Digoxin-like immunoreactivity of a circulating $(Na^+ + K^+)$ATPase inhibitor

WE wish to dispute a statement made by Hamlyn *et al.*[1] concerning the use of anti-digoxin antibodies to detect a circulating inhibitor of $(Na^+ + K^+)$ATPase. They claim to be using a technique which is "essentially as described by Gruber *et al.*". In reality the procedure they used is not our technique. We use plasma (4–5 ml) isolated from 10 ml of blood. The plasma is deproteinized, Diafiltered through Um-10 membranes, then the filtrate is lyophilized and redissolved in 500 μl of water[2]. Our approach purifies the extract as we concentrate it. In the method described by Hamlyn *et al.* it is unclear whether they concentrate or dilute the deproteinized extracts. In addition, they do not mention a Diafiltration procedure.

In our experience, concentrating a deproteinized plasma extract without first Diafiltering it produces a sample with little immunoreactivity. In addition, the digoxin-like activity present is very variable, will not dilute out, and thus does not give parallel displacement to digoxin in serial dilutions.

Parallelism is the cornerstone of any radioimmunoassay (RIA), especially when attempting to measure a cross-reacting factor. In our original publication[2], we demonstrated that as plasma extracts were purified, their serial dilution displacement curve approached that of standard digoxin. A Diafiltrate was the crudest extract with which we could obtain an acceptable displacement curve.

Hamlyn *et al.* go to great lengths to verify their $(Na^+ + K^+)$ATPase assay. It is a pity they did not go to similar lengths to verify that their RIA was detecting the same substance that we first reported on. As they provided no evidence or inference that they were able to dilute out their endogenous digoxin immunoreactivity, it is conceivable that their measurements were made on the nonlinear part of the displacement curve; it is impossible to quantify immunoreactivity unless it is read on the linear segment of the dilution curve. Other investigators have approached the detection of an endogenous digoxin-like factor in the appropriate manner[3], and have confirmed our original findings.

In view of the comments made by Hamlyn *et al.*[1] concerning the results of their digoxin RIA, we feel it is important to draw attention to the fact that the excellent correlation they show between $(Na^+ + K^+)$ATPase inhibition and blood pressure in normotensive and hypertensive humans is strikingly similar to that which we first obtained by measuring digoxin-like immunoreactivity in monkeys with spontaneous hypertension[4]. Elevated digoxin-like activity has also been found in plasma of rats with coarctation hypertension[5]. Finally, injection of anti-digoxin antibodies lowers the blood pressure of deoxycorticosterone acetate–salt hypertensive rats[6].

The measurement of a cross-reacting substance by a radioimmunoassay is at best a difficult procedure. To not follow an established technique is to invite failure unless one wishes to establish the validity of one's own assay procedure.

Note added in proof: Hamlyn *et al.*[7] have recently reported that endogenous digoxin (endoxin) levels directly correlate with plasma noradrenaline and inversely correlate with aldesterone. Thus, endoxin appears to have some physiological variability.

KENNETH A. GRUBER
VARDAMAN M. BUCKALEW JR

Department of Medicine,
Section on Nephrology,
Bowman Gray School of Medicine of
Wake Forest University,
Winston-Salem,
North Carolina 27103, USA

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HAMLYN *ET AL.* REPLY—Gruber and Buckalew are correct in asserting that the method we recently used[1] to measure digoxin-like immunoreactivity (DLI) in samples derived from the plasma of normotensive and hypertensive individuals, is not identical to the one they described[2]. The differences between the two procedures, however, are smaller than claimed by Gruber and Buckalew and hence led us to use the phrase "essentially as described"[1].

The methods we used to prepare the plasma samples for the digoxin radioimmunoassay (RIA) are clearly stated in the legends of Figs 2 and 3 of ref. 1 and differ from the Gruber *et al.* procedure[2] only in that we omitted the ultrafiltration step used by them. This omission was based on an earlier report by Buckalew[3] which showed that an inhibitor of active sodium transport obtained from dog plasma can bind to the polypropylene membrane support used in the ultrafiltration apparatus. It seemed unwise to use such a protocol without the benefit of a stainless steel support[3] because the recoverability of active material is then difficult to determine. Furthermore, the claim that Diafiltration removes interfering substances appears to compound the difficulty in quantitating the immunoreactivity of native plasma. It is noteworthy that we did not experience any difficulty in detecting, quantitatively, the small amounts of digoxin (50–100 pg ml^{-1}) that were added to either native or deproteinized plasma samples, as 'internal standards' for the digoxin RIA. Thus, any interfering substances must inhibit only the interaction of the digoxin-like material, and not digoxin itself, with the antibody.

The claim that other investigators have used appropriate methods to quantitate DLI is not directly relevant. The source of material (guinea pig heart versus plasma from volume-expanded dogs) and the extraction protocols used by others in the preparation of DLI[4] were very different from that reported by Gruber *et al.*[2]. Consequently, the work by DePover *et al.*[4] cannot be considered direct confirmation of the original findings of Gruber *et al.*[2].

The specificity of the anti-digoxin antibody may also be a problem. This antibody shows little or no cross-reactivity with other cardiac glycosides. In contrast, the $(Na^+ + K^+)$ATPase will bind, and is inhibited by, a wide variety of structurally different cardiac glycosides. This implies that the anti-digoxin antibody is not recognizing the structurally important determinants involved in inhibition of $(Na^+ + K^+)$ATPase. Thus, the relationship of the digoxin-like material to inhibition of the ATPase by endogenous factors is not clear.

We found[1] that human plasma (when deproteinized and concentrated) contains a DLI substance. However, as reported, we found no significant difference in plasma DLI between normotensives and patients with essential hypertension. The immunoreactive factor did dilute out and, in contrast to the experience of Gruber and Buckalew, was not variable in repeated assays.

The significance of the relationships between blood pressure and DLI in spontaneously hypertensive monkeys[5], and between $(Na^+ + K^+)$ATPase inhibition and blood pressure in human essential hypertension[1], alluded to by Gruber and Buckalew, are complicated by the use of different assays, and by the uncertainty that the primate (or rat) model is an appropriate model of the human disease. In our view, the appropriate way to quantitate endogenous inhibitors of the sodium pump is to use the pump molecule itself in the assay system.

J. M. HAMLYN
R. R. RINGEL
B. P. HAMILTON
A. A. KOWARSKI
M. P. BLAUSTEIN

Department of Physiology,
University of Maryland
School of Medicine,
Baltimore, Maryland 21201, USA

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