Reversal of Advanced Digitoxin Toxicity and Modification of Pharmacokinetics by Specific Antibodies and Fab Fragments

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ABSTRACT The effects of Fab fragments of high-affinity specific antibodies have been studied in a canine experimental model of lethal digitoxin toxicity. Selected antiserum from sheep immunized and boosted with a digoxin-serum albumin conjugate contained antibodies that cross-reacted with digitoxin with an average intrinsic association constant of $1.4 \times 10^{10} \text{ M}^{-1}$ as determined by equilibrium dialysis. Rapid second-order association kinetics ($k_f = 3.7 \times 10^6 \text{ M}^{-1} \text{ per s}$) and slow dissociation kinetics ($k_r = 1.9 \times 10^{-4} \text{ per s}$) were documented for the antibody-digitoxin complex. Eight dogs given 0.5 mg/kg digitoxin intravenously developed ventricular tachycardia after 23 ± 4 (SEM) min. Control nonspecific Fab fragments were then given. All animals died an average of 101 ± 36 min after digitoxin administration. Another eight dogs given the same digitoxin dose similarly developed ventricular tachycardia after 28 ± 3 min. This group then received a molar equivalent dose of specific Fab fragments intravenously over 3 min, followed by a 30-min infusion of one-third of the initial dose. All dogs survived. Conducted sinus beats reappeared 18 ± 4 min after initial Fab infusion, and stable normal sinus rhythm was present at 54 ± 16 min. Plasma total digitoxin concentrations increased threefold during the hour after initial Fab infusion, while plasma free digitoxin concentration decreased to less than 0.1 ng/ml. Effects on digitoxin pharmacokinetics of these Fab fragments and the antibody population from which they were derived were further investigated in a primate species. Unlike common laboratory animals previously studied, the rhesus monkey was found to have a prolonged elimination half-life, estimated at 135

and 118 h by radioimmunoassay and [^3H]digitoxin measurements, respectively, similar to man and thus providing a clinically relevant experimental model. Intravenous administration of 2 mol of specific Fab fragments per mole of digitoxin 6 h after 0.2 mg of digitoxin produced a rapid 4.3-fold increase in plasma total digitoxin concentration followed by a rapid fall ($t_{1/2}$ 4 h) accompanied by a 14-fold enhancement of urinary digitoxin excretion over control values during the 6-h period after Fab was given. Analytical studies were consistent with increased excretion of native digitoxin rather than metabolites, and the glycoside was found in equilibrium dialysis studies to be excreted in the urine in Fab-bound form. Administration of 2 mol of specific antibody binding sites per mole of digitoxin as intact IgG caused a greater and more prolonged increase in plasma total digitoxin concentration, peaking 13-fold above control levels. In contrast to the effects of Fab, however, specific IgG reduced the rate of urinary digitoxin excretion substantially below control values. We conclude that Fab fragments of antibodies with high affinity for digitoxin are capable of rapid reversal of advanced, otherwise lethal digitoxin toxicity, and are capable of reducing the plasma half-life and accelerating urinary excretion of digitoxin.

INTRODUCTION

Severe digitalis toxicity resistant to conventional therapy remains an important clinical problem (1, 2). Substantial experimental literature now exists demonstrating reversal of established effects of digoxin (3-5) as well as ouabain (6, 7) by specific antibodies or their Fab fragments, and Fab fragments of digoxin-specific antibodies have recently been used clinically to reverse toxicity after suicidal digoxin ingestion with high-

Dr. Ochs was a Fellow of Deutsche Forschungsgemeinschaft.
Received for publication 20 January 1977 and in revised form 5 July 1977.

grade atrioventricular block and intractable hyperkalemia (8).

The cardiac glycoside digitoxin is used in 16–20% of digitalis-treated patients in the United States (9, 10) and is in even more common use in several other countries (2, 11, 12). Substantial differences exist in plasma protein binding and pharmacokinetics of digitoxin compared with shorter-acting glycosides (13, 14). The apolar nature of the digitoxin molecule results in a high degree of binding to serum albumin and presumably accounts as well for substantially higher myocardial tissue to medium glycoside concentration ratios compared with digoxin or ouabain in *in vitro* studies (15, 16). Qualitative as well as quantitative differences in myocardial binding of digitoxin compared with more polar glycosides have been documented by Dutta et al. (17). Potentially important pharmacodynamic differences, including differences in neurally mediated effects, have been observed in recent studies (18, 19).

These differences in pharmacokinetics and pharmacodynamics of digitoxin raise serious questions regarding the extent to which prior studies of antibody reversal of digoxin toxicity can be extrapolated to digitoxin. Therefore, in the present study we have produced and characterized antibodies with high affinity for digitoxin and have examined in a canine experimental model the efficacy of Fab fragments of these antibodies in the reversal of advanced, potentially lethal digitoxin toxicity.

Because the dog, like other common subprimate laboratory animals, displays a pattern of digitoxin pharmacokinetics profoundly different from man (20), we determined that the rhesus monkey (*Macaca mulatta*) is a suitable experimental model for digitoxin pharmacokinetic studies in man. We then used this species to investigate the effects on digitoxin pharmacokinetics of these Fab fragments and the antibody population from which they were derived, and to test the hypothesis that excretion kinetics of a slowly excreted molecule could be enhanced by the administration of specific Fab fragments.

METHODS

Antibody production and characterization. Digoxin was conjugated to bovine serum albumin (BSA)¹ by periodate oxidation and Schiff's base formation and reduction (21, 22) as previously described (23). Sheep were immunized with the BSA-digoxin conjugate in complete Freund's adjuvant and serially boosted and bled (4). Preliminary studies identified an animal that responded to immunization with a high titer of antibodies that crossreacted strongly with digitoxin, and pooled antiserum from consecutive bleedings of this animal was used in subsequent experiments.

The IgG fraction was isolated by ammonium sulfate precipitation (24), and Fab fragments were prepared by

papain digestion as described by Nisonoff (25). Undigested IgG was removed by gel filtration chromatography on Sephadex G-150 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) equilibrated with 150 mM NaCl, 10 mM Na phosphate, pH 7.4 (phosphate-buffered saline [PBS]). Sodium dodecyl sulfate polyacrylamide-gel electrophoresis performed by the method of Weber and Osborn (26), but omitting the 2-mercaptoethanol step, confirmed the absence of detectable IgG in pooled Fab peaks. Digitoxin-binding capacities of Fab fragment and IgG preparations were determined by a dextran-coated charcoal method for separation of antibody-bound and free hapten as previously reported (23, 27). All antibody preparations were centrifuged at 20,000 *g* for 20 min and passed through a sterile 0.22 μ m Millipore filter (Millipore Corp., Bedford, Mass.) just before use. Control (nonspecific) IgG and Fab fractions were identically prepared from sera of sheep not previously immunized with cardiac glycoside conjugates.

Association and dissociation rate constants for interactions between antibody and digitoxin were determined as previously described (28, 29). The average intrinsic association constant (K_a) of the antibody population studied was determined by equilibrium dialysis as described in detail elsewhere (23), using [³H]digitoxin of specific activity 20 Ci/mmol (New England Nuclear, Boston, Mass.).

Toxicity reversal experiments. 16 mongrel dogs (mean weight 13.3 $\pm$ 1.2 [SEM] kg) were anesthetized with *i.v.* pentobarbital (30 mg/kg) and ventilated with a Harvard respirator at 12 cycle/min with a tidal volume adjusted to the weight of the animal. Arterial PO₂ was maintained in the range of 95–100 mm Hg, and electrocardiograms were continuously recorded. Preliminary dose-response experiments were carried out to determine a digitoxin dose that would elicit reproducible endpoints of advanced toxicity. For the studies reported here, 0.5 mg of digitoxin/kg body weight was injected intravenously over 10 min. When ventricular tachycardia had ensued for 5 min, eight control dogs were then given nonspecific (control) Fab fragments intravenously. A second group of eight dogs received an amount of specific Fab fragments representing the molar equivalent of the digitoxin dose over 3 min, followed by a 30-min infusion of one-third of the initial dose. Assuming a molecular weight of 50,000 for Fab fragments, the total dose of specific Fab fragments given was 44 mg/kg. Blood samples for determination of plasma digitoxin concentrations were drawn at the onset of ventricular tachycardia and at hourly intervals after administration of Fab fragments. After 3 h in stable sinus rhythm, surviving dogs were allowed to breathe spontaneously and to awaken. Electrocardiograms were again recorded 24 h after digitoxin administration.

Pharmacokinetic studies. Three male rhesus monkeys weighing 4.6, 7.4, and 13.6 kg were sedated 1 h before digitoxin administration with ketamine, 20 mg/kg, to facilitate handling. This set of experiments comprised four parts. Initially, animals received 0.2 mg digitoxin in saline intravenously over 5 min. In the subsequent three phases, each separated in time by 4–6 wk, the same dose of digitoxin included 33 μ Ci [³H]digitoxin (sp act 20.0 Ci/mmol, New England Nuclear) to permit determination of plasma and urine digitoxin or metabolite concentrations by direct counting of radioactivity as well as by radioimmunoassay. Blood samples in all experiments were obtained at 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 24, 28, 48, 52, 72, 96, and 100 h after digitoxin administration and also on the 7th, 9th, 11th, 14th, and 21st days.

The four experimental phases for each animal were as follows: (a) Digitoxin administration followed by control (nonspecific) Fab fragments 6 h later; (b) Digitoxin administration

¹Abbreviations used in this paper: BSA, bovine serum albumin; PBS, phosphate-buffered saline.

followed by a calculated dose of 26.2 mg of specific Fab fragments 6 h later (2 mol of binding sites per mole of digitoxin administered); (c) Digitoxin administration followed by a calculated dose of 39.3 mg of specific IgG 6 h later (2 mol of binding sites per mole of digitoxin administered); and (d) Digitoxin administration without subsequent IgG or Fab infusion for comparison with phase *a* above.

No untoward effects of infusion of sheep IgG or Fab fragments were noted.

In the second and third phases, additional blood samples were drawn 6.5, 7, 9, and 11 h after digitoxin administration (0.5, 1, 3, and 5 h after specific Fab or IgG injections, respectively). Spontaneously voided urine was collected during time intervals between 0 and 6, 6 and 12, 12 and 24, 24 and 48, 48 and 72, and 72 and 96 h after digitoxin administration. All plasma and urine samples were stored at -20°C until analyzed.

Plasma and urine digitoxin measurements. Digitoxin concentrations in plasma samples from dogs and in serum samples from rhesus monkeys were determined by radioimmunoassay as previously described (30). Identical results were obtained for samples prepared as plasma or serum from either species, and are therefore reported as plasma values for convenience. Samples obtained after administration of specific antibody or Fab fragments were diluted 1:5 with PBS, heated for 1 h in a water bath at 100°C , and centrifuged for 30 min at 5,000 *g* to remove coagulated protein. Suitable amounts of the clear supernatant phase were then used to determine the total digitoxin content. Initial studies showed 95–98% recovery of known amounts of digitoxin by this method whether or not an excess of specific antibody or Fab fragments was present before the heating step. The results to be reported have not been corrected for these minor losses.

To determine the effect of [^3H]digitoxin present in some doses administered to monkeys on the radioimmunoassay determination of unlabeled digitoxin, standards consisting of aliquots of suitable dilutions of the administered digitoxin mixture were used as well as conventional unlabeled standards. Comparison of results using these two types of standards documented the absence of measurable interference in the radioimmunoassay system by the relatively low specific activity of [^3H]digitoxin administered to animals.

For the direct determination of ^3H counts in samples, 0.2 ml of urine or plasma was counted in 10 ml of the liquid scintillation medium described by Bray (31). Quenching corrections were based on internal standards.

Separation of antibody-bound or Fab fragment-bound from free and albumin-bound digitoxin in plasma and urine samples was accomplished by equilibrium dialysis (23). Dialyses were carried out in plastic chambers (Technilab Instruments, Inc., Pequannock, N. J.) with 1-ml volumes on either side of a sheet of washed dialysis tubing (average pore diameter 48 Å, Arthur H. Thomas Co., Philadelphia, Pa.). Plasma samples were dialyzed against equal volumes of normal rhesus or dog plasma, as appropriate, at 4°C with gentle mixing for a period of 6 days. Preliminary studies were carried out to ensure that equilibration had fully occurred by this time. Tritium counts on each side of the dialysis membrane were determined by liquid scintillation counting as described above. 1-ml urine samples were dialyzed against PBS using the method just described.

Since the digitoxin radioimmunoassay as used was limited to a sensitivity of about 1 ng/ml, 1-ml samples of plasma obtained from dogs 1–6 h after specific Fab fragment administration were also equilibrated for 4 h with 2 ng of [^3H]digitoxin added in vitro and then dialyzed against 1 ml of normal dog plasma. This extended the sensitivity of detection of free digitoxin to levels of at least 0.05 ng/ml.

Analytical studies of digitoxin and metabolites excreted in urine. Urine samples from rhesus monkeys were heated at 100°C for 1 h in a boiling water bath to denature excreted immunoglobulins or immunoglobulin fragments. 1 ml of urine was then added to 3 ml of dichloromethane (Aldrich Chemical Co., Inc., Milwaukee, Wis.) and mixed for 5 min with a Vortex-Genie (Scientific Industries, Inc., Bohemia, N. Y.) in a glass-stoppered tube (32). After centrifugation, aliquots of aqueous and dichloromethane phases were subjected to liquid scintillation counting as described above.

After extraction, another aliquot of the dichloromethane phase was evaporated in a water bath at 50°C to dryness and the residue redissolved as described by Storstein (33). Appropriate amounts of the tritiated digitoxin solution administered to animals in the second, third, and fourth experimental phases and [^3H]digoxin (New England Nuclear) were identically treated and used as reference standards, applied in parallel with aliquots of dichloromethane extracts to silica-gel chromatographic sheets (Eastman Chromagram, Eastman Kodak Co., Rochester, N. Y.). The sheets were run in cyclohexane:glacial acetic acid:chloroform, 49:2:49, developed with chloramine-T followed by heating, and read under ultraviolet light (34). Tracks along which applied materials were chromatographed were then cut into 1-cm-long sections and subjected to liquid scintillation counting as noted above.

Pharmacokinetic analysis. Plasma pharmacokinetic data were fitted by computer using weighted nonlinear-squares regression analysis to a function of the form

$$C = Ae^{-\alpha t} + Be^{-\beta t},$$

where *C* = plasma concentration and *t* = time after the dose (35, 36). The coefficients *A*, *B*, α , and β are “hybrid” quantities related to parameters of a two-compartment open model (37, 38). Each residual error was weighted by a factor equal to the reciprocal of the concentration. Goodness of fit was assessed by comparison of actual data points to the computer-generated line to determine randomness of scatter. All analyses were then repeated using triexponential functions consistent with a three-compartment open model. The most appropriate model (biexponential for the data reported here) was chosen according to which yielded the smallest sum of squares of residual errors. The following pharmacokinetic parameters were then calculated: distribution half-life ($t_{1/2\alpha}$), apparent elimination half-life ($t_{1/2\beta}$), volume of central compartment (V_1), total apparent volume of distribution using the “area” method (V_d), and total clearance.

RESULTS

Characterization of antibodies with high affinity for digitoxin. A sheep immunized and repeatedly boosted with a digoxin-bovine serum albumin (BSA) conjugate produced antibodies that crossreacted strongly with digitoxin. The average intrinsic association constant (K_a) for digitoxin of the pooled antiserum used in experiments reported in this paper, as determined by equilibrium dialysis and Scatchard analysis, was $1.4 \times 10^{10} \text{ M}^{-1}$ (Fig. 1). Association and dissociation rate constants for the reaction

were $k_f = 3.7 \times 10^6 \text{ M}^{-1} \text{ per s}$ and $k_r = 1.9 \times 10^{-4} \text{ per s}$.

FIGURE 1 Scatchard plot of data obtained from equilibrium dialysis of [^3H]digitoxin against specific antibody. B denotes the molar concentration of digitoxin bound to antibody; F is the molar concentration of free digitoxin. The average intrinsic association constant (K_o) of the antibody population for digitoxin is $1.4 \times 10^{10} \text{ M}^{-1}$.

The resulting k_f/k_r ratio of $1.9 \times 10^{10} \text{ M}^{-1}$ is in satisfactory agreement with the K_o value of $1.4 \times 10^{10} \text{ M}^{-1}$ obtained under equilibrium conditions.

Digitoxin toxicity reversal studies in dogs. Preliminary studies demonstrated that i.v. digitoxin doses between 0.15 and 0.3 mg/kg given over 10 min usually caused ventricular tachycardia within 1 h, lasting 30–90 min. Both time of onset and duration of arrhythmias were variable, however, and the dose of 0.5 mg/kg was chosen for subsequent studies because of the reproducible and unequivocal endpoint of death in all eight dogs receiving this dose (Table I) without specific Fab infusion.

The usual pattern of digitoxin intoxication was an

TABLE I
Digitoxin Toxicity Reversal in the Dog

	Control (non-specific) Fab $n = 8$	Specific Fab $n = 8$
Time to VT,* min	23.4 ± 3.8	28.1 ± 2.8
Incidence of death	8	0
Time to death, min	101 ± 36	—
Time to reappearance of conducted sinus beats, min	—	18 ± 4
Time to stable normal sinus rhythm, min	—	53.9 ± 16.5

All values are given as mean $\pm$ SEM.

* VT = ventricular tachycardia.

initial sinus bradycardia with varying degrees of atrio-ventricular block shortly after digitoxin administration, sometimes followed by runs of supraventricular tachycardia. Ventricular tachycardia ensued shortly after the appearance of the first premature ventricular depolarizations, at an average time of 23.4 ± 3.8 (SEM) min after digitoxin injection. Ventricular fibrillation occurred terminally in six of eight control animals; ventricular standstill occurred in the other two. Average survival time of the eight control dogs was 101.4 ± 36.1 min after digitoxin injection (Table I).

The group of eight dogs subsequently treated with specific Fab fragments developed ventricular tachycardia an average of 28 ± 3 min after digitoxin administration, similar to the time course of toxicity in control animals (Table I). In marked contrast to the control group, however, conducted sinus beats reappeared in all animals given specific Fab fragments, an average of 18 ± 4 min after initial Fab administration. Stable sinus rhythm without ventricular or supraventricular ectopic activity was present an average of 54 ± 16 min after Fab injection (Table I).

Animals treated with specific Fab fragments were observed over a 24-h period after the acute phase of the experiment, and all appeared healthy. Electrocardio-

FIGURE 2 Plasma total digitoxin concentrations in individual dogs before (left) and 1 h after specific Fab infusion. Horizontal bars indicate mean values.

FIGURE 3 Time course of plasma total digitoxin concentrations of eight dogs treated with specific Fab fragments (upper curve); brackets denote 1 SE above and below the mean. The lower curve shows the expected decline in plasma digitoxin levels in an animal that received nonspecific Fab fragments and died 220 min after digitoxin administration.

grams recorded 24 h after digitoxin administration showed normal sinus rhythm in all instances.

Fig. 2 shows the effect of specific Fab fragments on plasma total digitoxin concentration. Each of the eight animals so treated showed a substantial increase

in plasma total digitoxin concentration between the time of onset of ventricular tachycardia (just before Fab infusion) and 1 h after Fab infusion. The mean value of 762 ± 68 ng/ml at onset of ventricular tachycardia was similar to that of 832 ± 56 for control dogs, and increased to $2,143 \pm 144$ ng/ml at 1 h ($P < 0.0001$).

As illustrated in Fig. 3, peak plasma total digitoxin concentrations were present 1 h after initial Fab infusion and remained at these high levels over the ensuing 2 h. The only control animal surviving beyond 1 h after onset of ventricular tachycardia showed the expected decline in plasma concentration from 1,000 to 600 ng/ml at 3 h (Fig. 3). Despite these striking rises in plasma total digitoxin concentrations, free digitoxin levels in samples obtained from 1 to 6 h after specific Fab fragment administration were less than 1 ng/ml as determined by radioimmunoassay and were less than 0.1 ng/ml by direct measurement of dialyzable [^3H]digitoxin added in vitro.

Digitoxin pharmacokinetic studies. The rhesus monkey was studied with the hope that the digitoxin elimination rate in this species would be similar to that in man. This proved to be the case. As shown in Fig. 4, semilogarithmic plots of digitoxin plasma concentration vs. time showed a biexponential disappearance pattern. The mean distribution half-life ($t_{1/2\alpha}$) of the three animals studied on two occasions by radioimmunoassay (phase 1 and phase 4) was 0.59 h (Table II). The apparent elimination half-life ($t_{1/2\beta}$) was 135.5 h in the absence of specific antibody or Fab fragment infusion. Infusion of nonspecific (control) IgG or Fab fragments produced no

FIGURE 4 Time course of mean serum digitoxin concentrations after i.v. injection of 0.2 mg digitoxin. Values from radioimmunoassay are shown in the left panel (open circles) for six studies in three rhesus monkeys. The right panel (solid circles) shows data obtained by direct tritium counting after administration of [^3H]digitoxin to the same three animals. The lines of best fit illustrated were derived by computer as described in the text.

TABLE II
Digitoxin Pharmacokinetics in the Rhesus Monkey*

	Radioimmunoassay Mean ($\pm$ SEM) (n = 6)	Tritium counts Mean ($\pm$ SEM) (n = 3)
$t_{1/2}$, h	0.59 ($\pm$ 0.23)	0.86 ($\pm$ 0.05)
$t_{1/2}$, h	135.5 ($\pm$ 21.9)	117.6 ($\pm$ 10.9)
V_1 , liter/kg	0.20 ($\pm$ 0.11)	0.26 ($\pm$ 0.03)
V_d , liter/kg	1.23 ($\pm$ 0.05)	1.26 ($\pm$ 0.12)
Clearance		
ml/min	0.90 ($\pm$ 0.22)	0.79 ($\pm$ 0.36)
ml/min per kg	0.113 ($\pm$ 0.024)	0.126 ($\pm$ 0.017)

V_1 = volume of central compartment; V_d = total apparent volume of distribution.

* Data analyzed on the basis of a two-compartment open model.

discernible change in digitoxin pharmacokinetics compared with experimental phase 4, in which digitoxin alone was administered.

Samples analyzed by direct tritium counting yielded results similar to those obtained by radioimmunoassay (Table II). Fig. 4 illustrates the good agreement between pharmacokinetic data derived from the disappearance of radioactivity from plasma with data obtained by radioimmunoassay.

Effects of specific Fab fragments on serum digitoxin concentrations. As shown in Fig. 5, administration of 2 mol of Fab fragments per mole of digitoxin 6 h after digitoxin infusion led to a rapid 4.3-fold increase in total plasma radioactivity, compared with control values. Plasma total digitoxin concentrations then declined

FIGURE 5 Effect of i.v. administration of specific Fab fragments on serum total digitoxin concentrations. 2 mol of Fab binding sites per mole of administered digitoxin was given 6 h after an i.v. dose of 0.2 mg digitoxin. Solid circles show mean values for control experiments in three rhesus monkeys. Open circles show similar mean values until administration of Fab at 6 h (arrow). Note break in time scale after 96 h.

FIGURE 6 Effect of i.v. administration of specific IgG on serum total digitoxin concentrations. 2 mol of antibody binding sites per mole of digitoxin was given 6 h (arrow) after an i.v. dose of 0.2 mg digitoxin. Values shown are means from studies of three rhesus monkeys as shown in Fig. 2. Solid circles: control experiments; open circles: specific IgG administration at 6 h. Note break in time scale after 96 h.

with an initial mean half-life of about 4 h. 12–16 h after specific Fab injection, plasma concentrations of digitoxin returned to levels found in control experiments in which no specific Fab fragments were given.

Effects of specific IgG administration on plasma digitoxin concentrations. Administration of 2 mol of specific binding sites per mole of digitoxin in the form of intact IgG resulted in a substantial increase in total plasma radioactivity to a mean peak value 12.9-fold above pre-IgG infusion levels at the 28th hour of the experiment, as shown in Fig. 6. This was followed by a gradual decline of plasma radioactivity until the 4th day, after which a more rapid fall was observed between the 4th and 9th days after specific IgG administration. Plasma levels of radioactivity were 10-fold higher 6 and 12 h after specific IgG administration when compared with the last sample obtained before antibody infusion. 2 wk after specific IgG administration, mean plasma radioactivity concentrations had returned to levels comparable with those observed in control experiments.

Effects of specific Fab fragments on urinary excretion of digitoxin. Mean urinary excretion of radioactivity over 96 h in the absence of specific antibody or Fab fragments totaled $11.7 \pm 2.4 \mu\text{g}$, or 6% of the dose given, the largest amounts being excreted during the first and second 6-h collection periods (Fig. 7).

After infusion of specific Fab fragments, urinary excretion of radioactivity increased 13-fold in comparison to the first 6-h period of the experiment (before Fab administration) and 12-fold (from 165 ± 61 to $1,985 \pm 395 \text{ ng/kg}$, $P < 0.05$) over recovery values for the same time period in control experiments in which no Fab or IgG

FIGURE 7 Cumulative urinary excretion of digitoxin and metabolites, determined from tritium counts excreted after i.v. administration of 0.2 mg [^3H]digitoxin. Solid circles: mean values for control experiments in three rhesus monkeys; open triangles: mean values for the same three animals given a two-fold molar excess of specific Fab fragments at 6 h (arrow); open circles: mean values for the same three animals given a twofold molar excess of specific binding sites as intact IgG at 6 h.

was given. Tritium recovery in urine increased from a control value of 0.6% to 8% of the [^3H]digitoxin dose given for the 6-h time period after specific Fab infusion. Fig. 8 summarizes the urinary excretion of digitoxin and metabolites during the 6-h collection period immediately after specific Fab administration. The mean hourly excretion rate of radioactivity during the 12- to 24-h collection period (6–18 h after Fab infusion) remained elevated and exceeded values before Fab administration by a factor of 2.2 (mean 489 ng/h vs. 220 ng/h). Cumulative urinary excretion of ^3H counts over the 90 h after specific Fab infusion more than doubled when compared with control experiments, as shown in Fig. 7.

Effects of specific IgG on urinary excretion of digitoxin. In marked contrast to Fab infusion, administration of intact antibodies significantly reduced the urinary digitoxin excretion rate to 37% of control during the interval between the 6th and 12th hour of the experiment. These data are summarized in Figs. 7 and 8. During the 90 h after IgG infusion, only 1.5% of ^3H counts present in the digitoxin dose given was recovered in the urine, compared with 4.1% in control experiments and 11.4% after specific Fab administration.

Binding of digitoxin in plasma and urine by specific IgG and Fab. Table III summarizes results of equilibrium dialysis studies of plasma samples obtained after injection of specific IgG or Fab fragments.

FIGURE 8 Mean urinary excretion values for digitoxin and metabolites during the period from 6 to 12 h after i.v. administration of 0.2 mg [^3H]digitoxin in three rhesus monkeys. Brackets show 1 SEM. Values given were determined by tritium counts excreted in control experiments and after specific Fab or IgG, given at 6 h.

Whereas digitoxin fully equilibrated across the dialysis membrane in samples obtained before specific antibody or Fab injection or after nonspecific protein administration, the glycoside was more than 90% bound to specific IgG or Fab in samples obtained from 30 min through 5 h after specific Fab or IgG was given. Whereas the amount of digitoxin bound to IgG declined insignificantly from 99.6 to 98.7% over 90 h, the percentage of plasma [^3H]digitoxin bound to Fab declined relatively rapidly after 2 h and only a minor fraction

TABLE III
Equilibrium Dialysis of Rhesus Monkey Plasma Samples Obtained after Specific Fab or IgG Administration*

Hours after injection of specific Fab or IgG	Percent bound after Fab Mean ($\pm$ SEM)	Percent bound after IgG Mean ($\pm$ SEM)
h	%	%
0.5	99.0 ($\pm$ 0.1)	99.6 ($\pm$ 0.1)
1.0	98.6 ($\pm$ 0.2)	99.7 ($\pm$ 0.03)
2.0	97.2 ($\pm$ 1.0)	99.6 ($\pm$ 0.1)
3.0	93.5 ($\pm$ 5.2)	99.6 ($\pm$ 0.1)
4.0	94.3 ($\pm$ 3.8)	99.6 ($\pm$ 0.04)
5.0	90.6 ($\pm$ 4.1)	99.6 ($\pm$ 0.1)
6.0	82.8 ($\pm$ 5.9)	99.5 ($\pm$ 0.2)
18.0	62.6 ($\pm$ 1.9)	99.8 ($\pm$ 0.1)
42.0	12.7 ($\pm$ 1.6)	99.6 ($\pm$ 0.1)
66.0	1.6 ($\pm$ 0.9)	99.6 ($\pm$ 0.04)
90.0	0.5 ($\pm$ 0.4)	99.7 ($\pm$ 0.4)

* No binding other than that attributed to serum albumin was demonstrable in plasma samples obtained before antibody infusion.

TABLE IV
Equilibrium Dialysis of Rhesus Monkey Urine Samples Obtained after Administration of Specific Fab or IgG*

Collection period	Percent bound after Fab Mean ($\pm$ SEM)	Percent bound after IgG Mean ($\pm$ SEM)
h	%	%
0-6	2.7 ($\pm$ 1.6)	0.3 ($\pm$ 0.3)
6-12	98.7 ($\pm$ 0.5)	45.2 ($\pm$ 12.1)
12-24	77.7 ($\pm$ 7.9)	25.3 ($\pm$ 9.7)
24-48	58.5 ($\pm$ 9.7)	42.0 ($\pm$ 8.4)
48-72	40.5 ($\pm$ 1.8)	40.0 ($\pm$ 5.4)
72-96	17.7 ($\pm$ 5.6)	45.6 ($\pm$ 3.8)

* No binding was demonstrable in urine samples obtained before antibody infusion.

(12.7%) was still bound 42 h after specific Fab injection, decreasing to an average of 1.6% after 66 h.

The urine equilibrium dialysis results (Table IV) show a marked increase of the bound (nondialyzable) fraction of digitoxin during the 6 h after specific Fab administration to 98.7 $\pm$ 0.5%, compared with a negligible value of 2.7 $\pm$ 1.6% for urine excreted during the 6-h control period before Fab infusion. After specific IgG infusion, the small amount of ^3H activity excreted increased from negligible percent bound values during the control (0-6 h) period to values in the 25-45% range, presumably related to small amounts of immunoglobulin or immunoglobulin fragments excreted.

It should be noted that the percent bound values for urine samples after Fab or IgG administration represent lower limits of actual binding since some degree of degradation of antibody or antibody fragments during collection and storage periods cannot be excluded.

Analytical studies of digitoxin and metabolites excreted in urine. To test the hypothesis that enhancement of ^3H excretion in urine after specific Fab infusion was due to excretion of native digitoxin bound to Fab fragments, further analytical studies were done. Dichloromethane extraction according to the method used showed that 99% of ^3H counts in digitoxin samples before administration to animals were extracted into the dichloromethane phase. Table V summarizes the percentages of dichloromethane-extractable radioactivity from urine obtained during the control experiments (phase 4) and after administration of specific Fab fragments and specific IgG (phases 2 and 3). After administration of specific Fab fragments, the percentage of extractable radioactivity increased from a control mean of 40% (phase 4) to 92% during the first 6-h period, gradually decreasing to control levels by the time of the 72- to 96-h urine sample. This is consistent with accelerated excretion of native digitoxin and excludes the possibility that ^3H excretion was due to ^3H exchange

with water-soluble material or accelerated formation of water-soluble metabolites. Analysis of extracted material by thin-layer chromatography further demonstrated identical mobility of greater than 90% of ^3H counts present in this fraction with identically treated samples of [^3H]digitoxin standard.

DISCUSSION

Digitoxin is one of the two cardiac glycosides most widely used in clinical practice. A substantial number of instances of severe intoxication have been reported (2, 39). Due to its prolonged half-life compared with other glycosides (13), digitoxin intoxication poses a special therapeutic problem. In a series of 115 patients treated for advanced (usually suicidal) digitalis intoxication, 96% had taken digitoxin and the resulting mortality was 22% (2). Patients died as late as 4 days after injection of the drug, and doses as low as 3 mg were reported to cause mortality. Caldwell et al. have suggested a novel therapeutic approach using oral ingestion of a steroid-binding resin to interrupt enterohepatic cycling of digitoxin (40, 41). This approach was highly effective in the rat (40), a species with very active enterohepatic cycling of digitoxin, and some enhancement of digitoxin excretion was also documented in man (41). However, since no specific antagonist of demonstrated clinical effectiveness in the setting of advanced toxicity has yet been described, therapy is restricted to symptomatic management of the clinical manifestations of digitalis toxicity.

To provide a more specific and effective therapeutic approach, the present study was undertaken to determine whether specific Fab fragments of antibodies that bind digitoxin with high affinity can reverse advanced, life-threatening digitoxin toxicity in a canine experimental model. The feasibility of this approach was suggested by earlier studies using digitalis-specific antibodies or their Fab fragments for reversal of glyco-

TABLE V
Percent of ^3H Radioactivity Extractable into Dichloromethane from Rhesus Monkey Urine Samples*

Collection period	Phase 2 (specific Fab) Mean ($\pm$ SEM)	Phase 3 (specific IgG) Mean ($\pm$ SEM)	Phase 4 (no Fab or IgG) Mean ($\pm$ SEM)
h			
0-6	38.2 ($\pm$ 2.2)	37.8 ($\pm$ 5.1)	40.0 ($\pm$ 2.1)
6-12	91.8 ($\pm$ 2.4)	37.7 ($\pm$ 5.3)	49.7 ($\pm$ 0.2)
12-24	81.9 ($\pm$ 3.0)	25.6 ($\pm$ 7.7)	54.7 ($\pm$ 2.8)
24-48	61.6 ($\pm$ 10.8)	43.8 ($\pm$ 12.4)	52.8 ($\pm$ 5.2)
48-72	44.6 ($\pm$ 12.3)	43.7 ($\pm$ 3.6)	34.6 ($\pm$ 5.3)
72-96	37.4 ($\pm$ 9.0)	52.3 ($\pm$ 3.7)	36.2 ($\pm$ 4.2)

* 99% of ^3H activity from native [^3H]digitoxin was dichloromethane-extractable under the conditions used.

side-induced effects in vitro (4–7, 42–44) and toxic arrhythmias in vivo (3–5, 45). Differences in both pharmacokinetics (13, 46) and pharmacodynamics (15–19), however, make extrapolation of results from studies of one cardiac glycoside to another hazardous. The rationale for use of Fab fragments in preference to intact IgG for in vivo reversal of cardiac glycoside toxicity has been discussed in detail recently (5, 47) and includes lesser antigenicity of Fab compared with IgG as well as more rapid excretion of both binding protein and bound drug.

With regard to potential therapeutic use, the first requisite of an antibody population or the Fab fragments derived therefrom is high affinity and specificity for the drug molecule which is to be counteracted. In the case of cardiac glycosides such as digitoxin and digoxin, toxic effects may be manifest at free plasma concentrations of 10 nM or less (30, 48). Antibody affinity constants must therefore be at least 10^9 or more if free glycoside concentrations are to be lowered to negligible levels without the use of excessive amounts of antibody, an issue of obvious importance if heterologous antiserum is used as the source of cardiac glycoside-binding immunoglobulin (5).

In extending our earlier work to the reversal of digitoxin toxicity, we have taken advantage of the observation that selected antisera from animals immunized with BSA-digoxin conjugates contain antibody populations that strongly crossreact with digitoxin (23). Among the first three sheep screened, we identified an animal that developed a high titer of antibodies with a K_o value for digitoxin of $1.4 \times 10^{10} \text{ M}^{-1}$ by equilibrium dialysis, in satisfactory agreement with results of separate estimates of the kinetics of formation and dissociation of the hapten-antibody complex. The rapid second-order association kinetics ensure that the free fraction of serum digitoxin concentration will fall to less than 1 nM within a few seconds of the infusion of antibody in the amount used in the experiments reported here, assuming rapid mixing in the intravascular compartment. This rapid binding of digitoxin by antibody occurs in spite of extensive serum albumin binding of digitoxin (14) because of the very rapid dissociation rate of the digitoxin-albumin complex. It should be noted that the degree of digitoxin crossreactivity noted in the present studies cannot be expected for every digoxin-binding antibody population (23).

The dose of 0.5 mg/kg of digitoxin administered intravenously to dogs uniformly led to the death of the animals at a mean time of 101 min. Nonspecific Fab given to eight control animals did not protect from digitoxin toxicity. The administration of a small (33%) molar excess of specific Fab fragments not only prevented the death of all eight animals so treated, but also reversed cardiotoxic effects completely and led to reappearance of conducted sinus beats as early as 9–10 min after

injection. This relatively rapid reversal of established advanced digitoxin toxicity confirms and extends previous experience with antibody reversal of established digoxin toxicity (3–5) as well as our initial clinical experience in the treatment of advanced digoxin toxicity with purified specific Fab fragments (8).

A rapid increase in plasma total digitoxin levels took place within 30 min after the end of specific Fab infusion in all dogs (Fig. 2); during the subsequent 2 h, plasma concentrations did not change significantly (Fig. 3). A similar plateau of serum total digoxin concentration after the initial rise after administration of specific Fab fragments lasted for 10 h in a patient treated for advanced digoxin toxicity after a massive suicidal ingestion of 22.5 mg of the drug (8). The consistent increase in total digitoxin concentration in plasma observed after specific Fab administration in the studies reported here presumably reflects continuing removal of digitoxin from the tissues and sequestration in the extracellular space in an antibody-bound, pharmacologically inactive form, balanced by renal excretion of the Fab fragment-digitoxin complex. This mechanism was further supported by equilibrium dialysis studies showing free digitoxin concentrations of less than 0.1 ng/ml after specific Fab infusion in these dogs.

The prolonged half-life of digitoxin in man is associated with very slow excretion by the kidneys, which in turn is presumably due to the high degree of binding of digitoxin in the circulation by serum albumin. Lukas and DeMartino demonstrated that 97% of digitoxin at usual therapeutic concentrations is bound to serum albumin (14). An effective approach to the specific treatment of advanced digitoxin intoxication, then, ideally should enhance excretion of the drug as well as neutralize its effects before excretion.

Canine digitoxin excretion, like that of usual laboratory animals such as the mouse, rat, and cat, is substantially more rapid than is the case in man (11, 13, 20). The rhesus monkey (*Macaca mulatta*) was shown in the present studies to provide a more suitable model. The mean elimination half-times of 135 and 118 h found by two separate methods in this species (Fig. 4; Table II) are in satisfactory agreement with the values of 115 and 165 h in man reported by Lukas (13) and Gjerdum (11), respectively.

Prior studies of the effects of digoxin-specific IgG and Fab fragments on the pharmacokinetics of digoxin in the dog have documented substantial rises in plasma total digoxin concentration, with IgG causing about a sixfold greater rise than Fab (47). Analogous results were observed in the present rhesus monkey experiments. Administration of 2 mol of binding sites per mole of digitoxin in the form of an IgG preparation resulted in a greater and substantially more prolonged increase in plasma total digitoxin levels compared with the response to an equal number

of binding sites given as Fab fragments. The differences may be accounted for in part by a smaller distribution space of 160,000-dalton IgG molecules compared with 50,000-dalton Fab fragments, as well as by continuing urinary excretion of Fab fragments. The increases in plasma total digitoxin levels after both IgG and Fab administration were demonstrated in equilibrium dialysis studies to be due to high-affinity immunoglobulin binding, and were accompanied by rapid decreases in free digitoxin concentration to near-zero levels (Table III).

Administration of IgG reduced the urinary excretion of radioactivity to 37% of control values. Of this amount more than 40% was not dialyzable, indicating that digitoxin was partly excreted in bound form, possibly to low molecular weight antibody fragments formed in vivo. The serum t_1 of homologous and heterologous γ -globulin injected into rabbits or guinea pigs has been shown to be between 4.2 and 6 days (49), and only small amounts of intact IgG were excreted in the urine. The reduction in urinary excretion of radioactivity after specific IgG administration was therefore not an unexpected observation.

Binding of digitoxin to Fab fragments appears to account for the initial half-life of digitoxin elimination from serum of 4 h (Fig. 5). It has been reported that rabbit Fab fragments are excreted by the mouse, at least in part via the kidneys, with a half-time of 3.6 h (50). Although comparable data are not available for subhuman primates, the data of Janeway et al. (51) indicate elimination of immunoglobulin fragments of size and other properties similar to Fab by humans with a half-time of 5 h. One would therefore predict that relatively rapid renal elimination of sheep specific Fab fragments by the rhesus monkey kidney would enhance excretion of bound digitoxin, and this proved to be the case, as summarized in Figs. 7 and 8. The high affinity of this population of Fab fragments for digitoxin ensures that, once filtered by the glomerulus, the Fab-digitoxin complex will not dissociate to a major extent and the digitoxin excreted in the urine remains predominantly bound in early urine samples collected after specific Fab administration (Table IV). The analytical experiments using dichloromethane extraction and thin-layer chromatography suggest that administration of specific Fab fragments led to elimination of native digitoxin and not of metabolites.

Under the experimental conditions used in this primate model, digitoxin elimination rose 14-fold to 8% of the dose given over 6 h, compared with control values. When in severe digitoxin intoxication an even more pronounced acceleration of glycoside excretion is desirable, this may well be achieved by a constant infusion or repeated doses of purified Fab fragments over a more prolonged period of time. This, to our knowledge,

is the first demonstration of enhanced excretion of a drug by an immunoglobulin fragment and may represent only one example of a number of therapeutic opportunities wherein a higher clearance of a drug or hormone can be obtained.

In conclusion, the present studies demonstrate that high-affinity specific Fab fragments enhance urinary excretion of digitoxin, whereas administration of intact IgG leads to the opposite effect and retains the potentially toxic glycoside stores in the body. Taken together with the toxicity reversal studies in the dog, these data support the potential clinical use of purified specific Fab fragments in selected cases of advanced, life-threatening digitoxin toxicity unresponsive to conventional therapy.

ACKNOWLEDGMENTS

This work was supported in part by Award HL-18003 and Program Project Award HL-19259, both from the National Heart, Lung, and Blood Institute.

REFERENCES

1. Smith, T. W., and J. T. Willerson. 1971. Suicidal and accidental digoxin ingestion: report of five cases with serum digoxin level correlations. *Circulation*. 44: 29-36.
2. Bismuth, C., M. Gaultier, F. Conso, and M. L. Efthymiou. 1973. Hyperkalemia in acute digitalis poisoning: diagnostic significance and therapeutic implications. *Clin. Toxicol.* 6: 153-162.
3. Schmidt, D. H., and V. P. Butler, Jr. 1971. Reversal of digoxin toxicity with specific antibodies. *J. Clin. Invest.* 50: 1738-1744.
4. Curd, J., T. W. Smith, J-C. Jatton, and E. Haber. 1971. The isolation of digoxin specific antibody and its use in reversing the effects of digoxin. *Proc. Natl. Acad. Sci. U.S.A.* 68: 2401-2406.
5. Smith, T. W., V. P. Butler, Jr., and E. Haber. 1977. Cardiac glycoside-specific antibodies in the treatment of digitalis intoxication. In *Antibodies in Human Diagnosis and Therapy*. R. Krause and E. Haber, editors. Raven Books, Abelard-Shuman, Limited, New York. 365-389.
6. Smith, T. W. 1972. Ouabain-specific antibodies: immunochemical properties and reversal of Na^+ , K^+ -activated adenosine triphosphatase inhibition. *J. Clin. Invest.* 51: 1583-1593.
7. Gold, H. K., and T. W. Smith. 1974. Reversal of ouabain and acetyl strophanthidin effects in normal and failing cardiac muscle by specific antibody. *J. Clin. Invest.* 53: 1655-1661.
8. Smith, T. W., E. Haber, L. Yeatman, and V. P. Butler, Jr. 1976. Reversal of advanced digoxin intoxication with Fab fragments of digoxin specific antibodies. *N. Engl. J. Med.* 294: 797-800.
9. National Prescription Audit, IMS American Ltd., Ambler Pa.
10. Lukas, D. S. 1972. Of Toads and Flowers (Editorial). *Circulation*. 46: 1-4.
11. Gjerdrum, K. 1972. Digitoxin studies. *Acta Med. Scand.* 191: 25-34.
12. Storstein, O., V. Hansteen, L. Hatle, L. Hillestad, and L. Storstein. 1977. Studies on digitalis. XIII. A prospective

- study of 649 patients on maintenance treatment with digitoxin. *Am. Heart J.* 93: 434-443.
13. Lukas, D. S. 1971. Some aspects of the distribution and disposition of digitoxin in man. *Ann. N.Y. Acad. Sci.* 179: 338-361.
 14. Lukas, D. S., and A. G. DeMartino. 1969. Binding of digitoxin and some related cardenolides to human plasma proteins. *J. Clin. Invest.* 48: 1041-1053.
 15. Kuschinsky, K., H. Lahrtz, H. Lüllmann, and P. A. Van Zwieten. 1967. Accumulation and release of ^3H -digoxin by guinea-pig heart muscle. *Br. J. Pharmacol.* 30: 317-328.
 16. Kuschinsky, K., H. Lüllmann, and P. A. Van Zwieten. 1968. A comparison of the accumulation and release of ^3H -ouabain and ^3H -digitoxin by guinea-pig heart muscle. *Brit. J. Pharmacol.* 32: 598-608.
 17. Dutta, A., S. Goswami, D. K. Datta, J. O. Lindower, and B. H. Marks. 1968. The uptake and binding of six radio-labeled cardiac glycosides by guinea-pig hearts and by isolated sarcoplasmic reticulum. *J. Pharmacol. Exp. Ther.* 164: 10-21.
 18. Runge, T. M. 1977. Clinical implications of differences in pharmacodynamic action of polar and nonpolar cardiac glycosides. *Am. Heart J.* 93: 248-255.
 19. Runge, T. M., J. C. Stephens, P. Holden, D. F. Havemann, W. M. Kilgore, E. M. Dale, and R. E. Dalton. 1975. Pharmacodynamic distinctions between ouabain, digoxin, and digitoxin. *Arch. Int. Pharmacodyn. Ther.* 214: 31-45.
 20. Okita, G. T. 1967. Species difference in duration of action of cardiac glycosides. *Fed. Proc.* 26: 1125-1130.
 21. Butler, V. P., Jr., and J. P. Chen. 1967. Digoxin-specific antibodies. *Proc. Natl. Acad. Sci. U.S.A.* 57: 71-78.
 22. Erlanger, B. F., and S. M. Beiser. 1964. Antibodies specific for ribonucleosides and ribonucleotides and their reaction with DNA. *Proc. Natl. Acad. Sci. U.S.A.* 52: 68-74.
 23. Smith, T. W., V. P. Butler, Jr., and E. Haber. 1970. Characterization of antibodies of high affinity and specificity for the digitalis glycoside digoxin. *Biochemistry.* 9: 331-337.
 24. Campbell, D. H., J. S. Garvey, N. E. Cremer, and D. H. Sussdorf. 1963. Ammonium sulfate precipitation of gamma globulin. In *Methods in Immunology*. W. A. Benjamin, Inc., New York. 118-120.
 25. Nisonoff, A. 1964. Enzymatic digestion of rabbit gamma globulin and antibody and chromatography of digestion products. *Methods Med. Res.* 10: 134-141.
 26. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations of dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244: 4406-4412.
 27. Herbert, V., K. S. Lau, C. W. Gottlieb, and S. J. Bleicher. 1965. Coated charcoal immunoassay for insulin. *J. Clin. Endocrinol. Metab.* 25: 1375-1384.
 28. Smith, T. W., and K. M. Skubitz. 1975. Kinetics of interactions between antibodies and hapten. *Biochemistry.* 14: 1496-1502.
 29. Skubitz, K. M., and T. W. Smith. 1975. Determination of antibody-hapten association kinetics: a simplified experimental approach. *J. Immunol.* 114: 1369-1374.
 30. Smith, T. W. 1970. Radioimmunoassay for serum digitoxin concentration: methodology and clinical experience. *J. Pharmacol. Exp. Ther.* 175: 352-360.
 31. Bray, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* 1: 279-285.
 32. Gjerdrum, K. 1970. Determination of digitalis in blood. *Acta Med. Scand.* 187: 371-379.
 33. Storstein, L. 1976. Studies on digitalis. IV. A method for thin-layer chromatographic separation and determination of digitoxin and cardioactive metabolites in human blood and urine. *J. Chromatogr.* 117: 87-96.
 34. Stahl, E., editor. 1967. *Dünnschicht-Chromatographie*. Springer, Berlin, Heidelberg, New York, 2nd edition. 821.
 35. Usanis, R. A. 1972. NLIN-Nonlinear least squares estimation of parameters. Library Series Document No. LSR-089-1, Triangle Universities Computation Center. Research Triangle Park, N.C.
 36. Marquardt, D. W. 1963. An algorithm for least-squares estimation of non-linear parameters. *J. Soc. Ind. Appl. Math.* 11: 431-441.
 37. Gibaldi, M., and D. Perrier. 1975. *Pharmacokinetics*. Marcel Dekker, Inc., New York.
 38. Wagner, J. G. 1975. *Fundamentals of clinical pharmacokinetics*. Drug Intelligence Publications, Hamilton, Ill.
 39. Lely, A., and C. H. I. Van Enter. 1970. Large-scale digitoxin intoxication. *Br. Med. J.* 3: 737-740.
 40. Caldwell, J. H., and N. J. Greenberger. 1971. Interruption of the enterohepatic circulation of digitoxin by cholestyramine. I. Protection against lethal digitoxin intoxication. *J. Clin. Invest.* 50: 2626-2637.
 41. Caldwell, J. H., C. A. Bush, and N. J. Greenberger. 1971. Interruption of the enterohepatic circulation of digitoxin by cholestyramine. II. Effect on metabolic disposition of tritium-labeled digitoxin and cardiac systolic intervals in man. *J. Clin. Invest.* 50: 2638-2644.
 42. Mandel, W. J., J. T. Bigger, Jr., and V. P. Butler, Jr. 1972. The electrophysiologic effects of low and high digoxin concentrations on isolated mammalian cardiac tissue: Reversal by digoxin-specific antibody. *J. Clin. Invest.* 51: 1378-1387.
 43. Watson, J. F., and V. P. Butler, Jr. 1972. Biological activity of digoxin-specific antisera. *J. Clin. Invest.* 51: 638-648.
 44. Butler, V. P., Jr., J. F. Watson, D. H. Schmidt, J. D. Gardner, W. J. Mandel, and C. L. Skelton. 1973. Reversal of pharmacological and toxic effects of cardiac glycosides by specific antibodies. *Pharmacol. Rev.* 25: 239-248.
 45. Smith, T. W. 1974. Use of antibodies in the study of the mechanism of action of digitalis. *Ann. N. Y. Acad. Sci.* 242: 731-736.
 46. Smith, T. W., and E. Haber. 1973. Medical progress: digitalis. *N. Engl. J. Med.* 289: 945-952, 1010-1015, 1063-1072, 1125-1129.
 47. Butler, V. P., Jr., D. H. Schmidt, T. W. Smith, E. Haber, B. D. Raynor, and P. Demartini. 1977. Effects of sheep digoxin-specific antibodies and their Fab fragments on digoxin pharmacokinetics in dogs. *J. Clin. Invest.* 59: 345-359.
 48. Smith, T. W., V. P. Butler, Jr., and E. Haber. 1969. Determination of therapeutic and toxic serum digoxin concentrations by radioimmunoassay. *N. Engl. J. Med.* 281: 1212-1216.
 49. Spiegelberg, H. L., and W. O. Weigle. 1965. The catabolism of homologous and heterologous 7S gamma globulin fragments. *J. Exp. Med.* 121: 323-338.
 50. Wochner, R. D., W. Strober, and T. A. Waldmann. 1967. The role of the kidney in the catabolism of Bence Jones proteins and immunoglobulin fragments. *J. Exp. Med.* 126: 207-221.
 51. Janeway, C. A., D. Merler, R. S. Rosen, S. Salmon, and J. D. Crain. 1968. Intravenous gamma globulin. *N. Engl. J. Med.* 278: 919-923.