Elevated Plasma Binding Cannot Account for the Burn-related d-Tubocurarine Hyposensitivity


Hyposensitivity to the nondepolarizing muscle relaxant d-tubocurarine (dTc) has been reported during the treatment of burn injury. The authors present here evidence obtained by the technique of equilibrium dialysis, that a 1.7-fold increase in dTc binding by plasma constituents occurs one to two weeks following burn injury, a timing which coincides with the onset of clinical hyposensitivity to dTc. Plasma drawn from burned patients at least one week post-burn exhibited a mean dTc-binding capacity of 5.7 ± 0.9 nmol/g total protein compared to 3.4 ± 0.7 nmol/g for normal plasma at a final free concentration of 0.5 µM (0.34 µg/ml). Calculation suggests that increased plasma binding can account only for a fraction of the observed hyposensitivity; other unidentified factors must also be involved. The elevated binding capacity of plasma drawn from burned patients seems unrelated to any of the quantitative or gross qualitative changes in plasma protein profile reported here. Preliminary experiments with metocurine (mTc) show directional changes similar to dTc.

(Key words: Complications: burns. Neuromuscular relaxants: metocurine; d-tubocurarine. Protein: binding; plasma.)

Because of the known hyperkalemic response of burned patients to succinylcholine,1 nondepolarizing muscle relaxants such as d-tubocurarine (dTc) are normally employed to induce skeletal muscle relaxation in these patients.2 Increased intravenous dose requirement for dTc has been reported in certain exceptional but healthy normals,3 in liver disease patients,4 and in burned patients.2 In the latter group, total plasma dTc levels necessary to attain a given twitch depression approach five times that of the average patient population, yet standard doses of neo-

stigmine were sufficient to reverse curarization.2 The main question addressed in this paper is: what fraction of this abnormally high plasma dTc concentration in burned patients is free and available for transcapillary diffusion to the neuromuscular junction? Comparison of the binding of 3H-dTc and 14C-metocurine (mTc) to plasma drawn either from burned patients or from normal volunteers has resolved this question.

Materials and Methods

Plasma

Blood samples from patients were drawn into heparinized syringes only through existing venous or arterial cannulae. The mean ± SD together with range for age, size of burn, and post-burn interval at which blood samples were drawn, are shown in tables 1 and 2. Only one blood sample was drawn per patient in the first part of the study reported in tables 1 and 2. In an additional three patients, it was possible to obtain plasma soon after injury and at 4, 7 and 14 days post-injury so that the onset of elevated plasma binding of dTc could be compared to the changes in plasma protein fractions (table 3). Blood from nonburned controls was drawn into heparinized syringes from the antecubital vein through a 22-gauge needle following the application of a tourniquet. A volume of 5–7 ml of blood was obtained per study. The plasma was separated within 2 hours, refrigerated and stored in plastic tubes or Vacutainers® to test the effect of catalyst in rubber stoppers on binding (see Results and Discussion). Studies were performed within two weeks of sampling. Total protein was measured by refractometry,5 and protein profiles were quantitated densitometrically following standard cellulose acetate electrophoretic separation.

Equilibrium Dialysis

Equilibrium dialysis was accomplished in standard scintillation vials to which 14.4 ml phosphate buffer (pH 7.4) in saline was added. A 0.5-ml plasma sample was added to a smaller cellophane dialysis sack. These sacks were suspended in the buffer and the experiment initiated by the addition of 100 µl of 3H-d-tubocurarine chloride (New England Nuclear) or 14C-

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metocurine iodide (Amershams) of desired concentration and specific radioactivity to the surrounding buffer. Volume of buffer, plasma and labeled relaxant in the scintillation vial totalled 15 ml. The data reported in this study were collected at the clinically relevant final free concentration of 0.5 μM of neuromuscular relaxant in phosphate buffered saline. Vials were sealed and placed on a rotary shaker (Labline Junior Orbit Shaker©), covered with aluminum foil to exclude light, and shaken at 150 rpm for 18–24 h. At room temperature (20°C), equilibrium was reached in 18 h. Protein assay indicated that no protein leakage from the sack occurred during the experiment. Aliquots (50 μl) of plasma from the sack and of the surrounding buffer were pipetted with calibrated micropipettes (Micropet©; Clay Adams) into 2-ml Ultrafluor® scintillation cocktail (National Diagnostics) in glass vials. The samples were then counted with a Beckman® LS 8100 scintillation counter. Raw counts per min (cpm) were converted to disintegrations per min (dpm) on the basis of channels ratio data produced by the scintillation counter and predetermined efficiency curve for Ultrafluor®. The per cent fraction of dTc or mTc bound was calculated simply as the difference in dpm of bag and buffer aliquots divided by the dpm of the bag aliquot. Knowledge of the specific radioactivity of the dTc and mTc in stock solutions allowed the calculation of total moles of drug bound per bag. Quantitation of total plasma protein per bag permitted calculation of moles dTc bound per gram plasma protein. Three separate experiments for each sample of donor plasma were typically run and the resultant data averaged. Variations between experiments were small (0–5 per cent).

If the smaller molecular weight substances such as peptides or free fatty acids were responsible for the increased binding, these would not be retained within the dialysis sack and would pervade into the surrounding buffer causing a lowering of the binding capacity. To test this possibility, an aliquot of plasma was dialyzed exhaustively against three changes of phosphate buffered saline (1-liter changes every 12 h). Subsequently, binding analyses were undertaken in the well dialyzed and undialyzed fractions.

The unpaired Student's t test was used to test for significance. When appropriate, correlation coefficients were determined by least square regression.

**Results**

*dTc Binding*

The results of equilibrium dialysis with ³H-dTc are summarized in table 1. The burned patients studied

| Table 1. Binding of dTc by Plasma from Burned Patients and Normals at 0.5 μM (0.34 μg/ml) Final Free Concentration |
|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|
| Age (Yrs) | Extent of Burn (Per Cent) | Post-Burn Interval (Days) | Per Cent dTc Bound | Total Plasma Protein (g/dl) | Plasma Binding Capacity (nmol Tc/g Plasma Protein) |
| Burned Patients (n = 16) | | | | | |
| Range | 3–90 | 25–96 | 7–100 | 61–79 | 4.5–8.3 | 4.3–7.4 |
| Mean ± SD | 26.4 ± 22 | 59.5 ± 27 | 28 ± 26 | 71 ± 5* | 6.2 ± 1.1† | 5.7 ± 0.9† |
| Correlation with dTc binding | r = 0.24 | r = 0.01 | r = 0.11 |  |  |  |
| Controls (n = 11) | | | | | | |
| Range | 28–52 | 43–57 | 51 ± 5 | 7.3 ± 0.9 | 2.9–4.7 |
| Mean ± SD | 29.4 ± 8.9 |  |  |  | 3.4 ± 0.7 |

* P < 0.0005.

† P < 0.005.

| Table 2. Plasma Binding of mTc in Burned and Normal Subjects at 0.5 μM (0.45 μg/ml) Final Free Concentration |
|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|
| Age (Yrs) | Extent of Burn (Per Cent) | Post-Burn Interval (Days) | Per Cent dTc Bound | Total Plasma Protein (g/dl) | Plasma Binding Capacity (nmol Tc/g Plasma Protein) |
| Burned Patients (n = 7) | | | | | |
| Range | 3–46 | 40–80 | 5–19 | 44–54 | 5.3–7.7 | 1.8–4.9 |
| Mean ± S.D. | 20 ± 15.8 | 65 ± 13 | 12 ± 5.7 | 52 ± 4* | 6.3 ± 1.1† | 4.1 ± 1.1† |
| Correlation with mTc binding | r = 0.08 | r = 0.14 | r = 0.57 |  |  |  |
| Controls (n = 5) | | | | | | |
| Range | 28–35 | 30–48 | 43 ± 9 | 7.5–8.1 | 2.4–3.0 |
| Mean ± SD | 30 ± 3.0 |  |  | 7.9 ± 0.2 | 2.7 ± 0.2 |

* P < 0.005.

† P = 0.05.
were heterogeneous with respect to age, sex, surface area of burn, and the time post-burn when binding was studied. However, all patients in whom $dTc$ binding was studied and included in table 1 were at least one week post-burn. There was no apparent correlation between any of the clinical variables described and the extent of $dTc$ binding (table 1).

Despite significantly lower total plasma protein, burned patients bound an average $71 \pm 5$ per cent of the available drug at $0.5$ $\mu M$ (0.34 $\mu g/ml$) final free concentration as compared to $50 \pm 5$ per cent for control plasma. Expressed as moles $dTc$ bound per gram plasma protein, plasma from burned patients bound approximately $1.7$ times ($5.7 \pm 0.9$ nmol/g) the amount of $dTc$ as normal plasma ($3.4 \pm 0.7$ nmol/g) (table 1). These differences were significant and persisted up to thirteen weeks post-burn.

### mTc Binding

The smaller patient population used for the metocurine-binding study was likewise heterogeneous. No correlation was shown between age, or extent of burn, and per cent mTc bound (table 2). A slight correlation with post-burn interval was evident. Again, regardless of a lower total plasma protein, burned patients bound an average $52 \pm 4$ per cent of mTc at a total free concentration of $0.5$ $\mu M$ (0.45 $\mu g/ml$) as compared to $43 \pm 9$ per cent for normal plasma (table 2). Expressed as moles mTc bound per gram plasma protein, plasma from burned patients ($4.1 \pm 1.1$ nmol/g) bound approximately $1.5$ times the amount of available mTc as normal plasma ($2.7 \pm 0.2$ nmol/g). As in the case for $dTc$, the elevation in per cent mTc bound in burned patients persisted to the end of the observation period, 19 days (table 2).

### Contribution of Diffusible Components to the Binding

The dialysis technique assumes that the plasma components relevant to $dTc$ binding were large proteins retained within the dialysis sack. This assumption proved correct as the aliquot of burned patient plasma dialyzed exhaustively to deplete the plasma and buffer of any hypothetical small binding molecules and the native undialyzed plasma bound identical amounts of $dTc$ regardless of their prior treatment. If, in fact, small molecular weight substances were responsible for the increased binding, these would have pervaded into the surrounding buffer causing a lowering of the binding capacity by plasma.

The catalyst Tris-(2-butoxyethyl)-phosphate (TBE), found in the rubber stoppers of Vacutainer® tubes, has been shown to be responsible for spuriously low results regarding the binding of imipramine and alpranolol to $\alpha_1$-acid glycoprotein. Investigations of similar effects on $dTc$-plasma binding indicated only a slight (2–5 per cent) decrease in binding of the drug after Vacutainer® storage of both control and patient plasma relative to identical aliquots stored in plastic tubes.

### Protein Profiles

In an effort to define the contribution of each component of plasma protein to the increased binding in the larger population of sixteen patients studied at least one week post-burn and reported in table 1, $dTc$ binding was correlated with total protein measurements and its various electrophoretically separated components. Interestingly, total protein and plasma fractions correlated poorly with $dTc$ binding. Similarly, in the three patients in whom multiple samples were obtained, the onset of elevated plasma binding of $dTc$ was compared to the changes in plasma protein fractions. While changes in the relative contribution of each major protein fraction were observed, except for albumin, absolute values always remained within normal range² (table 3). The $dTc$ binding was similar to controls on day 1 post-burn and peaked about 7–14 days post-burn. It is of interest to note that there was unidirectional consistent change in the $dTc$ binding and the changes in the mean relative fractions of $\alpha_1$ and $\gamma$-globulin despite absolute amounts being within normal levels (table 3).
PLASMA BINDING OF dTc FOLLOWING BURNS

Discussion

Our data indicate that the proportion of dTc bound to plasma is 1.7-fold greater in burned patients compared to control subjects. Previous studies have demonstrated that the total plasma concentration of dTc at 50 per cent twitch depression is 2.3 \( \mu \)g/ml (3.3 \( \mu \)M) in burned patients, compared to 0.45 \( \mu \)g/ml (0.66 \( \mu \)M) in controls: a 5.1-fold increase. Can this decreased response be caused by increased plasma protein binding? Only a small fraction of this altered response could be accounted for by increased plasma binding of dTc. In fact, if binding were to completely explain the decreased response, a total concentration of only 0.78 \( \mu \)g/ml would result in 50 per cent twitch depression. The absence of changes in binding following exhaustive dialysis and the minimal changes in binding following storage in rubber stoppered Vacutainer\( ^{\circledast} \) tubes indicate that our experiments did not underestimate the extent of dTc binding by plasma. Thus our observation that binding of dTc by plasma is responsible for only a small part of the observed hyposensitivity seems secure.

The origin of this dTc hyposensitivity remains to be elucidated. It might be traceable to one or several lesions in the normal distribution and/or synaptic binding of the drug in response to burn trauma. It is clear that the dTc is maintained in the circulation of the burned patient.\(^2\) We have shown that a higher unbound dTc concentration is available in burned patients for transcapillary diffusion to the neuromuscular junction. Yet the standard dosage of neostigmine is sufficient to reverse curarization as determined by muscle-twitch tension recovery despite the abnormally high concentrations of circulating dTc,\(^2\) and arguing against the reduced availability of all drugs to the synapse.

The elevated total plasma concentration of dTc required for a given twitch depression\(^2\) suggests that neither a burn-related increase in volume of distribution as seen in liver disease\(^9\) nor leakage of the drug through the burn wound\(^8\) is relevant to dTc hyposensitivity. Increased glomerular filtration rate associated with major burns affects tobramycin pharmacokinetics.\(^9\) However, renal clearance affects only the duration and not the plasma level required for clinical curarization.\(^1\)

Thus, it appears as if the major burn-related defect would be expected to lie in the delivery of the dTc specifically to the neuromuscular junction, or in the function of the junction itself. In principle, alterations of either topography or permeability of a capillary bed might reduce the availability of intravenous dTc to the synapse. Alternately, a postulated increase in the number of junctional receptors in response to burn-induced denervation\(^1\) or decreased affinity of existing receptors for tubocurarine could easily explain the elevated demand for dTc. More detailed studies will be required to answer these and similar questions raised by this work.

Except for total albumin which decreased, quantitative changes in other protein fractions remained within normal limits. However, it is important to note that despite a lower total plasma protein there was a small but significant burn-related increase in dTc binding. Expressed as dTc bound per gram of plasma protein, burned patients bound approximately 1.7 times more than normals. Therefore, it is logical to conclude that a qualitative change in the subfraction(s) capable of causing increased binding is present without necessarily causing quantitative increases in the protein profile. Alpha\(_4\) and gamma-globulin are likely candidates. Alpha\(_4\) acid glycoprotein, a component of alpha\(_2\)-globulin, is an acute phase reactant reported to cause increased binding of cationic drugs following inflammation-induced changes in its concentration.\(^12\) Thus it is possible that alpha\(_1\)-acid glycoprotein is increased following thermal injury. Similarly, Stovner suggested that the free phenolic hydroxyl groups of dTc should allow binding of this drug to gamma-globulins and demonstrated a positive correlation between dose requirement and gamma-globulin levels.\(^13\) Although relative increases in alpha\(_4\) and gamma-globulins paralleled changes in dTc binding, both alpha\(_4\) and gamma-globulin fractions were within normal range on an absolute basis as is true for the other major plasma protein fractions we have studied (table 5). Therefore the possibility remains that the increase of a single subcomponent of a protein fraction which could cause increased binding is effectively masked by overall decline in the total protein.

We have presented data obtained by the equilibrium dialysis technique that indicate a 1.7-fold increase in dTc binding by plasma constituents occurring one to two weeks following burn injury which may contribute significantly towards, but only partially account for, the clinically observed dTc hyposensitivity documented previously.\(^2\) We cannot attribute these clinical observations primarily to increased retention of d-tubocurarine within the circulation; three times the necessary “free” concentration of dTc is available in the plasma even when the burn-related increase in plasma binding is accounted for. Either the circulating unbound dTc is unable to diffuse freely and quantitatively to the neuromuscular junction, or other more fundamental changes at the target tissue perhaps involving the synaptic membrane as
suggested by the succinylcholine-induced hyperkalemia, are responsible for the increased clinical demand for dTc in burned patients.

References