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AFFINITY AND DOSE-DEPENDENT DIGOXIN Na^+K^+ATPase DISSOCIATION BY MONOCLONAL DIGOXIN-SPECIFIC ANTIBODIES

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Abstract—The effect of three monoclonal digoxin-specific antibodies and of polyclonal Digidot® as reference on digoxin dissociation from rat brain Na^+K^+ATPase microsomes was studied to determine the role of the affinity constant (Ka) and dose of the antibody on the rate of digoxin dissociation from Na^+K^+ATPase. Stoichiometrical doses of 1C10, 6C9, 9F5 IgG, and Digidot® (Ka = 6 10^9, 3.1 10^8, 2.5 10^7, and 8.5 10^9 M^{-1}, respectively) resulted in digoxin dissociation related to Ka. When the IgG:digoxin molar ratio increased from 0.25 to 10, digoxin dissociation from Na^+K^+ATPase sites also increased according to the Hill equation, allowing comparative parameters among the three antibodies to be determined. 1C10 IgG was 2- and 10-fold more efficacious than 6C9 and 9F5, respectively. This *in vitro* model appears to be a useful predictive screening assay before *in vivo* experimentation.

Key words: digoxin; monoclonal antibodies, rat brain Na^+K^+ATPase; affinity; dissociation kinetics

Whole polyclonal or monoclonal immunoglobulins, Fab'_2 and Fab§ fragments are under active development for *in vivo* applications as therapeutic or diagnostic agents. Several factors such as species origin, size, affinity, and specificity of antibodies may be the source of wide variability in their clinical efficacy. Moreover, as a consequence of monoclonal production, a large number of antibodies can be prepared, and selection of the appropriate clinically efficacious antibody requires an *in vitro* and *in vivo* bioevaluation of their potency in the early preclinical steps of pharmaceutical development. As a first step, antibody immunoreactivity must be defined in terms of affinity, specificity, and percentage of specific active binding sites [1]. As a second step, *in vitro* tests using a small amount of material must be carried out before clinical efficacy can be predicted. Digoxin-specific Fab fragments have been shown to be powerful agents for digoxin detoxication in acute digoxin poisoning in humans [2]. However, their polyclonal origin has some limitations in terms of standardization; Fab fragments of monoclonal origin could be a valuable alternative. On the other hand, the preparation of several digoxin-specific monoclonal antibodies raises a crucial question: how to select preclinically the most efficacious antibody using an *in vitro* model? As digoxin has been shown to bind to specific membrane Na^+K^+ATPase sites [3, 4], our solution was to assess the extent and rate of digoxin dissociation from its binding to rat brain microsome Na^+K^+ATPase using three monoclonal digoxin-specific antibodies whose affinity constants spanned a 3-log range. Dissociation kinetic properties were also studied with a commercial polyclonal digoxin-specific Fab fragment (Digidot®) as reference.

MATERIALS AND METHODS

Reagents

^{3}H-digoxin (specific activity 18.3 Ci/mmol) was purchased from New England Nuclear Co. (Boston, MA, U.S.A.). All other reagents of analytical grade were from Merck (Nogent sur Marne, France). Activated charcoal powder Norit A was purchased from Serlabo (Paris, France). Dextran T70 was from Pharmacia (St. Quentin en Yvelynes, France). Monoclonal digoxin-specific IgG were produced by Wahyono *et al.* [5] by somatic cell fusion in ascites fluid from Balb C mice. The three monoclonal antibodies (Mab) used in this study, 1C10, 6C9, and 9F5 IgG, were selected by indirect ELISA according to their affinity and specificity. They were purified on a protein A-Sepharose CL-4B column (Pharmacia, Sweden). All the Mab were IgG1 isotypes. IgG specificity has been previously studied by an inhibition test using digoxin and structurally related analogs. 1C10, 6C9, and 9F5 IgG exhibited similar cross-reactivity with active metabolites [5]. Polyclonal digoxin-specific Fab fragments (Digidot®) were purchased from Boehringer (GmbH Mannheim, Germany). The polyclonal nonspecific immunoglobulins were purchased from Sigma (St. Louis, MO, U.S.A.).

Determination of antibody affinity

The intrinsic affinity constant (Ka) and the maximum concentration of active binding sites (Bm) of monoclonal digoxin-specific 1C10, 6C9, 9F5 IgG and polyclonal Digidot® were determined from saturation analysis (n = 3) by equilibrium dialysis as previously described [1].

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§ Abbreviations: EDTA, ethylene diamine tretraacetic acid; ELISA, enzyme linked immunosorbent assay; IgG, immunoglobulin G; Fab, fragment antigen binding; Mab, monoclonal antibody; Ka, affinity constant; SABS, specific active binding sites; AAC, area above the curve.

Briefly, increasing concentrations of ^{3}H-digoxin (0.1 μM to 5 μm) were incubated with a constant concentration of antibody (10.6 nM, 10.4 nM, 12.8 nM, and 10.2 nM for 1C10, 6C9, 9F5, and Digidot®, respectively) at 37°C. Total and free ^{3}H-digoxin concentrations were measured by liquid scintillation counting (Packard TRICARB 4530) after addition of 3 mL Picofluor 40 (Packard, Les Ulis, France). Saturation binding isotherms were converted to a linear plot according to Woolf's equation [6] using the Graphpad program (ISI, CA):

$$F/B = (1/Bm \cdot 1/Ka) + (1/Bm)F,$$

where B and F are the concentration of bound and free ligand, respectively. The percentage of specific active antibody binding sites (SABS) was defined as the ratio of the number of immunoreactive binding sites to the concentration of total binding sites, assuming that 1 mole of IgG binds 2 moles of digoxin.

Preparation of rat brain microsomes

The brain membrane fraction was prepared by differential centrifugation using the method of Sweadner *et al.* [7] as modified by Berrebi-Bertrand *et al.* [8]. Briefly, rat brains were homogenized with five strokes of a Teflon glass homogenizer in 15 mL of 0.32 M sucrose containing 1 mM EDTA, 0.1 μM phenylmethylsulfonyl fluoride, and 30 mM imidazole-HCl pH 7.2. The homogenate was centrifuged at 850 g for 20 min (Sorvall SS34 rotor) to remove nuclei and unhomogenized material, then at 8500 g (Sorvall SS34 rotor) for 20 min to remove mitochondria and myelin fragments. The supernatant was centrifuged at 100,000 g (Kontron TFT 65-38 rotor) for 30 min. The pellet (i.e. the microsomal fraction enriched in Na^+K^+ATPase activity) was resuspended in a buffer containing 0.32 M sucrose, 1 mM EDTA, and 30 mM imidazole-HCl pH 7.2 to obtain a protein concentration of approximately 2.5 mg/mL, then aliquoted and stored at –70°C until use. All microsomal samples were used within 3 weeks of preparation. The specific activity of this enzyme preparation, determined as described by Lelièvre *et al.* [9], was 60 μmol Pi released/mg protein/hr and accounted for more than 80% of the total Na^+K^+ATPase activities. Protein concentrations were determined according to Lowry *et al.* [10] with serum albumin as standard.

Characteristics of digoxin binding to rat brain microsomes

The specific binding of ^{3}H-digoxin was determined by incubating microsomal preparations of rat brain (28 μg/mL) with increasing concentrations of ^{3}H-digoxin (5–1000 nM) in 1 mM $MgCl_2$, 1 mM H_3PO_4, and 10 mM Tris buffer (pH 7.4) for 180 min at 37°C in a final volume of 550 μL. Specific binding of ^{3}H-digoxin to rat brain microsomes was linear at protein concentrations ranging from 3 to 50 μg/mL. Nonspecific binding was determined by incubating the microsomes with both ^{3}H-digoxin and an excess of unlabelled digitoxigenin (3 10^{-4} M). Bound ^{3}H-digoxin was quantified by a rapid filtration of the incubate (500 μL) through GVWP Durapore hydrophilic Millipore filters (0.25 μm) on a vacuum manifold (Millipore, St. Quentin en Yvelines, France). Filters were washed three times with 2 mL of ice-cold $MgCl_2$/(H_3PO_4)/Tris buffer (pH 7.4). After air drying for one minute, radioactivity retained on the filter was measured in Readyprotein® (Beckman, Gagny, France) by liquid scintillation. Apparent dissociation constants (Kd) and the maximum number of binding sites (B_{max}) were determined by Scatchard plots using the Inplot4 program (GraphPAD®, San Diego, CA). Kd and B_{max} values are mean ± SE from eight experiments.

Dissociation of microsome-bound ^{3}H-digoxin by unlabelled digoxin and digoxin-specific antibodies

Brain microsome preparations (28 μg/mL) were incubated with ^{3}H-digoxin (75 nM) at 37°C in an $MgCl_2$/H_3PO_4/Tris buffer (pH 7.4) for 120 minutes. The dissociation kinetics of ^{3}H-digoxin from rat brain microsomes were determined by addition of a 1600-fold excess of

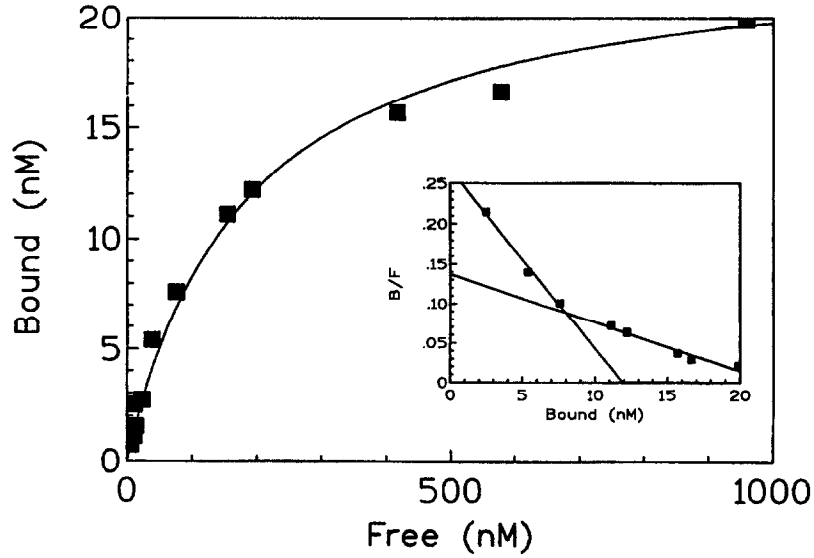

Fig. 1. Saturation isotherm and Scatchard analysis (insert) of ^{3}H-digoxin binding to rat brain Na^+K^+ATPase microsomes.

Table 1. Characteristics of monoclonal digoxin-specific IgG and polyclonal digoxin-specific Fab (Digidot®)

Digoxin-specific antibodies	Ka (M^{-1})	Specific active binding sites (%)
IgG 1C10	$6 \pm 0.7\ 10^9$	76
IgG 6C9	$3.1 \pm 0.5\ 10^8$	62
IgG 9F5	$2.5 \pm 1.2\ 10^7$	75
Fab Digidot®	$8.5 \pm 0.2\ 10^9$	85

unlabelled digoxin (0.12 mM) (n = 6) or by digoxin-specific antibody (n = 7).

Experiment 1: Influence of the antibody affinity. Dissociation of ^{3}H-digoxin from rat brain membrane was initiated by addition of polyclonal digoxin-specific Fab fragments (Digidot®) or monoclonal digoxin-specific IgG 1C10, 6C9, and 9F5 at the concentration of 75 nM of SABS, which represented an antibody binding site: digoxin molar ratio of 1.

Experiment 2: Influence of the antibody:digoxin molar ratio. Dissociation of ^{3}H-digoxin from rat brain membrane was initiated by monoclonal digoxin-specific IgG 1C10, 6C9, and 9F5 at 6 different concentrations, which represented successive antibody binding site: digoxin molar ratios of 0.025 (1.87 nM), 0.05 (3.75 nM), 0.1 (7.5 nM), 0.25 (18.75 nM), 1 (75 nM), 5 (375 nM), and 10 (750 nM).

A nonspecific immunoglobulin (0.4 μM) was added in the control experiments. At sequential times, aliquots (500 μL) of incubation medium were filtered immediately. After three washings, the radioactivity bound to the filters was measured as described above.

Dissociation kinetics of ^{3}H-digoxin from rat brain Na^+K^+ATPase was expressed as the ratio of the concentration of bound ^{3}H-digoxin after addition of digoxin-specific antibody (C) to the concentration of bound ^{3}H-digoxin after the simultaneous addition of nonspecific antibody (Co). (C/Co) was represented as a function of time and analysed by regression analysis (Graphpad®, San Diego, CA) to calculate the first-order dissociation rate constant (k_{-1}). The area above the bound percentage-time curve (AAC) from 0 to 480 minutes was calculated by the linear trapezoidal rule. Statistical analysis was performed using the one-way analysis of variance (ANOVA), followed by a Bonferroni test.

The relationship between the IgG:digoxin molar ratio

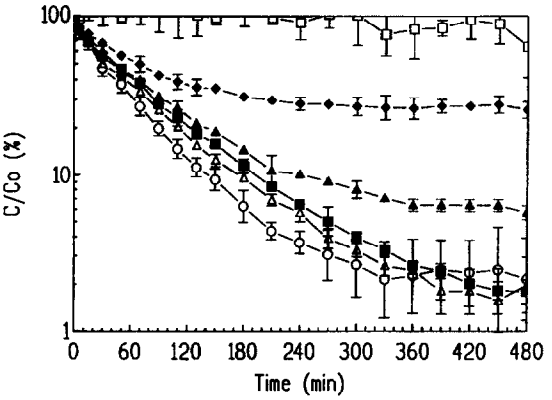

Fig. 2. Dissociation kinetics of ^{3}H-digoxin from rat brain Na^+K^+ATPase microsomes after addition of an antibody: digoxin molar ratio of nonspecific IgG (□), 9F5 IgG (♦), 6C9 IgG (▲), 1C10 IgG (■), Fab Digidot® (△), and a 1600-fold excess of unlabelled digoxin (○).

vs the two dissociation parameters was fitted with a Hill equation by the least square method:

$$AAC = (AAC_{max} \times R^n)/(R_{50}{}^n + R^n);$$

$$C/Co = (C/Co_{max} \times R^n)/(R_{50}{}^n + R^n)$$

where C/Co is the percentage of bound ^{3}H-digoxin at 480 minutes, AAC the area above the curve, n the Hill coefficient, R the IgG:digoxin molar ratio, and R_{50} the IgG:digoxin molar ratio corresponding to half the maximal effect.

RESULTS

Binding equilibrium of ^{3}H-digoxin was achieved within 120 minutes of incubation. The characterization of digoxin binding sites in rat brain microsomes indicates that digoxin interacts with two classes of specific binding sites in brain microsome: high-affinity sites (Kd = 3.4 ± 0.1 10^{-8} M) and low-affinity sites (Kd = 1.8 ± 0.13 10^{-7} M). B_{max} were 31 ± 4 pmol/mg and 87 ± 11 pmol/mg, respectively, for high- and low-affinity binding sites (Fig. 1). The three monoclonal digoxin-specific IgG were characterized by a difference of one log between each of their affinity constants, and the percentage of SABS ranged from 62% to 85% (Table 1).

Table 2. ^{3}H-digoxin dissociation kinetics parameters after addition of an excess of unlabelled digoxin or digoxin-specific antibodies

Compounds	k_{-1} ($10^{-4} \times sec^{-1}$)	AAC (% × min)/100	C/Co(%) (t = 480 min)
Unlabelled digoxin	1.05 ± 0.1	421 ± 3.2	2.7 ± 1.1
1C10 IgG	0.815 ± 0.05*	394.6 ± 14.5	3 ± 0.7
6C9 IgG	0.73 ± 0.14†	379 ± 9.5*	4.3 ± 1.2
9F5 IgG	0.68 ± 0.12‡	279.2 ± 40.5‡	33.3 ± 3.8‡
Fab Digidot®	0.89 ± 0.04	410.1 ± 3.3	3.2 ± 1.2

Values are means ± SD (n = 6). A one-way analysis of variance (ANOVA) followed by a Bonferroni test was used to compare the values obtained for 1C10, 6C9, 9F5 IgG, and Fab Digidot®.

* $P \leq 0.05$ compared to an excess of unlabelled digoxin.

† $P \leq 0.01$ compared to an excess of unlabelled digoxin.

‡ $P \leq 0.001$ compared to an excess of unlabelled digoxin.

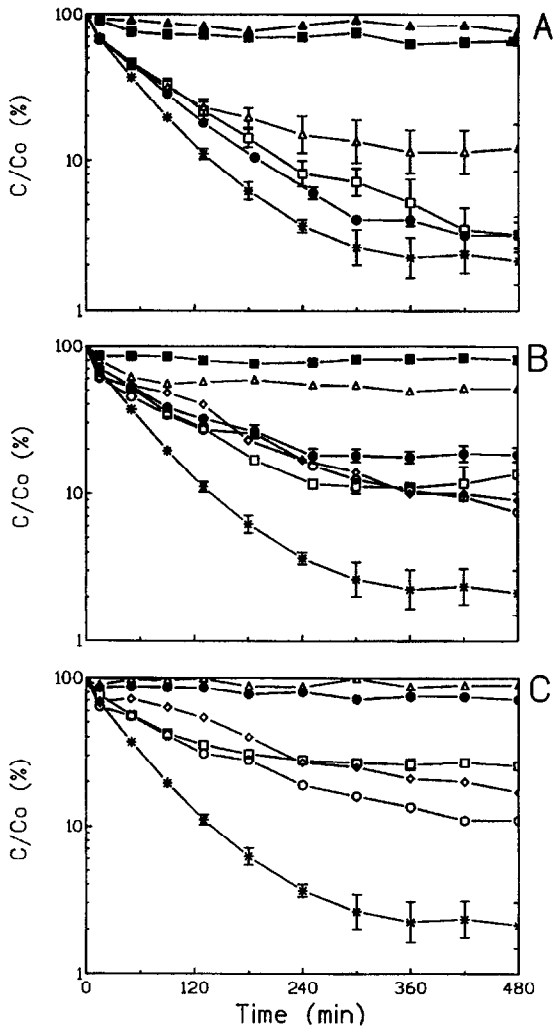

Fig. 3. Mean dissociation kinetics of ^{3}H-digoxin from rat brain Na^+K^+ATPase microsomes after addition of (A) IgG 1C10, (B) 6C9, or (C) 9F5 at an IgG:digoxin molar ratio of 0.025 (▲), 0.05 (■), 0.1 (□), 0.25 (●), 1 (□), 5 (○), and 10 (◇), or an excess of unlabelled digoxin (*).

The dissociation kinetics of ^{3}H-digoxin from rat brain microsomes were determined by adding either a large excess of unlabelled digoxin or different doses of monoclonal digoxin-specific antibody. Addition of nonspecific immunoglobulin in the control experiments did not result in dissociation of bound ^{3}H-digoxin. Control experiments confirmed that bound ^{3}H-digoxin remained stable over 480 minutes (Fig. 2). Addition of a 1600-fold excess of unlabelled digoxin resulted in a quasi-complete dissociation of ^{3}H-digoxin from Na^+K^+ATPase in 330 minutes, with kinetics parameters as shown in Table 2. When a stoichiometrical antibody binding site:digoxin molar ratio of digoxin-specific antibodies was assayed, the rate and extent of ^{3}H-digoxin dissociation from brain Na^+K^+ATPase were very similar to the previous data with the two antibodies whose affinity was 8.5 10^9 M^{-1} (Fab Digidot®) and 6 10^9 M^{-1} (1C10 IgG). Therefore, when the digoxin antibody Ka decreased, the dissociation became progressively less rapid and complete. In the presence of equimolar 6C9 and 9F5 IgG doses, the decrease in AAC was 90% and 66.2%, respectively, of that found in the presence of excess digoxin (Table 2), and dissociation ended at 240 (6C9) and 180 (9F5) minutes (Fig. 2).

By increasing the antibody binding site:digoxin molar ratio from 0.025 to 10, the ^{3}H-digoxin dissociation efficacy appeared dose-dependent (Fig. 3). Plots of the relationship between the dissociation kinetic parameters vs the IgG:digoxin molar ratio were well fitted by Hill functions for the three antibodies (Fig. 4a and b). Maximal ^{3}H-digoxin dissociation was observed at 0.5, 1, and 5 IgG binding site:digoxin ratios for 1C10, 6C9, and 9F5 IgG, respectively. Comparison of R_{50} values showed that 1C10 IgG was 2-fold and 10-fold more potent than 6C9 and 9F5 IgG, respectively (Table 3).

DISCUSSION

The treatment of life-threatening digoxin overdose with digoxin-specific Fab fragments is a successful immunotherapy. The detoxication depends on the stability of the binding of digoxin by the specific Fab fragments in their common body distribution volume (i.e. the extracellular body water space). The consequent reduction

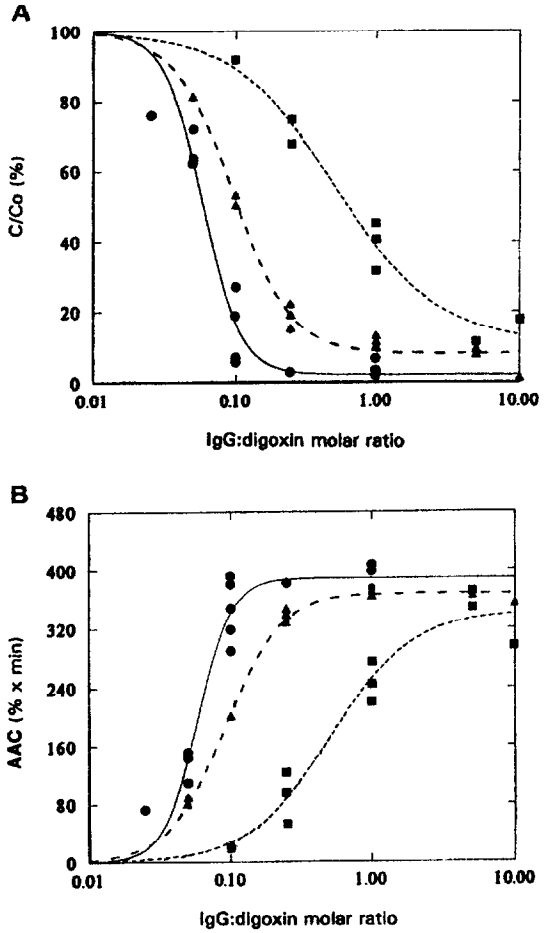

Fig. 4. Relationship between the digoxin dissociation kinetic parameters (A) *C/Co* and (B) AAC (individual data) from rat brain Na^+K^+ATPase microsomes vs the IgG:digoxin molar ratio of 1C10 (●), 6C9 (▲), and 9F5 (■) IgG.

Table 3. Parameters of Hill equation for the relationship between dissociation kinetic parameters and IgG:digoxin molar ratio

		Digoxin-specific IgG	
Parameters	1C10	6C9	9F5
AAC			
AAC_{max}(% × min)/100	389	368	344
R_{50}	0.058	0.090	0.51
n	3.66	2.20	1.50
C/CO			
C/CO_{max}(%)	2.3	8.2	11.2
R_{50}	0.058	0.095	0.51
n	3.22	2.08	1.21

AAC_{max} represents the maximal value of the area above the curve, R_{50} the IgG: digoxin molar ratio corresponding to half the maximal effect, C/CO_{max} the maximal percentage of bound ^{3}Hdigoxin at 480 minutes, and *n* the Hill coefficient.

of free digoxin in the extracellular space produces a concentration gradient that promotes release of digoxin from its specific binding sites on the Na^+K^+ATPase [11]. This mechanism that occurs *in vivo* can be reproduced in *in vitro* models. The effect of digoxin-specific antibody on digoxin inhibition of myocardial Na^+K^+ATPase microsomes has been studied by recording ATPase enzyme activity [12] and by the reversion of digoxin binding in isolated human erythrocytes [13]. However, in both studies, only one digoxin-specific antibody was used. In the present study, three monoclonal digoxin-specific antibodies, differing in their affinity constants, which spanned a 3-log range, were compared with an antibody in current clinical use: polyclonal digoxin-specific Fab (Digidot®), which can be considered as reference. The three digoxin-specific monoclonal IgG were not cleaved into their Fab fragments because early *in vitro* experiments showed no difference in efficacy between Fab and IgG [14]. Moreover, as the aim of the study was to predict the efficacy of the antibodies rapidly, the papain cleavage step to yield the Fab fragments was dropped to keep time loss to a minimum. The rat is relatively insensitive to the cardiac effect of digitalis, because most rat Na^+K^+ATPase molecules outside the central nervous system contain the 1 isoform [15], which has a relatively low affinity for digoxin. Despite this, rat brain microsomes were chosen for the development of the *in vitro* model because the dissociation constants of rat brain ATPase for digitalis ($Kd_1 = 1.8\ 10^{-7}$ M and $Kd_2 = 3.4\ 10^{-8}$ M) were similar to those observed in dog heart ($Kd = 10^{-7}$ M) [16], human heart ($Kd_1 = 1.7\ 10^{-7}$ M and $Kd_2 = 2\ 10^{-8}$ M) [17], and beef brain ($Kd = 1.7\ 10^{-7}$ M) [17]. As antibody potency is in part related to its ability to bind digoxin with an affinity constant higher than that of digoxin for the cardiac Na^+K^+ATPase, the rat brain microsome preparation, which has digoxin binding properties to Na^+K^+ATPase similar to those of hearts from different species, was used. Digidot® Fab, 1C10, and 6C9 IgG affinity constants were equal to or higher than those of digoxin for the high affinity Na^+K^+ATPase binding sites, whereas 9F5 affinity was lower.

The present study clearly indicates that following the stoichiometrical rule usually recommended for the clinical use of digoxin-specific Fab fragment, only Mabs with high affinity constants are able to dissociate digoxin from the Na^+K^+ATPase binding sites as efficiently (i.e. almost completely) as the reference antibody Digidot®. Usually, the lower the antibody affinity constant, the higher the dissociation rate of the antigen from the antibody binding sites [18]. As the digoxin-antibody complex is not stable, the released digoxin molecules can again bind the Na^+K^+ATPase sites, limiting the rate and extent of the ^{3}H-digoxin dissociation from the enzyme. In a previous report, we showed that a monoclonal digitoxin-specific Fab fragment with a Ka of 10^7 M^{-1} for digitoxin was not able to protect rabbits from the clinical manifestations of digitalis toxicity following a stoichiometrical dose ratio to digitoxin [19]. In contrast, Digidot®, which had a 2-log higher Ka, protected rabbits efficaciously from cardiac toxicity. In the same study we demonstrated that the monoclonal fragment was able to protect rabbits from toxicity as efficaciously as Digidot® if twice the initial amount of antibody was administered. The compensatory effect of increasing the antibody dose for antibodies with a low-affinity constant is well demonstrated by the present study using the *in vitro* model. Moreover, the relationship among the abilities of the three monoclonal digoxin-specific antibodies to dissociate digoxin from Na^+K^+ATPase and the amount of antibody is well fitted by the E_{max} sigmoid function. The modeling allows differences among the three antibodies to be established. If the Ka is high, (a) increasing values are observed for the Hill coefficient relative to the slope of the relationship, indicating that a small variation of the dose with the high-affinity antibody improves the dissociation of digoxin from Na^+K^+ATPase more than an antibody of lower Ka; (b) increasing maximal effect values in drug dissociation from the binding sites are also observed; and (c) decreasing R_{50} values characterize the efficacy of the antibodies, showing that the 1C10 antibody was approximately 2- and 10-fold more efficacious than 6C9 and 9F5, respectively. These results are extremely helpful for the selection of an efficacious antibody among several entities produced using hybridoma technology.

Production of monoclonal antibody to haptens raises the major problem of the wide range in their affinity constants. Chappey *et al.* [20] have recently reported that the affinity of 62 published digoxin-specific Mab varies from 10^6 M^{-1} to 10^{12} M^{-1}, and that only 8 Mabs to digoxin exhibited affinity constants equivalent to or higher than that of the polyclonal Digidot®. This large variability in the affinity constant required early selection of an appropriate antibody affinity in the preclinical

steps of pharmaceutical development. The *in vitro* model seems the more efficacious predictive assay, as it needs very small amounts of antibody and can be performed more rapidly than *in vivo* models. Moreover, the fact that a Hill function links the antibody dose to the digoxin dissociation parameters allows determination of comparative quantitative parameters that facilitate antibody screening.

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