PREPARATION OF IMMUNOGEN AND PURIFICATION OF HIGH AFFINITY AND SPECIFICITY FAB FRAGMENT OF ANTI-DIGOXIN POLYCLONAL ANTIBODIES

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ABSTRACT

In this study we produced and purified a high titer of specific and high affinity Fab fragments of anti-digoxin antibody. Immunization of rabbits with a conjugate of the cardiac glycoside digoxin, coupled by a periodate oxidation method to the amino group of lysine in bovine serum albumin resulted in the production of this type of high titer digoxin-specific antibodies with exceptionally high affinity \(10^9\) L/mol and specificity in immune response. Increase in titer was found in steps of purification ending up with the highest titer for Fab fragment to be at 1.75 \(\mu\)g of purified Fab (for 50% binding of \(^{125}\)I-digoxin). High specificity for antigenic determinants of the steroid nucleus of digoxin was observed such that much less cross-reaction with digoxin (2.3%) and no cross-reaction with ouabaine, estradiol, cortisol, progesterone and testosterone were detected.

Keywords: Anti-Digoxin, Polyclonal Antibody, High Affinity.

INTRODUCTION

Digoxin is a cardiac glycoside obtained from various plant sources, notably digitalis leaf and strophanthus seed. This compound is widely used in the treatment of congestive heart failure and various disturbances of cardiac rhythm. The use of cardiac glycosides in patients with congestive heart failure is based on their ability to increase the force of myocardial contraction, thus increasing the output of the failing heart. Since individual patients vary considerably both in the required dosage and sensitivity to the toxic effects, digitalis toxicity is reported to occur in 5-20% of all patients being treated with digitalis glycosides.1

In order to reverse advanced digitalis toxicity, digoxin specific antibodies are produced by animals immunized with synthetic conjugates.2 Digoxin specific Fab fragments rapidly reverse the toxic effects of digoxin or digitoxin with initial effects being observed within minutes and with reversal of all toxic effects usually being achieved within 2 to 3 hours.2 Using these antidigoxin Fab fragments, no interactions with other drugs were found.4

The use of Fab fragments rather than intact immuno-
globulin is based on three clinically important considerations: Fab molecules are considerably less antigenic and thus are safer than animal antitoxin preparations used previously for human therapy. Fab fragments diffuse more rapidly and more effectively into the interstitial space, thus providing an earlier onset of action than intact immunoglobulin and finally there is extensive renal excretion of Fab fragments by patients with intact renal function, thus permitting excretion of glycosides in a bound inactive form.

In this article we report the preparation of specific antisera to digoxin conjugated to bovine serum albumin (BSA) via the end sugar group. Fab fragments were prepared, purified and characterized for further investigation as a digibinding reagent.

**MATERIALS AND METHODS**

Digoxin, BSA, diethyl aminoethyl cellulose (DEAE-C), carboxymethyl cellulose (CM-C), protein G, ouabain, digitoxin, steroids, polyethylene glycol (PEG), Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA), papain and sodium periodate were all obtained from Sigma Chemical Company, St. Louis, MO, USA. Radioactive labeled digoxin was obtained from Incstar Corporation, Stillwater, MN 55082, U.S.A.

Other reagents and solvents were obtained from Merck Chemical Company, Germany.

**Preparation of immunogen**

Digoxin was conjugated to BSA following the procedure reported by Butler and Chen. The steps are shown schematically in Figure 1 and are explained briefly as follows. 43.8 mg digoxin was dissolved in 2 mL of 95% ethanol, added with 2 mL of freshly prepared sodium methaneperiodate (drop by drop within 5 minutes).

The reaction was allowed to proceed for 30 min. At the end of reaction time, 0.06 mL of a 1 molar solution of ethylene glycol was added and incubated for 5 minutes. The product was then added to a solution of BSA containing 56 mg of protein in 2 mL of distilled water (pH adjusted with a 5% carbonate solution to 9.5). After 30 min when the reaction between digoxin and periodate was completed, 0.06 mL of ethylene glycol (1 M) was added and allowed to stand for five minutes. Oxidized digoxin was then added to BSA solution. After 45 minutes, 2 mL solution of 30 mg sodium borohydride was added and allowed to stand at room temperature for 4 more hours, at the end of which pH was adjusted to 6.5 by addition of a 1 M solution of formic acid.

The resulting solution was incubated for 1 hr, after which ammonium hydroxide (1 M) was added such that pH was adjusted to 8.5. The product was extensively dialysed against distilled water. The content of the dialysis bag was transferred to another container and the protein content was precipitated using a 0.1 molar solution of HCl. The suspension was centrifuged at 1000 g and the precipitate was collected. The absence of protein in the supernatant was confirmed using a 1.5% solution of trichloroacetic acid and the presence of digoxin in the supernatant was confirmed with sulfuric acid test. Finally the precipitate collected and confirmed in the above ways (for the presence of digoxin) was dissolved in a 0.15 molar solution of sodium bicarbonate and dialysed against distilled water at 45°C, freeze-dried and stored at 4°C until use.

**Fig. 1. Schematic representation of conjugating digoxin to bovine serum albumin (BSA) by periodate oxidation method.**

**The number of digoxin molecules conjugated to each BSA molecule**

Five milligrams each of digoxin-BSA conjugate and pure BSA were dissolved in 4 mL of water and added to 20 mL of concentrated sulfuric acid and incubated for 3 hours. The optical density of both samples were measured at 470 nm. The digoxin content of the digoxin-BSA solution was calculated using the following equation.

\[
N = \frac{\text{molar absorption of conjugate} - \text{molar absorption of BSA}}{\text{molar absorption of digoxin}}
\]

**Immunization**

Two New Zealand white rabbits were each immunized with 0.5 mg of digoxin-BSA conjugate using complete Freund's adjuvant following the procedure of Vaitukaitis et al. Booster injections and bleedings were carried out in the same way and dosage as reported before.

**Antiserum characterization**

Titer: Antidigoxin coated polystyrene tubes were added with different dilutions of antibody obtained in the above way. Radioactive digoxin (125I-digoxin) was also added to every tube and incubated at 37°C for 3 hr. For nonspecific
bindings, normal rabbit serum (NRS) was added in place of antibody. Percent binding was calculated when taking NRS containing tubes as 100% binding of \(^{125}I\)-digoxin to antidigoxin antibody coated on the walls of the polystyrene tubes, using the following equation:

\[
N = \frac{\text{cpm of NRS containing tubes} - \text{cpm of antibody containing tubes}}{\text{cpm of NRS tubes}} \times 100
\]

**Fab fragment preparation**

*Immunoglobulin preparation:* Ammonium sulphate (17.5 g) was added to 100 mL of stirring rabbit antiserum and precipitates were collected by centrifugation at 1000 g for 10 min at room temperature and dissolved in a minimum amount of a 17 mM solution of phosphate buffer (pH = 6.3) and dialysed against the same buffer extensively. The contents of the dialysis bag was then chromatographed on a DEAE-cellulose packed column equilibrated with the above buffer. Fractions containing more than 0.3 OD were collected, pooled and freeze-dried. Another portion of immunoglobulin was purified using a protein G affinity column eluted with 1 molar Tris-buffer. Both products were electrophoresed and characterized.

**Production and purification:** Purified immunoglobu-

![Graph](image1)

**Fig. 2.** Titration of antiserum raised against digoxin-BSA before purification and Fab formation. *50% inhibition is equal to 1:27000 (initial titer).*

![Graph](image2)

**Fig. 3.** Isolation of anti-digoxin IgG from rabbit serum on DEAE-cellulose following ammonium sulfate precipitation. Column dimensions: 2×26 cm.

![Graph](image3)

**Fig. 4.** Isolation of anti-digoxin IgG from rabbit serum on protein G column. *Column dimensions: 1×5 cm.*

![Graph](image4)

**Fig. 5.** Cellulose acetate electrophoresis of purified IgG anti-digoxin antiserum.
lin (150 mg) prepared in the above way was dissolved in 15 mL of PBS (10 mM) containing EDTA (1 mM), cysteine (2 mM) and NaCl (0.15 M) added with 1.5 mg papain and incubated for 18 hr at 37°C. The product was dialysed against distilled water with 10 changes each 1 hr until all proteolytic factors such as cysteine and EDTA were removed. The resulting solution in the dialysis bag contained Fab and Fc fractions. These two were separated from each other using a cation exchanger prepacked CM-cellulose column. Fab fragment was eluted with a gradient of 0.1-0.9 molar acetate buffer of pH 5.5. First and second peaks were pooled, dialysed (against a 2 mM phosphate buffer for 18 hours), freeze-dried and characterized by SDS-PAGE electrophoresis.

Characterization of Fab

**Titer:** Different dilutions of Fab fragments (0.125-25 µg/mL) were prepared in 250 µg of 10 mM PBS and added to a glass tube containing 400 µL of 125I-labeled digoxin and incubated for 2 hr at room temperature. One milliliter of a 20% solution of PEG (8000) was added to each tube and incubated for 10 min. The resulting immune-complex was centrifuged for 30 min at 2000 g at 4°C. Precipitates were counted for radioactivity content and 50% bindings was calculated.

**Affinity:** Different concentrations of digoxin (0.05-1.5 ng/mL) were prepared in 50 mM phosphate buffer pH 7.0 containing gelatin (0.1%). Fifty microliters of this solution plus 400 µL of Fab fragment of antidigoxin antibody (1.75 µg/mL) were added to each tube. However for total cpm index only radioactive material, and for non-specific binding (NSB), normal rabbit serum was added in place of different antibody dilutions. The tubes were incubated for 2 hr at room temperature. One milliliter of a 20% solution of PEG was added to each tube, incubated for 10 min and centrifuged for 30 min at 2000 g at 4°C. Tubes were decanted and precipitates were counted for radioactivity. Percent binding was calculated denoting zero dose tubes as 100% binding. Affinity constant (Ka) were calculated in nanomolar (nM) concentration.

**Specificity:** Some of the structurally related molecules (digitoxin, ouabain, estradiol, testosterone, progesterone and cortisol) were tested in parallel with different concentrations of digoxin following the procedure reported by Abraham.

**RESULTS**

The number of digoxin molecules conjugated to each molecule of BSA was calculated to be 11. This result shows that digoxin was successfully conjugated to BSA (Fig. 1). Fifty percent inhibition of antiserum dilution (1:27000) is shown in Fig. 2. The elution profile of DEAE cellulose chromatography and protein G chromatography are shown in Figures 3 and 4, respectively. Figure 5 shows the electrophoretic pattern of the immunoglobulin purified by ion-exchange and protein G chromatography. At this stage 50% inhibition was obtained to be at 1:54000 dilution of antidigoxin antibody fraction (Fig. 6).

Figure 7 shows the elution profile for CM cellulose chromatography of Fab purification; peaks I and II contained Fab fragments, while that of peak III contained Fc fragment only. These results were confirmed by SDS-PAGE electrophoresis and are shown in Figure 8 where only one band with a molecular weight of about 50 kDa is observed. The concentration of fifty percent binding of
radiolabeled digoxin with purified and freeze-dried Fab fragments is shown in Figure 9 and was found to be 1.75μg/mL.

Fig. 8. SDS-PAGE electrophoresis of digoxin-specific Fab purified by ion-exchange chromatography.

![Graph](image)

Fig. 9. Optimum dilution of anti-digoxin Fab. *50% binding of antigen (125I-digoxin) to Fab anti-digoxin.

mL. In Figure 10 the affinity of Fab fragments for the digoxin molecule is calculated as 10^9 L/mol.

Finally a parallel dose-response assay for digoxin and structurally related molecules is shown in Figure 11, the result of which shows that the antibody is totally specific toward the digoxin molecule only.

DISCUSSION

The treatment of life-threatening digoxin overdose with digoxin-specific Fab fragments is a successful immunotherapy practice. The detoxification depends on the stability of the binding of digoxin by the specific and high affinity Fab fragments in their common body distribution volume. The use of Fab fragments rather than intact immunglobulin is based on three clinically important considerations: 1) Fab molecules are considerably less antigenic and thus are safer than animal antitoxin preparations used previously for human therapy; 2) Fab fragments diffuse more rapidly and more effectively into the interstitial space, thus providing an earlier onset of action than intact immunglobulin; and 3) there is extensive renal excretion of Fab fragments by patients with intact renal function, thus permitting excretion of digoxin in bound, inactive form. Our previous studies showed that in producing high affinity and specific antibodies, the site of hapten molecule conjugation to protein is of importance.14

In this study we have produced and characterized a specific and high affinity binding polyclonal antibody to the cardiac glycoside digoxin. To obtain specific antibody which does not cross-react with other normal steroids of
physiological importance, we coupled the carrier protein through a long distance between the steroid nucleus and protein carrier interposed by three sugar residues which enhanced the specificity of the immune response. Our results indeed indicate the correctly chosen strategy and correlate well with other results reported by others. Hence we did not detect any cross-reaction with common steroids (testosterone, progesterone, cortisol, ouabain and estradiol) and much less cross-reaction with digitoxin. The presence of three digitoxyde residues between the steroid nucleus and the carrier protein of the antigen may also influence the affinity of antibody produced in this study. However the analysis of antibody affinity produced in this study shows that these antibodies have high affinity for binding to digitoxin (Ka 10^8 mol/L). These results correspond with those reported for polyclonal antibodies as well as monoclonal antibodies reported by others.

Our next attempt would be to try the clinical use of the “digibind” reagent prepared in this study. The same preparation is also being used to develop an assay technique to measure digoxin in blood samples.

REFERENCES


