Monoclonal Digoxin-Specific Antibodies Induce Dose-and Affinity-Dependent Plasma Digoxin Redistribution in Rats

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The effect of three monoclonal digoxin-specific antibodies on total and free digoxin plasma disposition was studied in rats in order to determine the role of affinity constant (Ka) and dose. Thirty minutes after digoxin infusion, administration of a stoichiometrical dose of the 1CIO, 6C9 and 9F5 IgG (Ka = 6 10^9, 3.1 10^8 and 2.5 10^7 M^{-1}, respectively) resulted in a plasma digoxin increase linearly related to Ka. The mean free plasma digoxin was 0.6±0.4, 7.8±3.3 and 43±22 % respectively after 1C10, 6C9, and 9F5 IgG infusion in comparison to 70±9% in the control group. When the IgG:digoxin ratio increased from 1 to 5, plasma digoxin Cmax and AUC_T also increased as a function of both affinity (Ka) and dose (N), but not linearly. The product of NKa defined an immunoreactivity factor that was well fitted to the digoxin redistribution parameters (Cmax and AUC_T) by a Hill equation.

KEY WORDS: digoxin; monoclonal antibodies; redistribution; immunoreactivity.

INTRODUCTION

The therapeutic use of drug-specific immunoglobulin G (IgG) or Fab fragments represents a powerful tool for the reversal of drug toxicity. Polyclonal digoxin-specific Fab fragments are effective in the treatment of acute cardiac glycoside poisoning or overdose (1,2). Antibody therapy appears to reverse toxicity by binding extracellular digoxin which also facilitates the release of membrane Na^+K^+ATPase-bound digoxin (3). From a pharmacokinetic point of view, administration of specific antibodies results in sequestration of unbound drug and drug redistribution from tissues to the antibody distribution space. The ability of the antibody to alter the drug pharmacokinetics is related to the dose of antibody and the stability of the drug-antibody complex, which depends on the antibody affinity constant. Infusion of specific IgG or Fab fragments with affinity constants ranging from 10^8 to 10^{11} M^{-1} produces an increase in total drug plasma concentration, i.e., plasma drug redistribution, for digoxin (4,5), desipramine (6), phencyclidine (7) and colchicine (8). Pentel *et al.*, (9) were the first to demonstrate that the increase in total plasma desipramine concentrations was related to the antibody dose in rats. However, no comparative study concerning the influence of antibody affinity and dose on the efficiency of drug redistribution has been conducted. The aim of this study was to evaluate the influence of antibody affinity constant and dose on plasma digoxin redistribution in rats using three monoclonal digoxin-specific IgG with increasing affinity constants.

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MATERIALS AND METHODS

(^{3}H)Digoxin (12α^{3}H(N), 18.3 Ci/mmol) was purchased from New England Nuclear (Du Pont de Nemours, Les Ulis, France). Pentobarbital sodium (60 mg/ml) was from Clin-Midy (St Jean de la Ruelle, France). Polyclonal colchicine-specific IgG1 used in controls have been developed in our laboratory (10). Pico-Fluor 40 scintillation liquid was from Packard (Rungis, France). Radioactivity was measured in a Tri-Carb model 4530 liquid scintillation spectrophotometer.

Preparation and purification of digoxin-specific fragments.

Monoclonal digoxin-specific IgG were prepared as described by Wahyono *et al.*, (11) by somatic cell fusion and raised in ascites fluid from BALB/c mice. The monoclonal IgG_1 used in this study were 1C10, 6C9 and 9F5. The affinity constant (Ka) and concentration of specific active binding sites (SABS) of the three IgGs were determined by saturation analysis as previously described (12). The affinity constants of 1C10, 6C9, 9F5 IgG were, respectively, 6×10^9, 3.1×10^8 and 2.5×10^7 M^{-1}. The concentrations of SABS for 1C10, 6C9 and 9F5 IgG were, 0.61, 0.7 and 0.72 mg/ml, respectively, corresponding to 76, 62 and 75% of total IgG.

The active site concentration of IgG was calculated assuming a molecular weight of 150 Kd and taking into account the percentage of SABS in each antibody preparation.

Experimental model.

Male Sprague-Dawley rats (290 ± 20 g) (Iffa Credo, Lyon) with free access to food and water before the experiments were used. They were anaesthetized with pentobarbital sodium (60gm/kg, i.p.), and an additional dose (5mg/kg) was given when necessary. Femoral vein and artery were cannulated with PE-58 tubing (Biotrol Paris, France) for digoxin administration and blood sampling (0.4 ml), respectively. To compensated for the blood drawn, 0.4 ml of blood from rat donor was infused via the femoral vein. Digoxin was infused after a control period of 10 min after cannulation.

Both digoxin and antibody solutions were prepared in physiologic saline and administered in a volume of 3 ml/kg body weight. In the two protocols, rats were injected with 0.62 μg/kg of ^{3}H-digoxin. A control group (n = 5) receiving colchicine-specific IgG_1 (0.2 mg/kg) was included in each protocol.

Protocol 1. *Influence of affinity*. Thirty minutes after digoxin infusion, three groups of rats (n = 5) received 0.12 mg/kg of active binding sites, 1C10, 6C9, or 9F5 IgG. Based on the drug-Fab interaction (1 mol Fab for 1 mol drug), the

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amount of antibody represented a dose stoichiometrically equivalent to the amount of digoxin infused.

Protocol 2. Influence of the dose. Thirty minutes after digoxin infusion, 9 groups of rats (n=3) received three different doses of 1C10, 6C9 or 9F5 IgG, which represented molar IgG:digoxin ratios of 5 (0.6 mg/kg), 1 (0.12 mg/kg) and 0.2 (0.024 mg/kg), respectively.

Analytical methods.

50 µl aliquots (in duplicate) of plasma samples were mixed with 3 ml Pico-fluor 40 scintillation liquid in a minivial and radioactivity was measured by liquid scintillation counting using automatic external standardization for the quench correction. Total radioactivity, which may included unchanged digoxin plus metabolites, was expressed as digoxin equivalent.

The unbound fraction of digoxin was determined by equilibrium dialysis. Plasma was dialyzed against isotonic phosphate buffer in a system consisting of two 0.2 ml Teflon dialysis cells (Dianorm, B. Braun ScienceTec, Les Ulis, France) separated by a semipermeable membrane (M_r cutoff 12 000, Union Carbide, Chicago, IL). Dialysis was carried out at 37°C over a four hour period. The cells were rotated at 20 rotations per min. An aliquot of each dialysate was immediately assayed. Preliminary experiments established that (1) the equilibrium was achieved within 4 hours, (2) digoxin did not bind to the dialysis system and (3) no volume shift occurred during equilibrium dialysis.

Pharmacokinetic analysis.

Maximum total digoxin concentration (Cmax), time to reach Cmax (Tmax), minimum free digoxin (Cmin) and time to reach Cmin (Tmin) were from experimentally observed values. The area under the plasma concentration-time curve of total (AUC_T) and free (AUC_F) digoxin was calculated from 30 to 140 min by the trapezoidal rule. Mean free plasma digoxin percentage (fu) was calculated as following: $fu = AUC_F/AUC_T \times 100$.

Each pharmacokinetic parameter was plotted against the IgG dose or Log Ka by linear regression using InPlot 4 (GraphPAD, San Diego, CA).

Total digoxin AUC_T or Cmax plotted versus an immunoreactivity factor expressed as NKa/V, where N is the IgG dose infused and V the mean distribution volume of IgG in rats (35 ml/kg) (5). The experimental data were fitted using the MKModel^R program (Biosoft, Cambridge, UK) and Hill equation as follows:

$$Cmax = Cmax_{max} \times (NKa/V)^{\delta}/(Cmax\ 50^{\delta} + (NKa/V)^{\delta})$$

$$AUC_T = AUC_{Tmax} \times (NKa/V)^{\delta}/(AUC_T50^{\delta} + (NKa/V)^{\delta})$$

where δ is the Hill coefficient, Cmax50 and AUC_T50 are the (NKa/V) values corresponding to 50% of $Cmax_{max}$ and AUC_{Tmax} respectively.

All experimental data are expressed as mean ± SD. Statistical analysis was performed using Student's t-test. Significance was set at $p \leq 0.05$.

RESULTS

Influence of antibody affinity and dose on total plasma digoxin redistribution. While colchicine-specific antibody did not alter the plasma digoxin disposition, infusion of digoxin-specific 1C10, 6C9 and 9F5 IgG resulted in affinity- and dose-related increases in total plasma digoxin concentrations (fig 1 and 2). Table 1 summarizes the total digoxin pharmacokinetic parameters (AUC_T, Cmax, Cmin and Tmin) in the IgG-infused groups. AUC_T and fu were, respectively, 15±0.9 nMxmin and 70±9% for the control group. At a IgG stoichiometrical dose, a linear relationship was found between Log Ka and Cmax (r=0.99), AUC_T (r=0.99) and Tmax (r=0.99) within the IgG affinity range (2.5×10^7 to 6×10^9 M^{-1}) (fig. 3B). Therefore, at IgG:digoxin ratios of 5 and 0.2, digoxin redistribution increased but not linearly. No statistical differences in Cmax or AUC_T were observed between the two highest affinity 6C9 and 1C10 IgG at an IgG:digoxin ratio of 5 ($p>0.05$) (table 1 and fig. 3C). In the same manner, only a slight difference in Cmax and AUC_T was observed between the two lowest affinity 9F5 and 6C9 IgG at a IgG:digoxin ratio of 0.2 (fig. 3A). As the digoxin redistribution parameters depended both on the antibody dose and affinity, the intensity of total digoxin redistribution can be related to both antibody characteristics by the Hill equation (fig. 4) where:

$$Cmax = \frac{8.09 \times (NKa/V)^{0.81}}{11.63^{0.81} + (NKa/V)^{0.81}}$$

and $$AUC_T = \frac{7.49 \times (NKa/V)^{0.99}}{12.5^{0.99} + (NKa/V)^{0.99}}$$

Influence of antibody affinity and dose on free plasma digoxin. As early as 3 min after IgG infusion, free digoxin plasma concentration decreased and remained constant over 140 min (fig. 1). Free digoxin Cmin and Tmin were inversely related to Log Ka. At a IgG:digoxin ratio of 1, the mean free plasma digoxin was 0.6±0.4, 7.8±3.3 and 43±22% for 1C10, 6C9 and 9F5 IgG, respectively, compared to 70±9% in the control group (table 1). Moreover, a 5-fold increase in IgG dose produced a significant reduction of the mean free digoxin percentage for 6C9 (0.74±0.06%) and 9F5 (13.4±1.5%) IgG but only a slight decrease for 1C10 IgG (0.3±0.04%) (table 1).

DISCUSSION

Reversal of digitalis toxicity by the use of polyclonal specific antibodies has been successfully demonstrated in both animals (13, 14) and humans (1, 2). The binding of digoxin to antibody reduces the extracellular free pool of digoxin and produces a concentration gradient that promotes dissociation of digoxin from its Na^+K^+ATPase binding sites. From a pharmacokinetic point of view, the binding of digoxin by specific antibodies results in an increase in total digoxin and a decrease in free digoxin in the vascular compartment and consequently in the reduction of its distribution volume (15). This plasma drug redistribution after IgG or Fab infusion has been previously reported with phencyclidine (7) in dogs, digoxin (5, 16), desipramine (6) and colchicine (8) in rats and rabbits. The same phenomena are

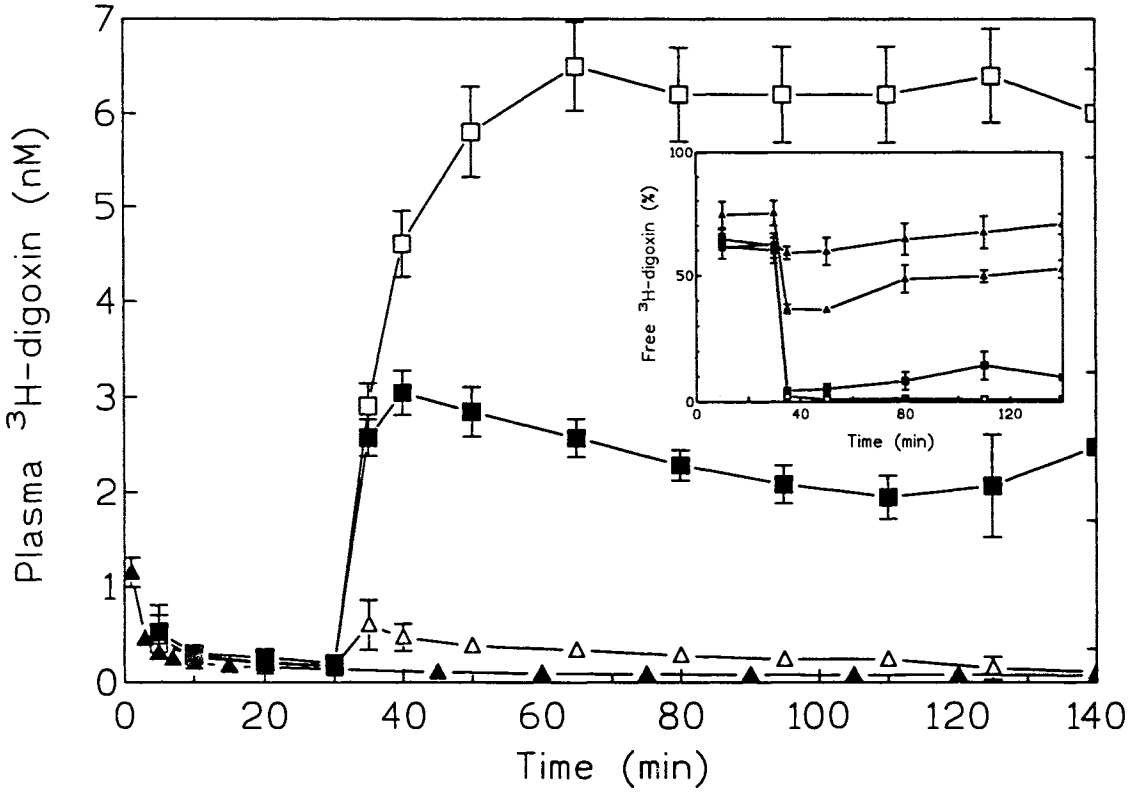

Fig. 1. Total digoxin plasma concentration-time profiles in control with colchicine-specific IgG (▲), 9F5 IgG- (△), 6C9 IgG- (■) and 1C10-IgG (□) infused rats. Digoxin-specific IgG were administered 30 min post-digoxin infusion at a IgG:digoxin molar ratio of 1. Insert: Plasma digoxin free fraction in the same groups (mean±SD, n=5).

currently observed following Fab infusion in the treatment of cardiac glycoside intoxication in humans (1, 17). This redistribution effect may depend on two factors: the dose ratio of digoxin to antibody and the affinity constant of the antibody that defines the stability of the neutralized complex. The administration of low affinity monoclonal digitoxin-specific Fab fragments ($Ka = 10^7 M^{-1}$) at a stoichiometrical dose ratio to digitoxin did not reverse digitoxin toxicity in rabbits, but increasing the Fab dose resulted in the reduction of digitoxin toxicity (18). In contrast, monoclonal digoxin-specific IgG and Fab fragments of high affinity ($Ka = 5\times10^9 M^{-1}$) were effective at a stoichiometrical dose in the reversal of advanced digitalis intoxication in guinea-pigs (19). Pentel *et al.*, (9) have shown that plasma redistribution of desipramine in rats was related to the dose of monoclonal specific IgG. However, the *in vivo* influence of both antibody affinity constant and dose has not been previously investigated. Alteration of plasma digoxin disposition by antibodies or Fab frag-

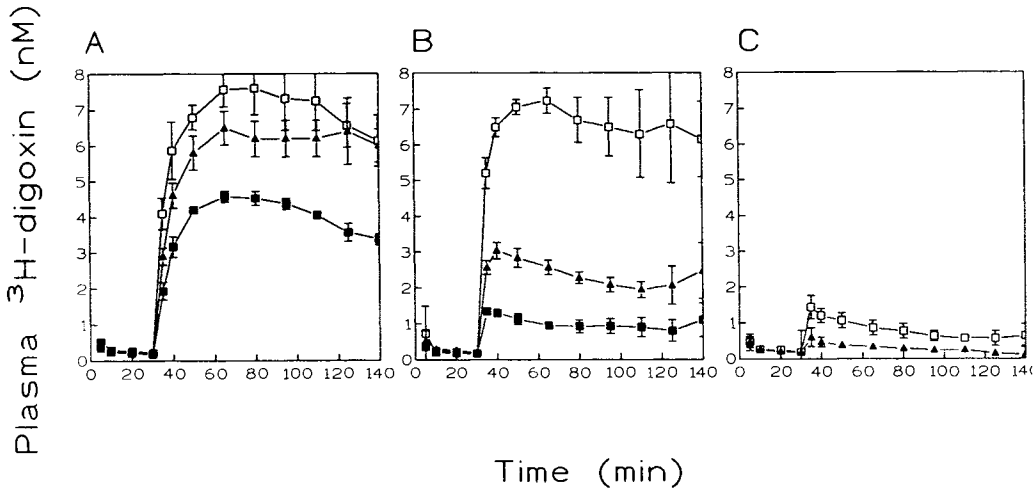

Fig. 2. Total digoxin plasma concentration-time profiles in 1C10 (A), 6C9 (B) and 9F5 (C) IgG-infused rats at a IgG:digoxin molar ratio of 0.2 (■), 1 (▲) and 5 (□). Digoxin-specific IgG were administered 30 min post-digoxin infusion (mean ± SD, n=3 to 5).

TABLE 1. Pharmacokinetic Parameters of Total and Free Plasma Digoxin in the IgG-Infused Groups

Anti-Digoxin	1C10 (6×10^9 M^{-1})			6C9 (3.1×10^8 M^{-1})			9F5 (2.5×10^7 M^{-1})		
Total Digoxin	AUC_T (nM × min)	Cmax (nM)	Tmax (min)	AUC_T (nM × min)	Cmax (nM)	Tmax (min)	AUC_T (nM × min)	Cmax (nM)	Tmax (min)
IgG:digoxin ratio									
5	742 ± 69	7.7 ± 0.5	75 ± 8.7	715 ± 75	7.5 ± 0.8	55 ± 8.6	85 ± 23	1.4 ± 0.2	35 ± 0
1	642 ± 55	6.7 ± 0.6	67.5 ± 6.1	253 ± 32	3 ± 0.3	40 ± 0	36 ± 13	0.8 ± 0.3	33 ± 2
0.2	453 ± 56	5.2 ± 0.9	80 ± 15	106 ± 21	1.3 ± 0.4	36.7 ± 2.9	15 ± 0*	0.4 ± .1	35 ± 0
Free Digoxin	fu (%)	Cmin (μM)	Tmin (min)	fu (%)	Cmin (μM)	Tmin (min)	fu (%)	Cmin (μM)	Tmin (min)
IgG digoxin ratio									
5	0.3 ± 0.04	16 ± 5	35 ± 0	0.74 ± 0.06	46 ± 6	35 ± 0	13.4 ± 1.5	86 ± 20	100 ± 17
1	0.6 ± 0.4	35 ± 17	40 ± 8	7.8 ± 3.3	100 ± 70	72 ± 29	43 ± 22	90 ± 40	92 ± 27
0.2	1.1 ± 0.3	20 ± 0	35 ± 0	13.1 ± 2.9	120 ± 3	35 ± 0	67 ± 5*	100 ± 50	130 ± 9

* not significant compared to the control group (AUC_T = 15 ± 0.9 nM min and fu = 70 ± 0.9%).

ments has been demonstrated to reflect the detoxification process (15, 20). We therefore considered that a pharmacokinetic approach would be of interest using a set of monoclonal digoxin-specific antibodies with different affinity constants to investigate the combined effects of both dose and affinity. Despite the resistance of the rat to digitalis toxicity, rat was selected as a convenient animal model frequently used by pharmaceutical manufacturer in preclinical drug development. Moreover, digoxin disposition in rats is characterized by a high (4.2 L/kg) and rapid distribution which is almost complete in 30 min (21). Thirty minutes after digoxin infusion, administration of a stoichiometrical dose of three monoclonal digoxin-specific IgG with Ka ranging from 2×10^7 to 6×10^9 M^{-1} resulted in an affinity-dependent increase in total digoxin concentrations. Plasma digoxin redistribution was linearly related to the IgG affinity constant. A high affinity constant ($6\times10^9 M^{-1}$ for 1C10) resulted in a maximal drug plasma redistribution. In contrast, a lower affinity constant ($2.5\times10^7 M^{-1}$ for 9F5), which is only 10-20 fold higher than that of rat Na^+K^+ATPase for digoxin (2.7×10^5 to $3.7\times10^6 M^{-1}$ (22)), did not result in a concentration gradient large enough to redistribute digoxin. Moreover, *in vivo* antigen-antibody affinity constant for desipramine-specific antibody was found two orders of magnitude less than the value obtained *in vitro* (23). We cannot preclude that the *in vivo* affinity constants of the three monoclonal digoxin-specific IgG were not lower than those we used to establish the relationships between digoxin redistribution and antibody affinity. This phenomenon could explain the weakness of 9F5 IgG to redistribute digoxin.

The extent of the digoxin redistribution effect was also kinetically assessed by measurement of the free drug. After administration of nonspecific IgG (control group), the mean free digoxin was 70±9%. This value is similar to the unbound digoxin in human plasma (70 %) (24). Infusion of digoxin-specific IgG resulted in a affinity-related decrease in Cmin. A low mean unbound plasma digoxin (0.6±0.04%) level was observed with the highest affinity antibody (1C10) which expressed the maximal digoxin redistribution capacity. The low efficacy of 9F5, which has 2 Log less affinity than 1C10, was confirmed by the mean 43±22% unbound plasma digoxin percentage. Moreover, Tmin was inversely related to the IgG affinity constant. Both effects are probably due to the higher dissociation rate 9F5 which was 25-fold higher than for 1C10 (unpublished observations). Thus, binding equilibrium was reached more rapidly and the immunocomplex stability was higher with 1C10 IgG.

Based on the drug-Fab interaction (1 mole Fab for 1 mole drug), a stoichiometrical dose of digoxin-specific antiboby or Fab fragments versus digoxin is usually proposed in most experimental studies (19) and for human treatment (2). However, no information is available on the relationship be-

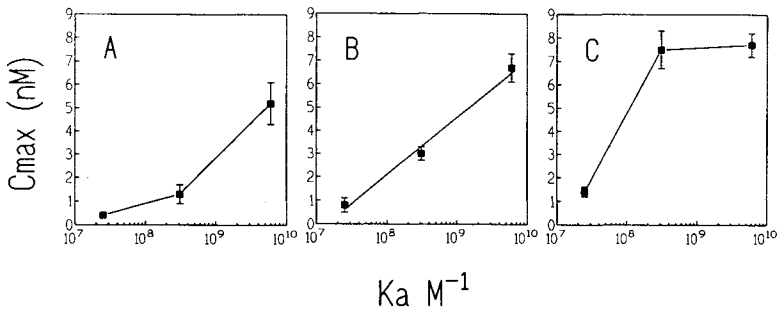

Fig. 3. Relationship between plasma digoxin Cmax and IgG 1C10, 6C9 and 9F5 affinity constant (Ka = 6 10^9, 3.1 10^8, 2.5 10^7 M^{-1} respectively) at IgG:digoxin ratio of 0.2 (A), 1 (B) and 5 (C).

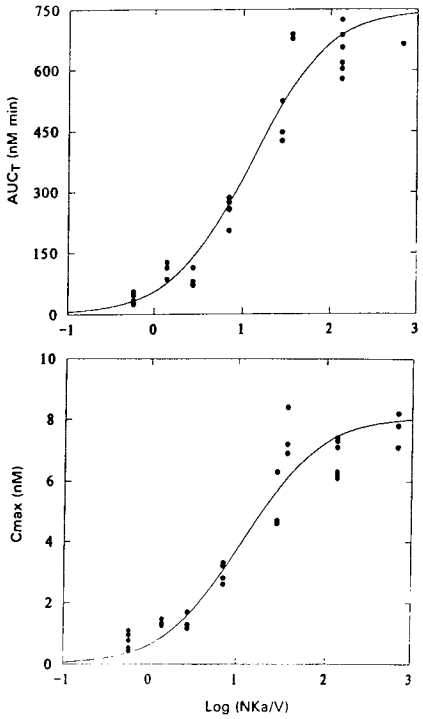

Fig. 4. Sigmoidal relationship between the immunoreactivity factor (nKa/V) and plasma digoxin AUC_T or Cmax. Each point corresponds to kinetics parameter of one rat.

tween the IgG dose and the redistribution efficacy according to the antibody affinity constant. The administration of a 5-fold higher dose of 1C10 IgG resulted in a 1.2-fold increase in Cmax (fig. 2). This small difference is in accordance with the low percentage of mean free plasma digoxin (0.6 and 0.3% at IgG:digoxin ratio of 1 and 5, respectively). In contrast, a 5-fold higher dose of 6C9 implies a 2.5-fold increase in Cmax to a value similar to that observed with 1C10 IgG, in accordance with the significative reduction of mean free digoxin (8 to 0.8%). These results show that the low affinity constant (Ka) of 6C9 could be compensated for by the increase, over the stoichiometry, in the number of binding sites. This effect is consistent with the redistribution of propranolol and desipramine following infusion of high α-1-acid glycoprotein doses in rats despite the low affinity (5×10^4 to 10^6 M^{-1}) of this plasma protein (25, 26). So, we applied this concept *in vivo* to describe possible relationships between kinetic parameters demonstrative of the IgG redistribution effect (AUC_T or Cmax) and the antibody properties (dose and affinity) which were combined to define an immunoreactivity factor. As most of the pharmacokinetic-pharmacodynamic relationships are well described by the sigmoid function which was originally proposed by Hill (27), the relationship between digoxin redistribution parameters and the antibody immunoreactivity factor was well fitted by the sigmoidal function. This relationship confirmed the existence of a maximal effect that cannot be exceeded despite the increase in antibody dose or affinity constant. The maximal effect expressed in terms of redistributed digoxin corresponds to an undetectable free digoxin. These observations are of prime importance in view of the current concept of stoichiometry in immunotherapy. In fact, the antibody dose is highly dependent on affinity. The higher the affinity, the smaller the dose and, inversely, a low affinity could be compensated for by a dose higher than the stoichiometrical dose.

In conclusion, our data clearly demonstrate that both affinity and dose define an immunoreactivity factor that can be considered to optimize antibody efficacy in immunotherapy drug intoxication.

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