Reversal of Ouabain and Acetyl Strophanthidin Effects in Normal and Failing Cardiac Muscle by Specific Antibody

HERMAN K. GOLD and THOMAS W. SMITH

From the Cardiac Unit, Medical Service, Massachusetts General Hospital, and the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02114

ABSTRACT Isolated cat right ventricular papillary muscles were used to study the effects of antibodies with high affinity for ouabain and acetyl strophanthidin on myocardium exposed to these cardioactive steroids. Antibodies with average intrinsic affinity constants for ouabain and acetyl strophanthidin of the order of 10^9 M^{-1} were raised in rabbits challenged by repeated injection of a conjugate of ouabain covalently linked to a poly D,L-alanyl derivative of human serum albumin. Effects were assessed in terms of time-course and extent of inotropy reversal, influence of experimentally induced ventricular failure, digitalis-antibody concentration relations, influence of digitalis-antibody complex on response to additionally added digitalis, and relation of antibody effects on digitalis-induced automaticity and contracture to reversal of inotropy. Specific antibody (but not control antibody) in 1.1–1.5-fold molar excess over cardioactive steroid concentrations blocked positive inotropic effects of ouabain and acetyl strophanthidin, and gradually reversed established contractile effects of these agents with a mean time for half-reversal of ouabain-induced inotropy of 124±6 (SEM) min and 37±3 min for half-reversal of acetyl strophanthidin-induced inotropy. Papillary muscles from cats with right ventricular failure induced by chronic pulmonary artery constriction responded similarly. Both normal and failing muscles returned to but not below levels of contractility existing before cardioactive steroid exposure, and time for half-reversal of inotropy by antibody was significantly shorter than time for half-reversal after removal of ouabain or acetyl strophanthidin by muscle bath washout alone. Presence of ouabain- or acetyl strophanthidin-antibody complex did not alter the myocardial contractile response to subsequently added cardioactive steroids.

Spontaneous automaticity occurring as a toxic response to ouabain or acetyl strophanthidin in eight muscles was rapidly reversed by specific antibody at a time when positive inotropic effects were still fully manifest. Early contracture was also reversed by specific antibody. These studies provide further support for the concept that cardiac glycoside-specific antibodies are capable of reversing established cellular effects of cardioactive steroids.

INTRODUCTION

Several recent studies have shown that cardiac glycoside-specific antibodies can reverse established effects of digitalis glycosides in biological systems (1–3). In addition to reversal of cardiac glycoside-induced effects on in vitro systems, it has been demonstrated that advanced cardiac digoxin toxicity in the intact dog can be reversed by digoxin-specific antibody (4, 5).

To assess the effect of cardiac glycoside-specific antibody on digitalis-treated myocardium, we have studied the effects of ouabain-specific antibody on isolated cat right ventricular papillary muscles exposed to ouabain and acetyl strophanthidin. In the experiments to be described, special attention has been directed to (*a*) the time-course of reversal of digitalis-induced inotropy by antibody and its relation to reversal of inotropy by washout alone; (*b*) the effect of congestive heart failure, induced by chronic pulmonary artery constriction, on papillary muscle responses to digitalis and specific antibody; (*c*) the relation of cardioactive steroid concentration to specific antibody concentration required to reverse established effects; (*d*) the question of whether

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addition of specific antibody will adversely affect contractility beyond the effect expected from removal of cardioactive steroid from myocardial receptor sites; (*e*) the question of whether the presence of digitalis-antibody complex will alter the response to additional digitalis added to the bath; and (*f*) the relation of antibody effects on digitalis-induced automaticity and contracture to reversal of inotropy.

METHODS

Crystalline ouabain and acetyl strophanthidin were donated by Dr. C. T. Chiu, Eli Lilly & Co., Indianapolis, Ind.) [^{3}H]ouabain (11.7 Ci/mmol) was obtained from New England Nuclear, Boston, Mass. Thin-layer chromatography on silica gel G with a chloroform : methanol : water (65 : 30 : 5) solvent system showed that 96% of counts were present in a single peak with the mobility of native ouabain.

Immunological methods. Antibodies of high affinity for ouabain and acetyl strophanthidin were raised in rabbits by repeated injection of a conjugate of ouabain covalently linked to a poly D,L-alanyl derivative of human serum albumin, as previously described (6). The average intrinsic affinity constant for ouabain of the antibody population used in the present studies, as determined by equilibrium dialysis, was 1.6×10^9 M^{-1} (6), and cross-reactivity experiments with acetyl strophanthidin demonstrated an affinity of the same order of magnitude (6, 7). Gamma globulin fractions were prepared by ammonium sulfate fractionation (8) and extensive dialysis against the modified Krebs solution used in papillary muscle experiments. Concentration of specific antibody binding sites was determined by studies in which constant amounts of antibody solution were added to test tubes containing varying amounts of [^{3}H]ouabain followed by incubation and separation of antibody-bound and free [^{3}H]ouabain with dextran-coated charcoal. Plots of reciprocal antibody-bound vs. reciprocal free [^{3}H]ouabain were used to determine concentrations of antibody binding sites (6).

Serum samples obtained from rabbits before immunization were treated exactly like those containing specific antibody and used as controls. Gamma globulin concentrations of control solutions were adjusted to equal those of specific antibody solutions by matching optical densities at 280 nm.

Papillary muscle studies. Right ventricular failure was produced in 13 2.0–3.2 kg adult cats by placing a clip of 3.1 mm diameter around the proximal main pulmonary artery as described by Spann, Buccino, Sonnenblick, and Braunwald (9), reducing the lumen to about 15% of the normal cross-sectional area. This technique has previously been shown to produce typical hemodynamic abnormalities of right ventricular failure (9, 10). At sacrifice 4–35 days later, right ventricular hypertrophy was evident in all hearts and the ratio of right ventricular to left ventricular weight averaged 0.70±0.05 (SEM), compared with 0.27±0.02 in the nonoperated cats ($P < 0.001$).

After anesthesia with 35 mg/kg intraperitoneal pentobarbital, hearts were rapidly excised and right ventricular papillary muscles were transferred to a myograph apparatus containing modified Krebs solution (11) at 37°C bubbled with 95% O_2 and 5% CO_2. Muscles contracting isometrically were stimulated by field electrodes at a frequency of 12/min by a 3-ms square wave stimulus 10% above threshold. Length-tension curves were determined for each muscle and preparations were maintained for the remainder of the experiment at one-half of the resting tension necessary for maximal tension development. Resting and developed tension were measured with a Statham P-23Db transducer (Statham Instruments, Inc., Oxnard, Calif.) and recorded on a Hewlett-Packard model 7700 multichannel recorder together with stimulus artifact (Hewlett-Packard Co., Palo Alto, Calif.). In addition to continuous recording at slow paper speeds, representative data were recorded at a paper speed of 100 mm/s and the maximum rate of tension development determined from these high-speed recordings. To compare the mechanical function of papillary muscles of differing sizes, tension measurements were corrected for cross-sectional area.

All preparations were allowed to equilibrate to a stable resting and developed tension for at least 1 h before further interventions were carried out. After the equilibration period, ouabain or acetyl strophanthidin was added to the muscle bath to a final concentration of 4×10^{-7} M and a steady state again allowed to develop for 1 h at the new level of tension development. Control or specific gamma globulin was added in a volume 1/50th or less of the total bath volume, with continuous recording of resting and developed tension. In washout experiments, after development of a stable response for 1 h, the muscle bath was rapidly emptied and refilled five times with warmed, oxygenated, modified Krebs solution to completely remove free ouabain or acetyl strophanthidin. Observation of contractile performance of muscles in the cardioactive steroid-free bath was then continued as in the case of antibody experiments. An additional 10 muscles (5 for ouabain and 5 for acetyl strophanthidin) were studied in these washout experiments.

Statistical significance of differences in data from various groups was evaluated by Student's *t* test of unpaired data, with a two-tailed distribution (12).

RESULTS

Baseline contractile performance values for papillary muscles from normal cats and cats with pulmonary artery constriction are included in Figs. 1 and 2. Active isometric tension and rate of tension development were significantly lower ($P < 0.001$) in muscles from cats with pulmonary artery constriction than in muscles from normal cats, as previously observed in this experimental heart failure model (9, 10).

Fig. 1 summarizes responses of normal and failing papillary muscles to 4×10^{-7} M ouabain. Mean developed tension increased from 4.8±1.1 (SEM) g/mm^2 to 9.9±1.2 ($P < 0.02$) in the eight normal muscles, and from 1.4±0.1 to 3.1±0.4 in six muscles from failure animals ($P < 0.005$). Relative increases in developed tension in response to ouabain were not significantly different for normal and failure animals, equaling 131±16% and 122±35%, respectively. Changes in rate of tension development (dT/dt) were similarly greater on an absolute scale for normal than failure muscles (from 33±6 (SEM) g/s per mm^2 to 76±11, compared with 12.5±0.6 to 26.0±5.0, respectively) but were not significantly different in terms of percentage changes.

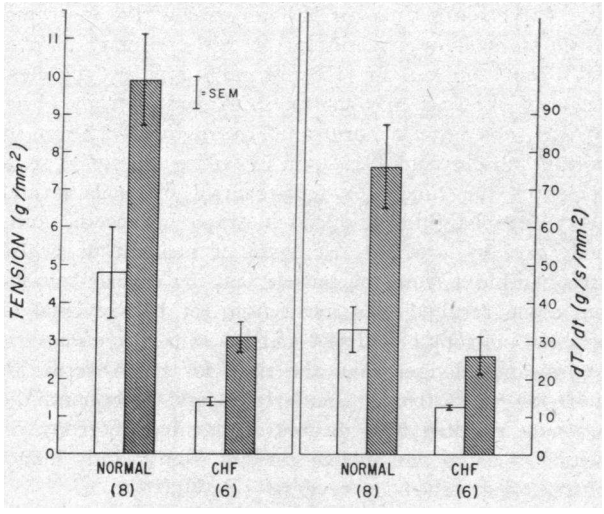

FIGURE 1 Maximum inotropic response to 4×10^{-7} M ouabain of papillary muscles from eight normal cats and six cats with chronic pulmonary artery constriction (CHF). Mean control values are shown in open bars and values at peak ouabain effect in cross-hatched bars. Developed tension is illustrated in the left panel and maximum rate of change of tension in the right panel. Vertical lines denote one SEM above and below the mean.

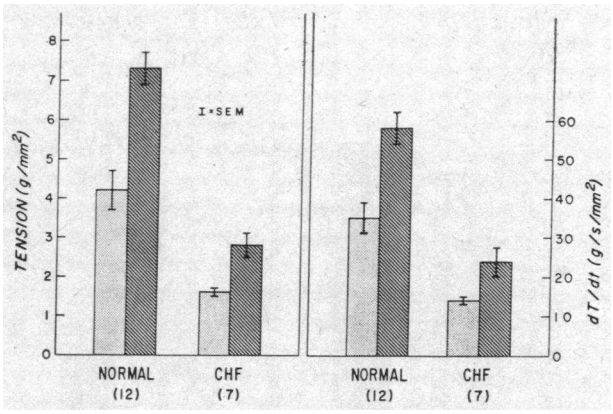

FIGURE 2 Maximum inotropic response to 4×10^{-7} M acetyl strophanthidin of papillary muscles from 12 normal cats and 7 cats with chronic pulmonary artery constriction (CHF). Mean control values are shown in lightly stippled bars and values at peak acetyl strophanthidin effect in cross-hatched bars. Developed tension is shown in the left panel and maximum rate of change of tension in the right panel.

Responses of 12 normal and 7 failure muscles to 4×10^{-7} M acetyl strophanthidin are summarized in Fig. 2. Developed tension increased from a mean of 4.2±0.5 to 7.3±0.4 g/mm² in normal muscles ($P < 0.001$) and from 1.6±0.1 to 2.8±0.3 ($P < 0.01$) in failure muscles. The percentage increments for normal and failure muscles were similar at 89±14% and 85±16%, respectively. As in the case of exposure to ouabain, increases in dT/dt were greater on an absolute scale for normal (from 35±4 to 58±4 g/s per mm²) than for failure muscles (from 14±1 to 24±3), but the percent increase in normal muscles was not significantly different from that in failure muscles.

Initial experiments showed that on each of five occasions, specific antibody added to the muscle bath in amounts that provided a 1.1–1.5-fold molar excess of antibody binding sites over subsequently added ouabain or acetyl strophanthidin concentration completely blocked the positive inotropic effects of these agents. Equivalent amounts of control gamma globulin allowed full expression of the expected contractile response. Experiments were then carried out to investigate antibody reversal of established positive inotropic effects of ouabain or acetyl strophanthidin. Fig. 3 shows the response of a normal papillary muscle to acetyl strophanthidin. When the positive inotropic effect had reached the plateau level, a 1.4-fold excess of antibody binding sites was added to the bath. This was followed by gradual reversal of the acetyl strophanthidin-induced inotropic effect (as

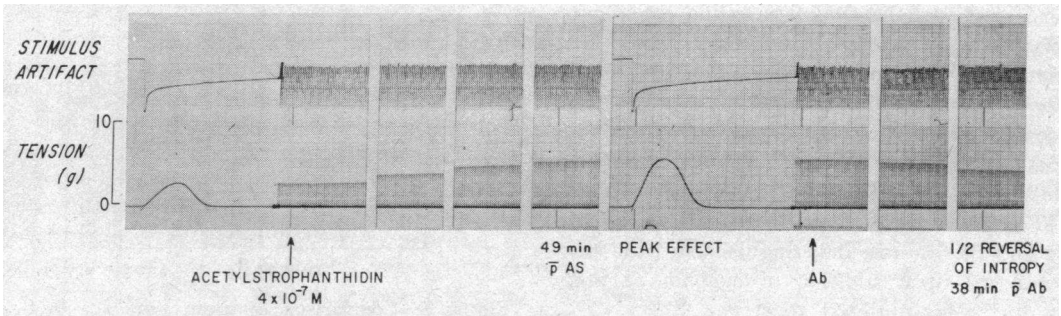

FIGURE 3 Recording of stimulus artifact (upper tracing) and tension (lower tracing) of a normal cat right ventricular papillary muscle. After stabilization for 1 h, 4×10^{-7} M acetyl strophanthidin was added to the muscle bath. Peak positive inotropic effect was manifest at 49 min. After stabilization in the presence of acetyl strophanthidin for 1 h, a 1.4-fold excess of specific antibody was added, resulting in a gradual fall in developed tension to half the peak increment after 38 min. AS, acetyl strophanthidin; Ab, specific antibody.

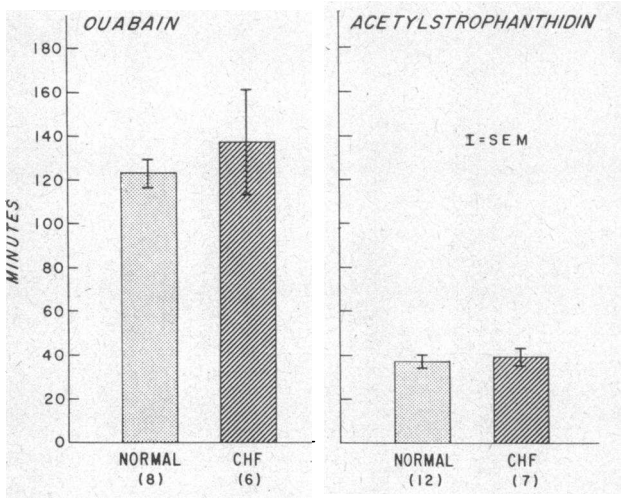

FIGURE 4 Mean times for half-reversal of inotropy by specific antibody. Values for papillary muscles from eight normal cats (stippled bar) and six cats with chronic pulmonary artery constriction (cross-hatched bar) exposed to ouabain (left panel) are not significantly different, nor are times for half-reversal of acetyl strophanthidin-induced inotropy in 12 normal and 7 pulmonary artery constriction muscles (right panel).

judged by decreases in both developed tension and rate of tension development) to the same base-line levels as before addition of acetyl strophanthidin. In each experiment, addition of specific antibody in 1.1–1.5-fold excess over acetyl strophanthidin or ouabain resulted in similar reversal of inotropy, with contractility gradually returning to but not significantly below base-line levels determined before cardioactive steroid addition. Control gamma globulin had no tendency to reverse acetyl strophanthidin or ouabain effects, and developed tension remained unchanged in its presence for periods up to 2 h. Failing muscles from cats with pulmonary artery constriction responded like normal muscles, with contractile force gradually returning to but not below pre-ouabain or acetyl strophanthidin base-line levels after addition of specific antibody.

After return to base-line levels of developed tension in response to specific antibody, addition of excess ouabain (three experiments) or acetyl strophanthidin (three experiments) to the muscle bath in quantities sufficient to provide a free cardioactive steroid concentration of 4×10^{-7} M resulted in positive inotropic effects with magnitude and time-course indistinguishable from those initially observed upon addition of ouabain or acetyl strophanthidin before antibody reversal.

Because full antibody reversal of the inotropic effects of ouabain or acetyl strophanthidin was approached asymptotically and was difficult to time precisely, time required for half-reversal of inotropy was used to characterize the time-course of antibody effect. As shown in Fig. 4, the mean time for half-reversal of the inotropic effect of ouabain was similar in both normal (124±6 SEM min) and failing (138±24 min) papillary muscles. Reversal of acetyl strophanthidin-induced inotropy (Fig. 4) was substantially more rapid, averaging 37±3 min in normal muscles and 39±4 min in failing muscles. Fig. 5 compares the times for half-reversal of ouabain and acetyl strophanthidin-induced inotropy by specific antibody and by washout. Removal of ouabain or acetyl strophanthidin from the muscle bath by repeated washout alone resulted in a mean time for half-reversal of ouabain inotropy of 181±24 (SEM) min in five muscles, significantly longer than the time for half-reversal by antibody ($P < 0.05$). Similarly, acetyl strophanthidin washout resulted in a mean time for half-inotropy reversal of 63±5 min in five muscles, significantly longer than that for antibody reversal ($P < 0.001$).

Augmentation of contractile force resulting from ouabain or acetyl strophanthidin was later accompanied by development of spontaneous automaticity in eight muscles, five of which were from normal animals and three from animals with chronic pulmonary artery constriction. Fig. 6 shows the course of events in an experiment in which exposure of the papillary muscle to 4×10^{-7} M ouabain resulted in development of spontaneous automaticity as peak inotropic effect was reached. Addi-

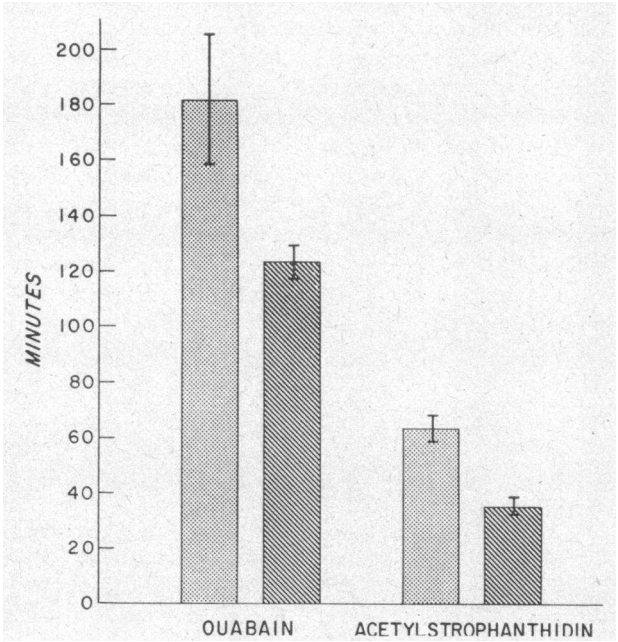

FIGURE 5 Comparison of mean time for half-reversal of ouabain and acetyl strophanthidin-induced inotropy by bath washout alone (stippled bars) and by addition of specific antibody (cross hatched bars). Differences in mean time to half-reversal of inotropy are significant for both ouabain ($P < 0.05$) and acetyl strophanthidin ($P < 0.001$). Vertical lines depict one SEM above and below the mean.

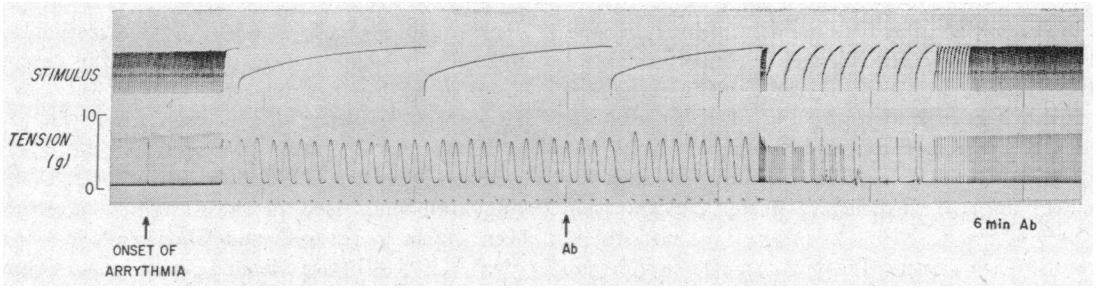

FIGURE 6 Continuous recording of reversal of ouabain-induced automaticity by specific antibody. Paper speed is varied to show relationship between stimulus artifact (upper tracing) and muscle contraction. Addition of specific antibody is followed, after 4 min, by a return to contractile activity only when a stimulus is applied. Positive inotropic effect is still fully manifest at the time when spontaneous automaticity ceases.

tion of a 1.5-fold excess of specific antibody resulted in disappearance of ouabain-induced automaticity after 4 min, at a time when the positive inotropic effect of ouabain was still fully manifest. In each of the eight such instances observed, addition of specific antibody abolished automaticity within 6.6 min (mean±SEM, 3.2±0.6 min), and the positive inotropic effect of ouabain or acetyl strophanthidin was still manifest at the time of automaticity reversal. Early mechanical toxicity resulting in progressive contracture was also reversed by specific antibody in each of three experiments in which antibody was added to the muscle bath within 5–10 min of the onset of progressive contracture. Addition of control gamma globulin had no effect upon cardioactive steroid-induced automaticity (three experiments) or contracture (two experiments) over observation periods of 30–60 min.

DISCUSSION

An interesting potential therapeutic approach to the problem of advanced, life-threatening digitalis toxicity has followed from the demonstration by Butler and Chen that digoxin coupled to suitable protein carrier molecules could elicit digoxin-specific antibodies in animals immunized with this conjugate (13). Schmidt and Butler showed that immunization with a digoxin-serum albumin conjugate substantially increased the resistance of rabbits to potentially lethal doses of digoxin (14). They further determined that passively administered digoxin-specific antibody could reverse established cardiac digoxin toxicity in the dog (4), a finding confirmed in our own laboratory (3, 5). Consistent with these data are the studies of Mandel, Bigger, and Butler who demonstrated antibody reversal of digoxin-induced electrophysiologic toxicity in isolated canine Purkinje fiber-ventricular muscle and rabbit atrioventricular node preparations (15). Digoxin-specific antibody was shown to reverse digoxin-induced electrical inexcitability, marked phase 4 depolarization in Purkinje fibers, and prolongation of atrioventricular nodal refractoriness. Butler et al. have recently summarized further experiments documenting reversal of digoxin effects by digoxin-specific antibodies, including reversal of digoxin-induced inotropy in isolated cardiac muscle by digoxin-specific antibody (2, 16).

The present observations provide further evidence that specific antibodies can reverse established cellular effects of cardiac glycosides, including electrophysiologic and mechanical toxic manifestations. We have demonstrated that antibodies with high affinity for ouabain and acetyl strophanthidin are capable of blocking effects of these cardioactive steroids on isolated cardiac muscle. Furthermore, established inotropic effects are gradually reversed by the addition of specific antibody to the cardioactive steroid-containing muscle bath, with inotropy reversal half-times of 124±6 (SEM) min and 37±3 min for ouabain and acetyl strophanthidin, respectively, in normal papillary muscles. Contractile performance returned to, but not below, predigitalis levels, consistent with removal of cardioactive steroid from cellular receptor sites. Similar responses were observed in both normal right ventricular papillary muscles and those from animals with cardiac failure induced by chronic pulmonary artery constriction. The latter model has been used in previous studies, which have documented typical pathophysiologic findings of right ventricular failure (9, 10) with alterations in right ventricular papillary muscle contractility, as observed in the present experiments.

It is of interest that near-stoichiometric amounts of specific antibody reverse inotropic effects of ouabain and acetyl strophanthidin more rapidly than removal of these agents from the muscle bath by washout. Formation of a stable antibody-hapten complex at the cardiac glycoside receptor site of the muscle, with resulting reversal of the effect, is rendered unlikely by the observation of responses to further addition of ouabain or acetyl strophanthidin after antibody reversal which were indistinguishable from the responses recorded after initial addition of cardioactive steroids. In addi-

tion, there is evidence that the dominant chemical determinants of both cardiac activity and antibody binding of these agents reside in the C–D rings and unsaturated lactone portions of the molecule (17, 18), making simultaneous receptor and antibody binding unlikely. We hypothesize rather that specific high-affinity antibody acts to lower the free cardioactive steroid concentration to near-zero levels, resulting in progressive removal of drug from receptor sites as the drug-receptor equilibrium is displaced in the direction of dissociation. A similar mechanism has been favored by Gardner, Kiino, Swartz, and Butler (19) for the removal of [^{3}H]digoxin from human erythrocytes by digoxin-specific antibody. The rate of antibody reversal of effect would thus be dependent on the rate constant for drug-receptor dissociation and the relative effective concentrations and association rate constants of myocardial receptors and specific antibodies. Effective antibody concentration, in turn, is dependent upon the rate of diffusion into the papillary muscle. More rapid reversal of acetyl strophanthidin- than ouabain-induced inotropy is probably related to more rapid acetyl strophanthidin-receptor dissociation kinetics compared with those of ouabain. Antibody reversal of inotropy at a rate more rapid than that resulting from drug washout alone could result from diffusion of antibody throughout the papillary muscle interstitial space, thus shortening the diffusion distance for effective removal and preventing rebinding to another receptor site. Very rapid kinetics of ouabain binding to ouabain-specific antibody have recently been documented in our laboratory,[1] with second-order association rate constants of the order of 10^7 M^{-1} s^{-1} at 22°C, consistent with this proposed mechanism. The observation that the effects of added ouabain or acetyl strophanthidin were not influenced by the presence of cardioactive steroid-antibody complex is also consistent with this hypothesis.

With due respect for the hazards of extrapolating these experimental results to potential clinical situations, it is reassuring that inotropy reversal proceeded to but not below pre-cardioactive steroid treatment levels in both normal and failing papillary muscles, and that the time course of inotropy reversal in both types of muscles was relatively slow in relation to reversal of toxicity as evidenced by automaticity and contracture. The observation that near-stoichiometric amounts of antibody binding sites were sufficient to reverse digitalis-induced automaticity and contracture is also important. Although cat papillary muscle has not been extensively studied in terms of electrophysiologic responses to cardiac glycosides, it seems likely that the typical toxic changes in transmembrane action potential observed in other cardiac tissues, including loss of resting potential and increased slope of spontaneous diastolic depolarization (15, 20) underlie the appearance of spontaneous automaticity observed in the present experiments. In view of circumstantial evidence implicating $(Na^+ + K^+)$-ATPase as a receptor for both inotropic and toxic effects of cardiac glycosides (21, 22), it is of interest that the specific antibodies used in these studies have previously been shown to reverse established ouabain inhibition of $(Na^+ + K^+)$-ATPase from canine myocardium (6). This phenomenon may underlie, at least in part, the findings observed in the present studies.

[1] K. Skubitz and T. W. Smith. Unpublished experiments.

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