Analytical Control Procedures of Immunoreactivity for IgG and Fab Fragments Specific to Haptens

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This study investigates immunoreactivity control procedures, i.e., specificity, affinity constant (K_a), and specific active binding sites (SABS), for polyclonal anticolchicine, monoclonal antidigitoxin IgG and Fab fragments, and antidigoxin Fab fragments (Digidot). Preliminary control procedures for IgG and Fab fragment purity indicated that all reagents were immunologically pure. All IgG and Fab fragments exhibited similar cross-reactivity and K_a. No decrease in percentage of Fab fragment SABS was observed after papain cleavage of anticolchicine and antidigitoxin IgG. Nevertheless, only 4.3 ± 1.2% of nonimmunopurified anticolchicine polyclonal Fab fragments and 76.2 ± 2.3 to 88.7 ± 2.5% of different batches of immunopurified anti-digoxin Fab (Digidot) were active, the latter percentage being in the range of the 85% specified by the manufacturer. Only 58 ± 3% of digitoxin-specific monoclonal IgG was active and 67 ± 7% of its Fab fragments. Results show the importance of determining the ratio of SABS to presumed total specific binding sites for pharmaceutical monoclonal and polyclonal antibody preparations against haptens.

KEY WORDS: immunoreactivity; IgG and Fab fragments; colchicine; digoxin; digitoxin.

INTRODUCTION

The utility of antibodies and their Fab fragments derived from polyclonal or monoclonal IgG antibodies in immunotherapeutics and immunodiagnostics in humans is well established (1,2). For example, polyclonal antidigoxin Fab fragments have been a recent successful addition to the management of digitalis intoxication in humans (3). Two commercial sheep antidigoxin Fab fragment reagents are currently available: Digidot (Boehringer, GmbH Mannheim, Germany) and Digibind (Wellcome, Burroughs Wellcome, Research Triangle Park, NC). The efficacy of detoxification depends mainly on three antibody characteristics: (i) their ability to recognize the toxic substances (toxin plus active metabolites), which defines the specificity; (ii) their ability to form stable Fab fragment–toxin complexes, which is dependent on affinity; and (iii) the number of specific active binding sites (SABS) present in the final pharmaceutical reagent necessary to neutralize a stoichiometrical amount of ingested toxin (4). In fact, specificity, affinity, and SABS concentration each represent a component of the immunoreactivity or the potency of antibody reagents. The preservation of these qualities for both total IgG and Fab fragments is crucial during the different steps of IgG production and purification. Moreover, these qualities have to be taken into account in deciding shelf-life and play an important role in the verification of reagent stability. While analytical techniques for the study of polyclonal and monoclonal antibody stability have been established as required by the United States and the European regulatory authorities, little information on adequate procedures for assessing immunoreactivity is available (5). The aim of this study was to investigate the specificity, affinity constant, and SABS concentration for polyclonal anticolchicine IgG and Fab fragments, monoclonal antidigitoxin IgG and Fab fragments, and polyclonal antidigoxin Fab fragments (Digidot).

MATERIALS AND METHODS

Drugs and Chemicals

All radioactive chemicals were purchased from New England Nuclear (Dupont de Nemours, Paris, France): ^{3}H-colchicine (sp act, 39.4 Ci/mmol), ^{3}H-digoxin (sp act, 26.4 Ci/mmol), and ^{3}H-digitoxin (sp act, 15.8 Ci/mmol). Colchicine (MW = 399) was from Fluka (Paris, France); 2-demethylcolchicine, *N*-deacetylcolchicine, and colchiceine were from Laboratoires Roussel (Paris, France). Digoxin (MW = 780.9), digitoxin (MW = 764.9), and ouabain were from Laboratoire Nativelle (Paris, France). Aqualyte from Baker (Deventer, Holland) was used for liquid scintillation counting. Bovine serum albumin (BSA) was from Boehringer (Mannheim GmbH, Germany). Cellulose membranes for dialysis were from Union Carbide (Chicago, IL). All other reagents of analytical grade were from Merck (Nogent sur Marne, France).

Preparation of IgG and Fab Fragments

Sheep antidigoxin polyclonal Fab fragments (Digidot) (batches 738922-04, 741904-04, and 745789-05) were purchased from Boehringer (Mannheim GmbH, Germany). The Fab fragments were produced by papain digestion of total IgG and purified by immunoabsorption as previously described by Roesh and Lenz (6).

The monoclonal antidigitoxin and polyclonal anticolchicine IgG and corresponding Fab fragments were produced in our laboratory. Briefly, monoclonal antidigitoxin IgG's were obtained by somatic cell fusion and produced in ascites fluid from BalbC mice as described by Edelmann *et al.* (7). The IgG were purified by Q-Sepharose fast-flow anion-exchange chromatography (Pharmacia, Les Ulis, France). The polyclonal anticolchicine antisera were collected from three immunized Alpine goats by a colchicine conjugate as described previously by Pontikis *et al.* (8). IgG were isolated from the alcohol fraction II according to the method of Cohn *et al.* (9).

Monoclonal antidigitoxin Fab fragments and polyclonal anticolchicine Fab fragments were prepared from the puri-

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fied IgG fraction by papain enzymatic hydrolysis according to the method of Porter *et al.* (10). Purification of antidigitoxin and anticolchicine Fab fragments from the mixture containing undigested IgG, Fc, and Fab fragments was carried out by ion-exchange chromatography on Q-Sepharose (Pharmacia, Les Ulis, France) and by DEA-Spherodex (IBF, Villeneuve la Garenne, France), respectively.

Control Procedures of Purity

Purity of antidigitoxin and anticolchicine IgG was checked by cellulose acetate electrophoresis, immunoelectrophoresis, and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using the Phastsystem method (Pharmacia, Les Ulis, France). Purity of antidigitoxin and anticolchicine Fab fragments was ensured by gel filtration chromatography respectively on Superose 12 Gel (Pharmacia, Les Ulis, France) and Gel ACA 44 (IBF, Villeneuve la Garenne, France) and by SDS-PAGE. The presence of papain in the final preparation was determined by the method of Ouchterlony (11). Purity of antidigoxin Fab fragments (Digidot) was checked by SDS-PAGE using the Phastsystem method. The concentration of antidigitoxin, anticolchicine IgG and Fab fragments in the final solution was determined by the method of Lowry *et al.* (12) or Kjeldahl (13). The molar concentration of total binding sites present in the preparation was expressed as Fab equivalents, assuming an average molecular weight of 150 kD for IgG and 50 kD for Fab fragments.

Specificity

The specificity of IgG and their corresponding Fab fragments was determined by testing the cross-reactivity of metabolites and structural analogues of colchicine, digitoxin, and digoxin. The cross-reactivity was expressed as the percentage ratio of colchicine, digitoxin, and digoxin concentration to the cross-reacting substance concentration at the 50% inhibition of maximum binding using radioimmunoassay (RIA) procedures described previously for colchicine (14), digitoxin, and digoxin (15). Precipitation of the IgG or Fab complexes at equilibrium was achieved by using, respectively, 50 and 60% ammonium sulfate saturation.

Binding Assay

The intrinsic affinity constants (K_a) of IgG–antigen and Fab–antigen complexes were determined from saturation binding experiments by equilibrium dialysis. The system consisted of two 1-ml Teflon dialysis cells (Dianorm, B. Braun ScienceTec, Les Ulis, France) separated by a cellulose membrane (M_r cutoff, 6000). One milliliter of ^{3}H-ligand (10^{-8} to 10^{-10} *M*) was dialyzed against the same volume of a constant concentration of IgG or Fab fragments (from 0.8 to 21 n*M* according to the different IgG or Fab fragments). Crystalline BSA (1 mg/ml) was added to the saline phosphate buffer (0.2 *M* NaH_2PO_4, 0.2 *M* Na_2HPO_4, 3 *M* NaCl) to inhibit nonspecific binding of antibody to Teflon according to the method of Smith *et al.* (16). Dialysis was carried out at 37°C with cells gently rotated overnight. Equilibrium was defined as the presence of equal radioactivity in each half of the cell in the absence of antibody. Total and free ^{3}H-ligand concentrations were measured by liquid scintillation counting (Packard TRICARB 4530) after the addition of 3 ml of scintillation liquid. Mean values (±SE) were obtained from three experiments. Preliminary experiments established that colchicine, digitoxin, and digoxin did not bind to the dialysis system. Nonspecific binding of tritiated colchicine, digitoxin, and digoxin, respectively, to antidigoxin and anticolchicine IgG and Fab fragments never exceeded 3%.

Analysis of Binding Data

Saturation binding isotherms were converted to a linear plot using the Graphpad program (ISI, California) and K_a was calculated according to Woolf's equation (17):

$$F/B = (1/B_m \times 1/K_a) + (1/B_m)F$$

where B and F are the concentrations of bound and free ligand and B_m is the maximal concentration of ligand binding sites. The concentrations of active binding sites of IgG or Fab fragments were calculated from the concentration of bound ^{3}H-ligand at saturation (B_m) corrected by the dilution of antibodies in the dialysis cell. The percentage of active binding sites of antibodies was defined as the ratio of the number of immunoreactive binding sites to the concentration of total binding sites.

RESULTS

Analysis of Purity of IgG and Fab Fragments

SDS-PAGE of polyclonal antidigoxin Fab fragments showed a single band of 50-kD molecular weight, confirming the result described by the manufacturer. SDS-PAGE of monoclonal antidigitoxin IgG revealed a single band of 150-kD molecular weight. Gel filtration chromatography indicated that antidigitoxin Fab fragments were 99.7% pure. Analysis of digestion products and purified Fab fragments by SDS-PAGE confirmed the purity of antidigitoxin Fab fragments. Analysis of polyclonal anticolchicine IgG by acetate cellulose electrophoresis showed that IgG was 99.1% pure. IgG had a molecular weight of 150 kD by SDS-PAGE. Purity of polyclonal anti-colchicine Fab fragments assessed by gel filtration was as follows: Fab, 91.9%; Fabc, 5.3%; peptides,

Table I. Specificity of IgG and Fab Fragments

Compound	Cross-reactivity (%)	
	Anti-colchicine[a]	
Colchicine	100	
2-Demethylcolchicine	14	
N-Deacetylcolchicine	0.8	
Colchiceine	0.07	
	Antidigoxin[b] (No. 741904-04)	Antidigitoxin[a]
Digoxin	100	1.2
Digitoxin	23	100
Ouabain	1.6	0.9

[a] IgG and Fab fragments.
[b] Fab fragments.

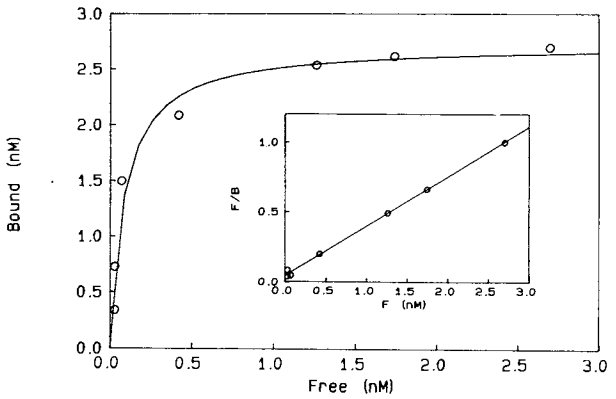

Fig. 1. Saturation curve and Woolf plot analysis (inset) of anti-digoxin Fab fragments (batch No. 741904-04) bound to ^{3}H-digoxin. The concentration of Fab fragments was $3.2 \cdot 10^{-9}$ *M*.

1%; and IgG, 0.7%. Under nonreducing conditions in SDS-PAGE, the Fab preparation appeared 100% pure and structurally intact (50 kD).

Specificity Determination of IgG and Fab Fragments

Results of cross-reactivity are presented in Table 1 for IgG and Fab fragments. No differences of cross-reactivity were observed between IgG and their corresponding Fab fragments.

Affinity Determination of IgG–Antigen and Fab–Antigen Complexes

Saturation curves of IgG and/or Fab fragments specific to digoxin, colchicine, and digitoxin bound by corresponding ^{3}H-ligand are shown in Figs. 1, 2, and 3, respectively. The affinity of polyclonal Fab–digoxin complex was 1.7 ± 0.3 10^{10} M^{-1} (batch 738922-04) (r = 0.99), 1.1 ± 0.1 10^{10} M^{-1} (batch 741904-04) (r = 0.99), and 8.5 ± 0.2 10^9 M^{-1} (batch 745789-05 (r = 0.99). The affinity of monoclonal IgG–digitoxin complex (6.4 ± 1.5 10^8 M^{-1}) (r = 0.95) was similar to that of Fab–digitoxin complex (7.2 ± 0.9 10^8 M^{-1}) (r = 0.95) (Table II). The same result was found for polyclonal IgG–colchicine complex (K_a = 7.5 ± 2.5 10^9 M^{-1}) (r = 0.97) and Fab–colchicine complex (K_a = 7.3 ± 0.8 10^9 M^{-1}) (r = 0.99).

Percentage of Active Binding Sites

Concentrations in the final product of total binding sites and percentage of SABS are presented in Table III. The percentage of SABS for polyclonal antidigoxin Fab fragments was 76.2 ± 2.3% (batch 738922-04), 88.7 ± 2.5% (batch 741904-04), and 85 ± 0.5% (batch 745789-05). The percentage of SABS for polyclonal anticolchicine Fab fragments (4.3 ± 1.2%) was similar to that of whole anticolchicine IgG (4.4 ± 1.5%). Monoclonal antidigitoxin IgG and Fab fragments had quite similar percentages of SABS, 58 ± 3 and 67 ± 7%, respectively.

DISCUSSION

With the recent advances of hybridoma and recombinant DNA technology, the applications for protein pharmaceuticals have increased dramatically. There is an evident need for a set of biological, physical, and chemical control procedures to assess the stability of such products. Recently, Manning *et al.* (18) documented the variety of chemical and physical processes which could affect proteins. The potential and limitations of analytical techniques involving changes in the molecular structure of monoclonal antibodies have been assessed by Jiskoot *et al.* (5). However, little information has been published concerning tests of immunoreactivity of antibody reagents. United States Food and Drug Administration guidelines state that the immunologic specificity, potency, and protein concentration of the monoclonal product should be quantified (19). Similarly, the International Association of Biological Standardization recom-

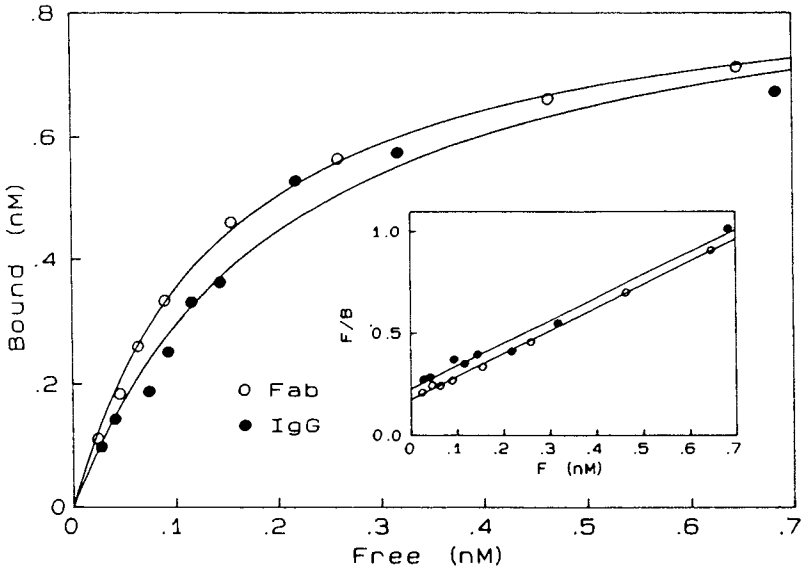

Fig. 2. Saturation curves and Woolf plot analysis (inset) of anticolchicine IgG and Fab fragments bound to ^{3}H-colchicine. The concentrations of IgG and Fab fragments were, respectively, $2.1 \cdot 10^{-8}$ and $2 \cdot 10^{-8}$ *M*.

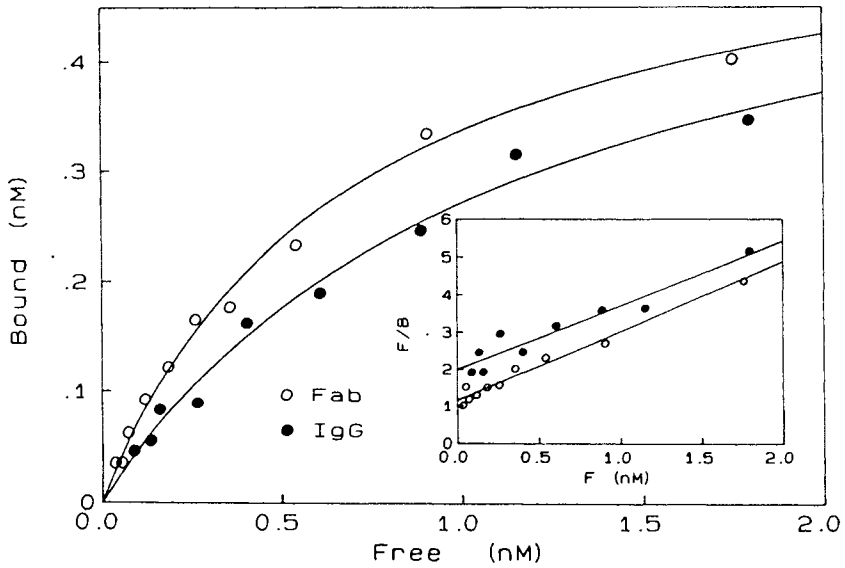

Fig. 3. Saturation curves and Woolf plot analysis (inset) of antidigitoxin IgG and Fab fragments bound to ^{3}H-digitoxin. The concentrations of IgG and Fab fragments were, respectively, 10^{-9} and $0.85 \cdot 10^{-9}$ *M*.

mends that immunological properties of the antibody should be described in detail including its antigenic specificity (20). Thus, appropriate analytical procedures need to be defined to establish pharmaceutical standardization of immunoreactivity for antibody reagents.

Antibody immunoreactivity is frequently defined by the specificity, affinity, and amount of protein assuming that all binding sites of the antibody are active. In this work, specificity was studied by a classical competitive radioimmunoassay, while both affinity and SABS concentration were assessed by a saturation binding method. Equilibrium dialysis was used because of the more complete separation of bound and free ligand than observed with precipitation methods. Our findings show that the specificity, the intrinsic association constant properties, and the SABS percentage remained similar between IgG and Fab fragments specific to colchicine and digitoxin. These data indicate that there were no alterations of Fab affinity and specificity during the preparation steps involving papain cleavage and purification by chromatographic procedures. In the same way, Bowles *et al.* (21) did not report modification of affinity constant between IgG and its corresponding Fab fragment specific to desipramine. However, a 10-fold decrease in affinity constant has been observed between IgG and Fab fragments specific to digoxin (22). As reported by Jiskoot *et al.* (5), the degradation processes might be antibody dependent. The most striking result was the SABS percentage in IgG and Fab products. Whatever the origin, monoclonal or polyclonal, we never found 100% of SABS for IgG and their corresponding Fab fragments. In fact, immunoreactivity levels found were only 58 to 67% of SABS for monoclonal antidigitoxin IgG and Fab, 76.2 to 88.7% for polyclonal antidigoxin Fab, and 4% for polyclonal anticolchicine IgG and Fab. This low percentage of SABS for anticolchicine Fab fragments can be easily explained by the polyclonal source of the reagent and the absence of immunoaffinity procedures for the selection of the specific Fab fragments to the hapten. In contrast, the polyclonal Digidot reagent has a higher SABS percentage range because of the use of an immunoaffinity procedure. Within the shelf-life period and during the months following the expiration date, the immunoreactivity remains very close to that described by the manufacturer. A 11% SABS decrease was observed only 32 months after the end of the shelf-life.

A similar percentage range was observed with the non-

Table II. Affinity Constants of IgG–Antigen and Fab–Antigen Complexes

	K_a (L/mol)	
	IgG	Fab fragment
Anticolchicine	$7.5 \pm 2.5 \cdot 10^{9a}$	$7.3 \pm 0.8 \cdot 10^{9}$
Antidigitoxin	$6.4 \pm 1.5 \cdot 10^{8}$	$7.2 \pm 0.9 \cdot 10^{8}$
Antidigoxin		
No. 738922-04	—	$1.7 \pm 0.3 \cdot 10^{10}$
No. 741904-04	—	$1.1 \pm 0.1 \cdot 10^{10}$
No. 745789-05	—	$8.5 \pm 0.2 \cdot 10^{9}$

[a] Mean values (±SEM) were obtained from three experiments.

Table III. Concentration of Total Binding Sites and SABS Percentage of IgG and Fab Fragments

	IgG		Fab fragments	
	SABS (%)	Conc. (*M*)	SABS (%)	Conc. (*M*)
Anticolchicine	4.4 ± 1.5^a	$7 \cdot 10^{-4}$	4.3 ± 1.2	$8 \cdot 10^{-4}$
Antidigitoxin	58 ± 3	$4.5 \cdot 10^{-5}$	67 ± 7	$5 \cdot 10^{-5}$
Antidigoxin				
No. 738922-04	—	—	76.2 ± 2.3	$8 \cdot 10^{-5}$
No. 741904-04	—	—	88.7 ± 2.5	$8 \cdot 10^{-5}$
No. 745789-05	—	—	85 ± 0.5	$8 \cdot 10^{-5}$

[a] Mean values (±SEM) were obtained from three experiments.

immunopurified, digitoxin-specific monoclonal IgG and Fab fragment. This last observation suggests that nonspecific IgG issued from ascites were probably present. Further control procedures will be needed to identify a nonquantitative recovery of SABS.

The results in this study show the necessity of establishing biological standardization of polyclonal and monoclonal IgG or Fab fragments specific to haptens as pharmaceutical reagents.

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