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THE EFFECT OF IMMUNIZATION WITH DIGOXIN-SPECIFIC ANTIBODIES ON DIGOXIN DISPOSITION IN THE MOUSE

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Abstract—After intravenous dosing, digoxin was rapidly distributed to tissues, with a distribution half-life of 3.0 min. The highest digoxin concentrations at 1 hr post dosing were found in lymph nodes, adrenals, gallbladder (including contents), liver and kidney respectively. Digoxin concentrations in the heart, spleen, brain, lung, skeletal muscle and fat were similar to, or lower than, those in the plasma. The apparent volume of distribution (AVd) was 1 l/kg, and the plasma elimination half-life and clearance (Cl) 2.8 hr and 0.25 l/kg per hr respectively. When digoxin was given one day after passive immunization with digoxin-specific immunoglobulin G (IgG) or Fab fragments the respective plasma digoxin concentrations were elevated some 26- and 5-fold respectively compared with control values. Consequently there were reductions in AVd (93 and 32%) and Cl (94 and 50%). The effect of IgG treatment on clearance was still apparent when the hapten was given up to 14 days after immunization, while the weaker effect of Fab-treatment was less persistent. Although tissue digoxin concentrations were slightly lower in the immunised mice, it was only in the lymph nodes at 10 and 14 days after IgG treatment that the reduction in hapten concentration was statistically significant.

Preliminary studies have indicated the potential use of digoxin-specific Fab† fragments, obtained from sheep antiserum, in reversing life-threatening digitalis toxicity [1–3]. More recently, their efficacy in such cases of poisoning has been confirmed in an interim report [4] of a clinical trial.

A similar antidotal effect of digoxin-specific antibodies either as whole antiserum, IgG or Fab fragments, has been demonstrated in laboratory animals [5–8]. In dogs both digoxin-specific IgG and Fab fragments have been shown to greatly increase total serum digoxin concentrations, but whereas IgG delayed digoxin urinary excretion it was unaffected by the Fab fragments [9]. Digoxin was found to be sequestered in the vascular compartment in the antibody-bound form thus reducing the concentration of free pharmacologically active drug. Although a resultant reduction in active cardiac digoxin concentration may account for the protective or antidotal effects of these antibodies, little work has been carried out on the influence of antibody treatment *in vivo* on digoxin pharmacokinetics, particularly relating to the levels of digoxin in the heart and other tissues.

To provide more information on the disposition of digoxin as a hapten, the effect of treatment with digoxin-specific antibodies on digoxin tissue distribution and elimination was examined. The mouse, although relatively insensitive to the cardiac effects of digoxin, was chosen as the experimental animal to facilitate the use of large numbers of animals necessary for such a tissue distribution study. Prior to the passive immunization studies, however, it was considered necessary to establish information on digoxin disposition in a wider range of tissues than so far reported for the mouse [10, 11].

MATERIALS AND METHODS

Materials. ^{3}H-Digoxin, specific activity, 6.4 Ci/mole, was obtained from Amersham International (Amersham, Bucks, U.K.). Tissue solubilizer (Protosol) was purchased from New England Nuclear (Dreiech, F.R.G.) and liquid scintillant (NE 260) was purchased from Nuclear Enterprises (Edinburgh, U.K.). All other reagents were obtained from British Drug Houses (Poole, Dorset, U.K.) and were of analytical grade.

Digoxin-specific IgG and Fab fragments were provided by Dr. A. Munro and Dr. R. Fraser, West of Scotland Blood Transfusion Service (Law Hospital, Lanarkshire, U.K.). These were prepared from digoxin-specific whole antiserum which had been produced in sheep following immunization with a digoxin–human serum albumin conjugate. IgG was prepared according to the method of Steinbuch and Audran [12] which separates IgG from serum by precipitating the bulk of the unwanted proteins with caprylic (octanoic) acid. The Fab fragments were obtained from IgG by proteolysis with papain [13] and were shown (Dr. R. Fraser, personal communication) both by immunoelectrophoretic and ultracentrifugation methods to contain no residual IgG. Antibodies (IgG and Fab fragments) were stored as 1 ml aliquots in phosphate-buffered (pH 7.4) saline at −20° until required.

Animals and treatment procedures. Female Swiss white mice weighing 25–40 g (Charles River Inc.,

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† Abbreviations: Fab, fragment antigen binding; IgG, immunoglobulin G.

Table 1. Tissue distribution of ^{3}H-digoxin 1 hr after intravenous administration* in the mouse

Tissue/body fluid	Digoxin concentration† (ng/g or ng/ml)	Total tissue digoxin (% of dose)
Lymph nodes	12.6 ± 2.9	0.76
Adrenals	10.4 ± 2.8	0.36
Gall bladder (plus contents)	7.8 ± 2.3	0.28
Liver	4.2 ± 2.4	5.6
Kidneys	3.75 ± 1.4	1.0
Thyroid	3.6 ± 1.6	0.76
Ileum (contents removed)	3.2 ± 1.4	4.8
Duodenum (contents removed)	3.02 ± 1.1	
Blood	2.9 ± 1.6	6.68
Lungs	2.9 ± 1.4	0.2
Plasma	2.7 ± 1.2	3.6
Spleen	1.9 ± 0.7	0.18
Heart	1.7 ± 1.2	0.18
Ovaries/uterus	1.3 ± 0.8	0.18
Skeletal muscle	1.06 ± 0.7	11.4
Fat	0.67 ± 1.6	2.54
Brain	0.43 ± 0.1	0.08
All tissues/fluids	—	35.0

* The dose was 3.75 μg/kg.

† Determined directly from radioactive content, mean ± S.D. from 3 mice.

Sussex, U.K.) were used in all experiments and received a normal diet and water *ad lib.* throughout the experimental period.

In experiments not involving passive immunization, mice received a single dose of ^{3}H-digoxin i.v. (3.75 μg/kg; 10 μCi/kg). Groups of three animals were then killed at 5, 10, 15, 30, 60, 120 and 180 min after dosing.

In passive immunization experiments, mice received a single dose of ^{3}H-digoxin i.v. (7.5 μg/kg; 20 μCi/kg) on days 1, 3, 5, 10 and 14, after digoxin-specific IgG (365 μg/kg) i.p., or digoxin-specific Fab fragments (65 μg/kg) i.p., or control treatment. The control treatment comprised 0.2 ml non-immune sheep serum or saline given i.p. Three animals from each treatment group were killed at 60, 120 and 180 min after ^{3}H-digoxin dosing.

Collection and treatment of blood and tissue samples for determination of digoxin content. Mice were killed by asphyxiation with CO_2, blood samples taken by cardiac puncture and the organs and tissues as listed in Table 1 were removed. The lymph nodes were the axillary, mesenteric and lumbar nodes pooled from each animal. Samples of skeletal muscle and fat were obtained from the "thigh" and abdomen respectively. The sample of duodenum was taken 1 cm from the stomach, and that of the ileum 5 cm from the caecum. Each tissue was rinsed with physiological saline, blotted dry and weighed. In addition, at death urine and faeces (voided material and taken from bladder or large bowel respectively) were collected.

Blood samples (0.05 ml) were solubilized with 0.5 ml Protosol:ethanol (2:1) by incubating at 60° for 1 hr. Samples were then decolorized by addition of 0.2 ml propan-2-ol followed by 0.2 ml hydrogen peroxide (30% v/v) and incubation for a further 30 min at 60°. After cooling, 10 ml scintillant was added and samples were counted for radioactivity for 10 min. The remainder of the whole blood was centrifuged at 3000 *g* for 10 min to obtain the plasma, and radioactivity determined in 0.1 ml plasma after the addition of scintillant. Urinary radioactivity was determined as for plasma.

Tissue or faecal samples (100 mg) were solubilized with 1 ml Protosol by incubating at 55° for 3–5 hr or until all the solid material had been digested. Following decolorization of samples, as described above, radioactive content was determined after addition of 10 ml scintillant.

Digoxin concentrations were calculated directly from the radioactive content, and are therefore strictly "digoxin equivalents". Although digoxin is considered a minimally-metabolised drug, digoxin concentration as determined could include metabolite concentration. For the estimation of total tissue digoxin content, blood and plasma volume, skeletal muscle and fat were assumed to be 8.5, 5, 40 and 14% of body weight respectively [14, 15].

Pharmacokinetic and statistical analyses. For the initial experiments not involving passive immunization, the curve of plasma digoxin concentration vs time was found to fit with a two-compartment model and the respective distribution and elimination half-lives were calculated. The distribution half-life was obtained using the method of residuals [16].

For the passive immunization experiments, only the elimination half-life was determined, and this was calculated from the slope of the regression line of the semilogarithmic plot of plasma digoxin concentration vs time after digoxin injection.

For both studies, the apparent volume of distribution was calculated by dividing the digoxin dose

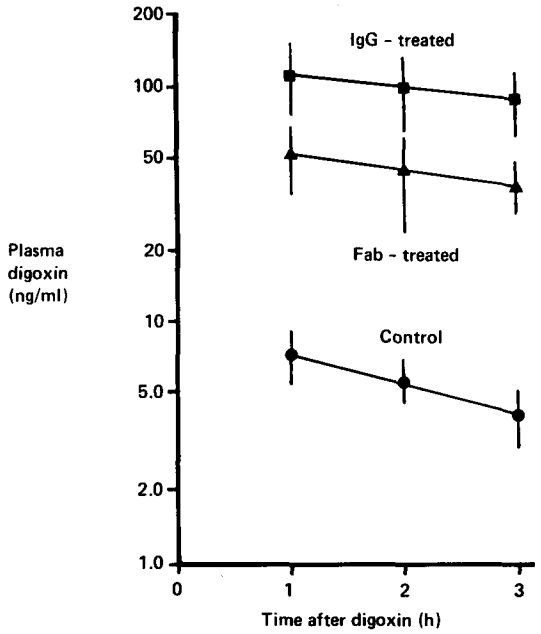

Fig. 1. The effect of passive immunization with digoxin-specific IgG or Fab fragments on plasma digoxin concentration. Animals were pretreated (i.p.) with saline, digoxin-specific IgG (365 μg/kg) or digoxin-specific Fab fragments (65 μg/kg) one day prior to ^{3}H-digoxin dosing (7.5 μg/kg, i.v.). The data points are the means ± S.D. for three mice.

by the zero-time plasma concentration (obtained by extrapolation of the "elimination" regression line). The plasma digoxin clearance was determined by dividing the volume of distribution by the elimination half-life and multiplying by 0.693.

All experimental results are means ± S.D. and data were analysed using a non-paired Student's *t*-test with a probability of <0.05 being taken as significant.

RESULTS

Digoxin disposition in non-immunized mice

Analysis of the data as described above gave a mean plasma digoxin distribution half-life of 3.0 ± 0.2 min and an elimination half-life of 2.8 ± 1.6 hr. The apparent volume of distribution was calculated to be 1.0 l/kg and plasma clearance was 0.25 l/kg per hr.

The tissue distribution of digoxin after 60 min is shown in Table 1. The highest concentrations of digoxin were found in the lymph nodes, adrenal glands, gallbladder (plus contents) and liver, and the lowest in the fat and brain. Heart digoxin concentration was some 60% of that in plasma. Although the results are not given in Table 1 this difference was maintained at 90, 120 and 180 min after digoxin injection.

The tissues which attained the highest total amounts of digoxin were skeletal muscle, blood and liver (Table 1). Of the digoxin dose given, 47.8% was recovered; 35% in the tissues examined (Table 1) and a further 8.7 and 4.1% in the faeces and urine respectively, collected at death.

The effect of passive immunisation with digoxin-specific IgG and Fab fragments

Preliminary experiments showed that digoxin disposition was similar using injection with either non-immune sheep serum or saline (i.p.) as a control procedure (results not shown). In subsequent experiments with IgG and Fab-fragments, saline injection was used as the control treatment.

The plasma digoxin concentration vs time profiles for control, IgG- and Fab-treated mice one day after

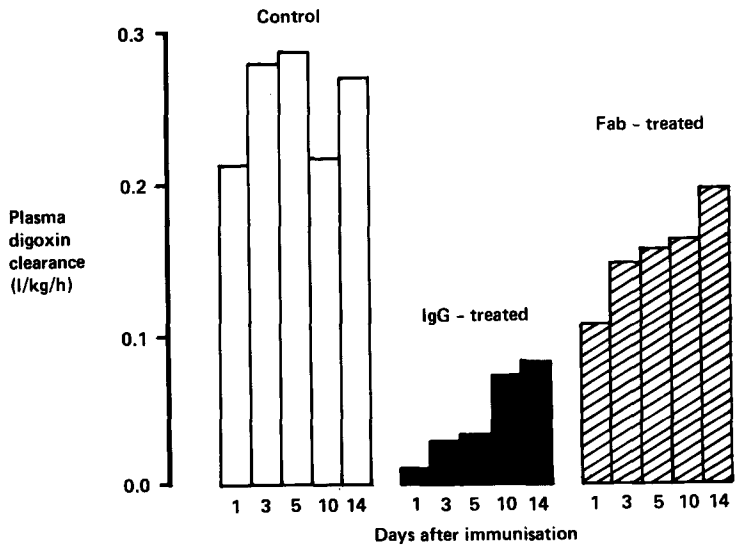

Fig. 2. The effect of passive immunization with digoxin-specific IgG or Fab fragments on plasma digoxin clearance. Animals were pretreated (doses as in legend to Fig. 1) with saline, digoxin-specific IgG or digoxin-specific Fab fragments. Plasma digoxin clearance was determined on days 1, 3, 5, 10 and 14 after treatment.

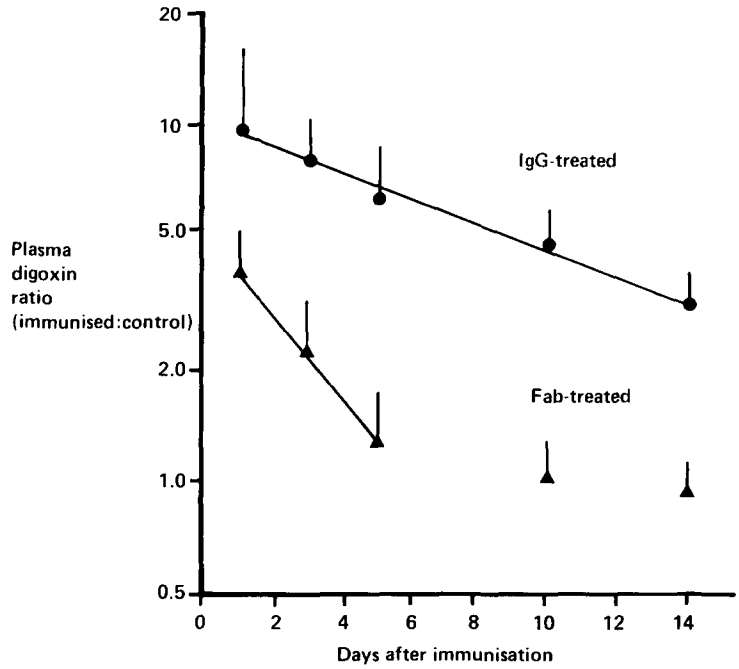

Fig. 3. The decline in plasma digoxin concentration ratio (immunized:control) with time. Animals were pretreated (doses as in caption to Fig. 1) with saline (control), digoxin-specific IgG or digoxin-specific Fab fragments. Plasma digoxin concentrations were measured 1 hr after injection of the drug.

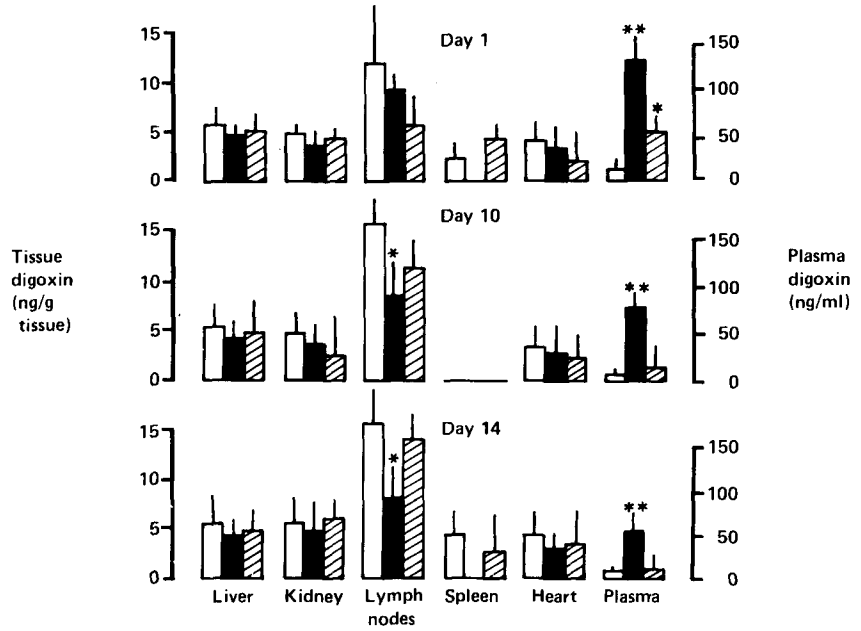

Fig. 4. The effect of passive immunization with digoxin-specific IgG or Fab fragments on tissue digoxin concentration (treatment details in legend to Fig. 1). The open, solid and hatched columns represent the saline, IgG and Fab treatments respectively. Tissue digoxin concentrations were determined 1 hr after a single dose of ^{3}H-digoxin (7.5 μg/kg, i.v.) on days 1, 10 and 14 after passive immunization. Each result is the mean with S.D. for three mice. The asterisks indicate a significant difference from control mice (*$P < 0.05$; **$P < 0.01$).

passive immunization are shown in Fig. 1. During the 3-hr period after digoxin injection the respective digoxin concentrations in the IgG- and Fab-treated mice were some 26-fold and fivefold those in controls. Plasma digoxin elimination half-life values were increased by passive immunization (control, IgG, Fab; 2.9, 5.3, 4.3 hr respectively), while the apparent volumes of distribution were reduced, greatly so in the case of the IgG-treated mice (control, IgG, Fab; 0.88, 0.063, 0.60 l/kg respectively). Consequently the plasma clearance was most markedly decreased after IgG treatment (control, IgG, Fab; 0.21, 0.008, 0.10 l/kg per hr respectively).

The effect of IgG treatment on plasma digoxin clearance was still apparent 14 days after passive immunization, while the weaker effect of Fab treatment appeared less persistent (Fig. 2).

To analyse further the duration of antibody effect, the ratio (immunized : control) of plasma digoxin concentration was determined for up to 14 days after immunization (Fig. 3). From the regression line, the "half-time" for the ratio calculated using Ig-treated mice data was 8.0 days. For the Fab-treated animals the half-time for the 1–5-day period was 2.4 days. After 5 days the ratio approached unity and the log ratio (Fab-treated : control) vs time relationship became non-linear (Fig. 3).

Figure 4 shows the digoxin concentration in the plasma and a selected number of tissues (1 hr after ^{3}H-digoxin administration) in the three treatment groups at 1, 10 and 14 days after immunization. Tissue concentrations of digoxin tended to be lower in the passively-immunized animals than in controls, but in most cases the differences did not attain statistical significance. The only statistically significant effect found with the tissues was the reduced digoxin concentration attained in the lymph nodes on days 10 and 14 after immunization with IgG. As indicated previously, plasma digoxin concentrations were significantly increased following antibody treatment.

DISCUSSION

The results obtained demonstrate that in the mouse, as previously reported for the rat [17, 18], digoxin is rapidly and widely distributed to the tissues. There is considerable accumulation of digoxin in the lymph nodes, adrenals, gall bladder/contents, and a less marked accumulation in the liver, kidneys and thyroid gland. However, in many tissues, digoxin concentrations tended to be similar to, or less than those in blood or plasma. This is reflected in the value for the apparent volume of distribution (about 1 l/kg). Similar results [10, 11] have been reported for the mouse with a smaller range of tissues (liver, heart, kidney, skeletal muscle, plasma).

About half the injected digoxin dose was not accounted for. Possibly appreciable amounts could have been present in biological material not examined. For instance, considerable digoxin could have been in the small bowel contents (via bile or direct excretion).

Considering tissues where digoxin was found to be concentrated, the high concentration of drug in the liver and gall bladder (plus contents) provides support for a biliary route of excretion for digoxin in the mouse, in common with other small rodents such as the rat and guinea-pig [19, 20]. Presumably the moderately elevated kidney digoxin levels also reflect a digoxin excretory role for this organ. The high adrenal concentration of digoxin is interesting, similar findings having been reported for digoxin [15, 21] and digitoxin [22, 23] in the rat. Like corticosteroids, cardiac glycosides possess a steroid moiety and this chemical similarity could account for their accumulation in adrenal tissue. For a number of years there has been speculation over the presence of an "endogenous cardiac glycoside" [24] and Schreiber *et al.* [25] have suggested that rat adrenal glands may be responsible for the synthesis of an endogenous digoxin-like compound. Therefore it is possible that in the rat and mouse there may be an adrenal uptake mechanism for digoxin. The very high concentration of digoxin found in the lymph nodes was unexpected, this being the first report of such a finding.

The effects on plasma digoxin concentrations, of specific passive immunization in mice are similar to those obtained using dogs [8, 9] and rats [26], the treatment with IgG giving a greater elevation of plasma digoxin concentration than that with Fab fragments [8]. In the present study, the effects of IgG were both greater in magnitude and duration than those of Fab fragments. The greater increase in plasma digoxin concentration found after IgG treatment may be partly explained by considering the relative molar doses of IgG and Fab fragments, and their digoxin binding capacity. On a molar basis, the doses of IgG and Fab fragments were 2.3 nmole/kg and 1.3 nmole/kg respectively. Since a molecule of IgG may bind two molecules of digoxin these doses are equivalent to 4.6 nmole and 1.3 nmole digoxin respectively, i.e. 48% and 14% of the administered digoxin dose. Assuming a similar binding affinity for IgG and Fab fragments, the ratio of plasma digoxin concentration (IgG : Fab; 1 hr post digoxin dosing, 1 day after passive immunization) would be approximately 3.5 (48/14). The actual value was about 5 (26/5). This is a reasonable agreement, the discrepancy possibly being explained on the basis of a faster elimination, resulting in a lower level in the plasma after day 1, and a larger volume of distribution of Fab fragments [27].

The more prolonged elevation of plasma digoxin concentrations after IgG treatment compared with Fab fragments is probably due to the slower excretion of IgG [28, 29]. On a few occasions we collected faecal and urine samples from passively-immunized mice (results not shown) and found that digoxin was excreted far more slowly in IgG-treated mice compared with Fab-treated animals or controls.

A similar half-time for heterologous IgG effect in the plasma to that found in the present study (8 days, Fig. 5) is indicated from the data of Berkowitz *et al.* [30], who passively immunized mice with rabbit anti-morphine serum (which could also bind dihydromorphine). The dihydromorphine binding ability (determined *in vitro*) of the mouse serum was determined at 2, 6 and 13 days after immunization. The calculated half-time for the decline of this binding ability is 8.8 days.

Using a more direct method, involving ^{131}I-labelled

IgG and Fab, Wochner *et al.* [29] reported the whole body elimination half-lives as 4 days and 3.6 hr respectively. While, perhaps surprisingly, indirectly assessing antibody elimination from the mouse by the binding of a specific hapten gives some agreement with the direct method of Wochner *et al.* [29] in the case of IgG (half-life, about 8 days vs 4 days), no such agreement is seen for Fab fragments (half-life, 2.4 days vs 3.6 hr).

The increased plasma digoxin concentrations following either IgG or Fab treatment resulting from binding to circulating antibody resulted in only a slightly lowered digoxin tissue concentration. Similarly in the rat, Campbell *et al.* [26] injecting much less antibody (whole antiserum) than in the present study, obtained a 10-fold rise in plasma digoxin concentration with no significant reduction in spleen, heart, liver or skeletal muscle digoxin concentration. It is rather surprising that, although the plasma only represents about 5% of the "volume" of the body, very marked elevations in plasma digoxin concentration are not associated with a more clearly seen reduction in digoxin tissue levels.

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