab fragments ($2.0\,mg\,kg^{-1}$, i.p.) and the other non-immune sheep serum (2 ml, i.p.).

[^{3}H]-digoxin ($250\,\mu g\,ml^{-1}$; $2.33\,\mu Ci\,ml^{-1}$) was infused at a rate of $3.8\,ml\,h^{-1}$ ($0.950\,mg\,h^{-1}$) until death. The electrocardiogram was monitored throughout the experimental period on a chart recorder. Initially recordings were made every 5 min, but when digoxin toxicity became clearly apparent, recordings were made every 0.5–1 min.

At death, blood was removed directly from the heart by cardiac puncture, allowed to clot and spun at 3,000 *g* for 15 min to obtain the serum. Bile and urine samples were collected from the gall bladder or urinary bladder respectively at the end of infusion. Any urine voided was also collected and added to the urine obtained from the bladder.

Samples of serum, bile and urine, as well as of tissues (see Table 1) were taken for determination of radioactive content by liquid scintillation counting.

After removal of cardiac tissue samples (pooled atrial and ventricular) for scintillation counting each heart was homogenized (10% v/v in 0.32M sucrose) and subcellular fractions prepared by differential centrifuguation. The homogenate was spun at 900 *g* for 10 min to remove nuclei and cell debris. The supernatant was then spun at 10,000 *g* for 30 min to remove the mitochondria and supernatant obtained from this was then spun at 100,000 *g* for 60 min to obtain the microsomal pellet. For each pellet and supernatant, the radioactive content was determined, and the protein was assayed by the method of Lowry *et al.* (1951).

Digoxin concentration was calculated directly from the radioactive content and is strictly 'digoxin equivalent' and could include concentrations of metabolites, particularly in the liver, bile and urine.

Experiments with HeLa cells

The influence of digoxin-specific antibodies, before or after the addition of digoxin, on digoxin binding or effect was examined in HeLa cells, the main methods being those described by Aiton *et al.* (1981). By incubating the cells at 37°C with digoxin and potassium-free (0K) or 15 mM potassium (15K) Krebs buffer solution, the respective total and non-specific digoxin binding was assessed (in 15K buffer no specific digoxin binding occurs because the sodium pump sites are saturated by K^+). Specific binding (Na pump-bound) was determined by subtracting non-specific from total binding. Washing with 5 mM potassium (5K) buffer removed extracellular, non-membrane bound digoxin.

In one series of experiments digoxin-specific antibodies (IgG, 1.88 nmol; or Fab fragments, 4.8 nmol) were added to cells (approximately 1.5×10^6) suspended in 3 ml 0K Krebs solution 5 min before incubation with [^{3}H]-digoxin (200 nM, $0.2\,\mu Ci\,ml^{-1}$) for 25 min. The second series of experiments was designed to assess the effect of antibodies on digoxin retention. After incubation for 30 min with the [^{3}H]-digoxin in 0K or 15K Krebs solution, the cells were washed with warm 5K Krebs solution and then incubated for 3 h in Eagles Basal Medium

(BME) containing added IgG or Fab fragments as above. After incubation. in both series of experiments, the cells were washed in ice-cold 5K Krebs solution to remove extracellular non-membrane bound digoxin.

In a third set of experiments designed to assess the influence of antibodies on residual digoxin effect, cells were firstly incubated with unlabelled digoxin (200 nM) in 0K Krebs solution for 30 min, washed with 5K Krebs solution and incubated in 3 ml BME for 3 h with or without digoxin-specific IgG (1.88 nmol). The number of free pumps was then measured (after 3 h) following a pulse label with ^{3}H-ouabain (200 nM, 0.2 μCi ml^{-1} for 15 min). Pump activity was assessed both by measuring intracellular Na^+ and K^+ contents and by measuring ^{86}Rb influx for 10 min in the presence and absence of ouabain (1 mM). The difference between ^{86}Rb influx with and without the Na pump inhibitor ouabain, gives a measure of active pump-mediated K^+ influx (Aiton *et al.*, 1981).

In the three sets of experiments, control plates were provided by adding 0.15 ml non-immune sheep serum instead of specific antibody. After each incubation of control and experimental plates, the cells were washed appropriately, treated with trypsin to form a single cell suspension and cell number and volume determined using a Coulter Counter (Aiton *et al.*, 1981). The ^{3}H or ^{86}Rb content in a known volume of cells was determined by liquid scintillation spectrometry. Intracellular Na^+ and K^+ contents were measured by flame photometry following rapid washing with ice-cold isotonic sorbitol solution to remove extracellular ions (Aiton & Lamb, 1984).

Materials

[^{3}H]-digoxin, [^{3}H]-ouabain and ^{86}Rb were purchased from Amersham International, Bucks. Digoxin solution for injection (Lanoxin) was purchased from the Wellcome Foundation, Ltd, Beckenham, Kent. Digoxin and ouabain were purchased from Sigma (London) Poole, Dorset. All other reagents were obtained from BDH Poole, Dorset, and were of Analar grade. Digoxin-specific IgG and Fab fragments were obtained from Dr A. Munro and Dr R. Fraser of the West of Scotland Blood Transfusion Service, Law Hospital, Lanarkshire, Scotland. These had been prepared using standard techniques (Steinbuch & Audran, 1969; Porter, 1959) from digoxin-specific whole antiserum which had been produced in sheep following immunization with a digoxin-human serum albumin conjugate. The Fab fragments were shown

Table 1 The effect of pretreatment with digoxin-specific Fab fragments on tissue digoxin concentration in guinea-pigs receiving a lethal digoxin infusion

Tissue/fluid/subfraction	*Digoxin concentration at death*	
	Control	*Fab-treated*
Kidney	3.7 ± 0.8	2.6 ± 0.8
Liver	3.0 ± 0.4	1.8 ± 0.7
Heart	1.3 ± 0.2	0.86 ± 0.07*
Lung	0.56 ± 0.1	0.25 ± 0.02*
Adrenal	0.45 ± 0.05	0.3 ± 0.03*
Skeletal muscle	0.44 ± 0.06	0.26 ± 0.04*
Spleen	0.38 ± 0.05	0.28 ± 0.06
Serum	0.94 ± 0.11	1.88 ± 0.3*
Urine	0.29 ± 0.09	2.35 ± 0.7**
Bile	16.2 ± 0.7	34.1 ± 0.94**
Heart whole homogenate	1.2 ± 0.2	0.72 ± 0.15*
Heart nuclei, cell debris (900 *g* pellet)	0.15 ± 0.03	0.09 ± 0.02
Heart mitochondria (10,000*g* pellet)	0.09 ± 0.05	0.05 ± 0.02
Heart microsomes (100,000 *g* pellet)	0.13 ± 0.01	0.08 ± 0.02*
Heart cytosol (100,000 *g* supernatant)	0.55 ± 0.07	0.27 ± 0.06*

The lethal dose of digoxin was 0.62 and 1.0 mg kg^{-1} in control and Fab-treated animals, respectively. The dose of Fab fragments was 2 mg kg^{-1} (i.p.). The values shown (means ± s.e.mean) are from 5 animals in each group. The units of digoxin concentration were μg g^{-1} wet weight, μg ml^{-1} and μg g^{-1} protein for the tissues, fluids and subfractions, respectively. The digoxin concentration was determined directly from the concentration of radioactivity. The asterisks indicate value is significantly different from control. ($^{*}P<0.05$; $^{**}P<0.01$).

by both immunoelectrophoretic and ultracentrifugation methods to contain no residual IgG [Dr R. Fraser, personal communication].

Statistical analysis

Experimental results are presented as the mean ± s.e.mean or s.d. as appropriate. Data were analysed using a non-paired Student's t test with a probability of <0.05 being taken as significant.

Results

Experiments in guinea-pigs

Digoxin caused characteristic changes in the electrocardiographic recordings of both groups of guinea-pigs. Early abnormalities were depression of the S-T segment and prolongation of the P-R interval, while ventricular tachycardia and ventricular fibrillation were common at the later stages of the infusion. The Fab-treated animals developed the fatal signs of ventricular fibrillation later than the controls (48.0 ± 5 min as compared with 29.8 ± 1.3 min). This slower onset of digoxin toxicity in the Fab-treated animals was reflected in the time to death (49.6 ± 4.7 min compared with 31.0 ± 1.2 min). The corresponding digoxin lethal doses were 1.01 ± 0.09 and 0.62 ± 0.03 mg kg^{-1}, in Fab-treated and control animals respectively (means ± s.e.mean; significant difference, $P<0.01$).

The disposition of digoxin at death is shown in Table 1. The highest concentrations of digoxin were found in the kidney and liver, followed by the heart and serum, and then lungs, adrenals, skeletal muscle and spleen. In the bile and urine collected, a far greater concentration of digoxin was found in the bile. With the heart subcellular fractions, the highest concentration of digoxin was found in the cytosol, with lower and roughly similar concentrations in the microsomes, mitochondria and nuclei/cell debris.

In the Fab-treated animals, despite a 1.6 fold greater digoxin dose, the drug concentrations attained in the tissues were lower, significantly so in the case of heart, lungs, adrenals and skeletal muscle. In contrast, the serum digoxin concentration was elevated 2 fold. The bile and urine concentrations were also elevated, very markedly so in the case of the latter. The percentage of the digoxin dose excreted in the control and Fab-treated animals respectively was 8.4 and 10.3% for the bile, and 0.25 and 1.16% for the urine. The reduction in cardiac digoxin concentration associated with Fab treatment appeared to be reflected in whole heart homogenate and the subcellular fractions examined, but only attained significance in the case of the whole homogenate, microsomes and cytosol.

Experiments in HeLa cells

The pretreatment of HeLa cells with IgG or Fab fragments reduced both non-specific and specific digoxin binding (Figure 1a). Pretreatment with IgG and Fab fragments reduced total, non-specific and specific binding by 70 and 42%, 60 and 35%, and 87 and 45% respectively. However, when HeLa cells were incubated with IgG or Fab fragments after treatment with digoxin, the total and specific digoxin binding associated with the cells was not significantly changed (Figure 1b). Furthermore, incubation with IgG after digoxin did not significantly reduce the number of sodium pumps and did not affect either intracellular Na^+ and K^+ concentrations, or ATPase mediated K^+influx (Table 2).

Table 2 The effect of digoxin-specific antibodies on sodium pump function and number in HeLa cells after digoxin pretreatment

Treatment	*Number of 'free' sodium pumps per cell*	*Measure of pump function*		
		Intracellular ion concentration (mM)		*Pump-mediated K^+ influx ($mmol^{-1}$ of cell water, min^{-1}]*
		Na^+	*K^+*	
Non-immune serum (control)	120,000 ± 2,600	29.0 ± 3.5	88.0 ± 6.9	0.97 ± 0.29
Digoxin-specific IgG	86,000 ± 12,000	26.0 ± 5.2	88.0 ± 6.9	1.00 ± 0.66

The HeLa cells were incubated with digoxin, washed to remove extracellular unbound drug, incubated with either non-immune sheep serum or digoxin-specific lgG, and then sodium pump function and number were determined as described in Methods. Means ± s.d. are given ($n=3$).

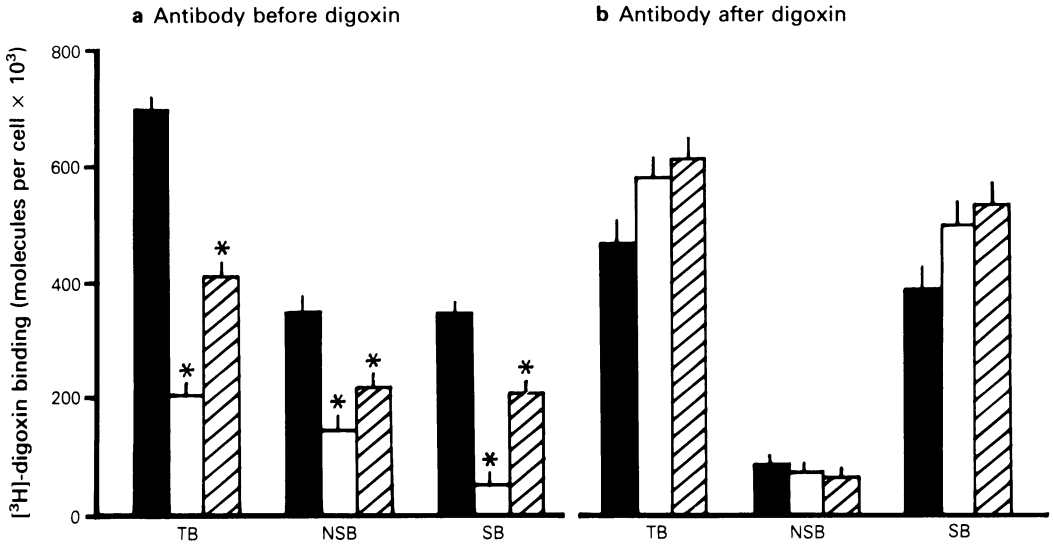

Figure 1 The effect of digoxin-specific antibodies added (a) before or (b) after digoxin, on its binding to HeLa cells. Total (TB), non-specific (NSB) and specific (SB) digoxin binding were determined as described in Methods. Solid columns, control (non-immune serum); open columns, IgG; hatched columns, Fab fragments. On each column the s.d. is shown by a vertical line ($n = 3$). The asterisks indicate significantly different from control ($P < 0.05$).

Discussion

The use of heterologous digoxin-specific Fab fragments provides a successful approach to the treatment of severe digitalis poisoning. The present study in guinea-pigs demonstrates that such antibody preparations given prophylactically also have a significant protective effect, increasing the lethal dose of digoxin by 60%. A similar finding was reported by Hess *et al.*, (1978), who showed that the prophylactic administration of digoxin-specific $F(ab^1)_2$ fragments (divalent IgG fragments as opposed to univalent Fab fragments) delayed the onset of digoxin-induced ventricular arrhythmias and also prolonged survival time after the onset of such arrhythmias. In the treatment of digoxin toxicity, digoxin-specific Fab fragments have advantages over the 'parent' IgG and $F(ab^1)_2$ fragments in that, although the affinity for the hapten is similar, Fab fragments have a faster and more extensive distribution to the interstitial space, are more rapidly excreted in the urine, and are less immunogenic (Smith *et al.*, 1979).

Despite the 60% higher digoxin dose given to the Fab-treated animals, digoxin concentrations attained in the tissues examined were 20–50% lower. Since the 100% increase in serum digoxin levels was greater than the dosage increase, it is likely that digoxin retention in the vascular compartment was responsible for the lower tissue concentrations. During the perfusion period, 8–10% of the injected dose was excreted in the bile and 0.2–1% in the urine. These findings are in agreement with those of Russell & Klaassen (1973) and Proppe (1975), who demonstrated that in small rodents such as the rat and guinea-pig a greater proportion of digoxin is initially excreted in the bile than in the urine. This contrasts with the situation in man and the dog where urinary excretion has the dominant role (Doherty & Kane, 1975; Butler *et al.*, 1977). Unlike the rat however, the relative adrenal digoxin concentration was not high. In the rat, digoxin (Dutta & Marks, 1966; Harrison *et al.*, 1975) and digitoxin (Castle & Lage, 1973; Boor *et al.*, 1976) adrenal concentrations are far greater than those found in most other tissues, including liver and kidney.

A surprising finding in the Fab-treated animals at death was the 30–40% lower cardiac digoxin concentration, seen in whole homogenate and in the subcellular fractions examined. This presumably means that, at the time of death, a lower proportion of non-specifically-bound (pharmacologically-inactive) digoxin was present in the hearts of Fab-treated guinea-pigs. A higher proportion of specifically-bound digoxin could be due to the fact that Fab fragments *in vivo* may prevent digoxin binding to non-specific sites (low affinity) to a greater extent than specific sites (sodium pumps with high affinity).

Considering the relative doses of Fab fragments and digoxin, it seems that, in molar terms, a much

lower dose of Fab fragments is required than would be expected. The dose of Fab fragments was 40 nmol kg^{-1}, while the lethal dose of digoxin in control guinea-pigs was 796 nmol kg^{-1}. Therefore, the dose of Fab fragments was only 5% of that required to bind 100% of a normally lethal dose of digoxin. An analogous finding has been reported in a study by Neri *et al.*, (1964), in which rats received testosterone-specific antibodies at the same time as testosterone. It was found that a molar ratio of antibody to testosterone of only about 1/50 was required to prevent the biological effects of the hapten.

Adding digoxin-specific antibodies to HeLa cells before incubating with digoxin, reduced both non-specific and specific digoxin binding, but, rather surprisingly, the specific binding was affected to a slightly greater extent. Presumably the free digoxin available for both types of binding was reduced. Despite possible differences in the relative effect on specific and non-specific digoxin binding, it is probable that in the *in vivo* guinea-pig study Fab fragments generally acted in the same way as *in vitro* and reduced the free digoxin concentration available for binding to active (and inactive) sites on the myocardial membrane. The greater *in vitro* effect of IgG was somewhat unexpected since, taking into account that (with respect to digoxin binding) IgG is divalent and Fab fragments monovalent, in the amounts added (IgG, 1.88 mmol; Fab fragments, 4.8 mmol) the digoxin binding capacities of the two antibody preparations were roughly similar. It is possible that in the Fab preparation used some denaturation could have occurred with regard to specific digoxin binding ability.

Adding digoxin-specific antibodies to the HeLa cells after digoxin treatment suggests that once digoxin is bound to HeLa cells neither treatment with IgG nor Fab fragments can remove the drug. In fact there is even an indication that antibody treatment may cause a slight retention of digoxin (Figure 1b). In line with the general lack of antibody effect on digoxin retention, antibody treatment does not restore the number of 'free' sodium pumps or the function of pumps once inhibited by digoxin.

This lack of effect of digoxin-specific antibodies on the binding of digoxin to HeLa cells appears to contradict the well-established antidotal effect of these antibodies shown in a number of *in vivo* and *in vitro* preparations. It is possible that the discrepancy can be explained by differences in cardiac glycoside binding to HeLa versus myocardial cells. The relative binding affinity of digoxin for the membrane compared with the antibodies could be very much greater in the case of the HeLa cells. Another possibility is that the internalization of sodium pumps (with associated digoxin) is a much more extensive process in HeLa cells compared with myocardial cells. In support of this it has been suggested (Werdan *et al.*, 1984), that differentiated myocardial cells have much lower internalization rates for cardiac glycosides than rapidy growing cancer-derived HeLa cells.

We are grateful to Dr A. Munro and Dr R. Fraser of the West of Scotland Blood Transfusion Service for supplying the antibody preparations. This work was carried out when N.M.G. held research assistantships from the Tayside Health Board and Scottish Hospital Endowments Research Trust.

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(Received June 2, 1984.
Revised July 24, 1984.)