Evidence for inhibitory and anti-ouabain-like factors in leukaemic blood

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Summary

1. At a high dilution in Ringer solution (1:200), leukaemic plasma significantly \( (P < 0.05) \) decreased the ouabain-sensitive and increased ouabain-insensitive components of sodium efflux from erythrocytes. At a low dilution (1:10) leukaemic plasma predominantly decreased the total and ouabain-insensitive component of sodium efflux \( (P < 0.01) \).

2. Erythrocytes from patients with leukaemia had a high affinity for the plasma factor which inhibited the total and ouabain-insensitive efflux (inhibitory factor).

3. Washings of leukaemic erythrocytes which had been incubated in leukaemic plasma contained a factor which significantly decreased the ouabain-sensitive and increased ouabain-insensitive components of sodium efflux (the anti-ouabain-like factor).

4. These studies show that leukaemic blood contains two factors which have opposite effects on sodium efflux from erythrocytes. These factors may contribute to the high incidence of multiple electrolyte disturbances in acute myeloid leukaemia.

Key words: electrolyte disturbances, erythrocytes, leukaemic plasma, ouabain, sodium transport.

Introduction

Plasma from patients with acute myeloid leukaemia inhibits sodium efflux from erythrocytes [1] and further experiments have shown that leukaemic plasma, diluted in Ringer solution (1:200), decreases the inhibitory effect of ouabain on sodium efflux. These findings raise the possibility that leukaemic plasma contains two factors: one inhibitory on sodium efflux and the other anti-ouabain-like in its effects. The purpose of the present study was to attempt to define these two factors further by investigating the effects of normal and leukaemic plasma on sodium efflux from erythrocytes.

Materials and methods

Blood samples

Blood samples were taken from 22 patients with acute myeloid leukaemia and from 42 healthy laboratory, nursing and medical staff. The samples were collected into heparinized syringes (100 units/ml of blood).

Sodium efflux from erythrocytes

The method is based on that of Villamil et al. [2] and has been described previously [1]. Briefly, washed erythrocytes in Ringer solution were loaded with \( ^{22}\text{Na} \) (0.3 \( \mu\text{Ci/ml of erythrocytes} \)) and added to the prewarmed (37°C) Ringer solution to give a packed cell volume (PCV) of less than 5%. Samples were taken in duplicate at 30, 60 and 90 min and the radioactivity was counted in portions of the suspension and supernatant after centrifugation at 5000 g. The values of \( 1 - (\text{supernatant counts/suspension counts}) \) were plotted against time; the slope was derived by the method of least-squares and the half-time \( (t_{1/2}) \) was calculated from this slope. The sodium efflux rate constant (that is the fraction of intracellular sodium extruded each
hour; $K_{Na}$ was derived from the equation,

$$K_{Na} = 0.693/t_{0.5},$$

The sodium efflux rate ($M_{Na}$ mmol h$^{-1}$ l$^{-1}$) was calculated as the product of the rate constant and the mean of the pre- and post-incubation erythrocyte sodium concentrations.

**Dilution experiments**

The effects of high and low dilutions of plasma in Ringer solution on the sodium efflux rate constant were investigated in five experiments each conducted in duplicate. In each experiment the leukaemic and normal plasmas were added to two separate pairs of flasks. For one pair the plasmas were used in a final dilution of 1:200 in Ringer solution, whereas for the other the plasma dilution was 1:10. Ouabain (0-1 $\mu$mol/l) was added to one of each pair of flasks to determine the ouabain-sensitive component of sodium efflux rate constant. Control flasks contained Ringer solution, but no plasma.

**'Baiting' protocol**

Leukaemic plasma, depending on its dilution in Ringer solution, either decreases sodium efflux or antagonizes the effect of ouabain on sodium efflux. These findings suggest the presence in leukaemic plasma of two factors which will be called the inhibitory factor and the anti-ouabain factor. Our previous studies showed that leukaemic erythrocytes have a high affinity for the inhibitory factor [1] so that initial washings from leukaemic erythrocytes should be rich in this factor. After the incubation of the washed leukaemic erythrocytes with leukaemic plasma, subsequent washes of these erythrocytes may contain both the factors, but would contain mostly the anti-ouabain-like factor as the inhibitory factor with a higher affinity for the erythrocyte membrane would have been removed in the previous wash. The following 'baiting' protocol was designed on these assumptions.

Leukaemic blood (50 ml) was obtained in a heparinized chilled syringe and immediately transferred to plastic tubes chilled on ice. Blood was centrifuged (1000 g) at 4$^\circ$C for 15 min and plasma was separated; 10 ml of this plasma was set aside as 'untreated' plasma (see Fig. 1). The erythrocytes were washed three times in Ringer solution by centrifugation and aspiration. In some experiments these three washes were pooled and saved as the source of this inhibitory factor. Washed erythrocytes were divided into 3 ml portions in separate tubes to which ABO compatible leukaemic or normal plasma was added and the mixture was incubated for 2 h at 37$^\circ$C with periodic shaking. These tubes were then spun at 1000 g for 5 min and the plasma was set aside as 'treated' plasma. The erythrocytes were then washed three times and the wash was pooled as the 'catch' containing mostly anti-ouabain-like factor. It was anticipated that the washes from the erythrocytes would contain plasma, but the erythrocyte portions (3 ml) and the quantities of washing Ringer solution (5 ml) were both kept constant in all the washing procedures. These steps ensured that the contamination with plasma was the same in the 'wash' containing the inhibitory factor and in the 'catch' containing the anti-ouabain-like factor.

Sodium efflux experiments were carried out on normal group O erythrocytes (unless otherwise stated). The test sample (2 ml of 'treated/untreated' leukaemic/normal plasma or 7 ml of normal/leukaemic 'wash/catch') was added to 1 ml of erythrocytes and the total volume was made up to 20 ml with Ringer solution. 'Baiting' and flux experiments were carried out in the same week, because preliminary experiments showed that the inhibitory and anti-ouabain-like factors were not stable for more than a week at 4$^\circ$C.

**Erythrocyte sodium determination**

Erythrocytes were washed three times with choline chloride solution and after the final wash choline chloride was aspirated leaving 1 ml of cell volume with a PCV of about 40%. The PCV was measured precisely in a micro glass-tube after centrifugation. To the erythrocyte sample was added 10 ml of saponin solution (20%, w/v) and the sodium concentration in the haemolysate was measured directly with a Corning Eel 450 flame photometer. Standards contained K$^+$ and Na$^+$ (36 and 2-4 mmol/l respectively). Haemoglobin was estimated as cyanmethaemoglobin at 541 nm with Drabkin's reagent.

**Solutions**

All solutions were made up to an osmolality of 290 ± 5 mmol/kg of water. The Ringer solution (pH 7.4) contained (mmol/l): NaCl, 131; KCl, 4; MgSO$_4$, 1; Na$_2$HPO$_4$, 7.2; NaH$_2$PO$_4$, 1.8; CaCl$_2$, 2; glucose, 10. This concentration of KCl is approximately the same as has been used by other workers in flux experiments [2-4] and was chosen because it is normal for human blood.

The choline chloride that was used to wash the loaded erythrocytes contained (mmol/l): choline chloride, 151; MgCl$_2$, 1; CaCl$_2$, 2-2 (in Tris buffer at pH 7.4).

Ouabain (strophanthidin-G) was dissolved in
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Ringer solution without glucose to give a final concentration of 0.1 mmol/l. The saponin solution (20%) was 2 g of saponin in 10 ml of choline chloride.

Statistical methods

All values are expressed as means ± SEM. Experiments were conducted in pairs (normal against leukaemic) and results were compared by Student’s paired t-test [5].

Results

Effects of diluted leukaemic and normal plasmas on sodium efflux

Leukaemic plasma at a 1:10 dilution in Ringer solution compared with normal plasma significantly decreased the ouabain insensitive sodium efflux rate constant (P < 0.01). There was a small but statistically insignificant decrease in the total rate constant and increase in the ouabain-sensitive rate constant (Table 1). Conversely, at a high dilution (1:200) leukaemic plasma significantly decreased (P < 0.05) the ouabain-sensitive efflux rate constant to 0.169, as compared with 0.247 in normal plasma at the same dilution. Thus at a low dilution leukaemic plasma inhibits the ouabain-insensitive efflux rate constant, whereas at a high dilution leukaemic plasma has an anti-ouabain-like effect. ‘Baiting’ experiments were performed to separate these two factors.

'Baiting' experiments

Treated and untreated plasmas. In this series of experiments the effect of leukaemic and normal plasmas (1:10 dilution) on sodium efflux were compared with the effects these plasmas had after they had been incubated with group O leukaemic erythrocytes at a PCV of 40%. The results are set out in Table 2.

Treatment of normal plasma decreased its effect on the ouabain-sensitive component by

<table>
<thead>
<tr>
<th>Table 1. Effects of normal and leukaemic plasmas in two dilutions on sodium efflux rate constant (O(^{K_{Na}}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>The plasma/Ringer solution ratio is shown against each set of data. Results are means ± SEM of five experiments in each group. Significance of differences: *P &lt; 0.05; **P &lt; 0.01.</td>
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<tr>
<td><strong>K_{Na}</strong></td>
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<tr>
<td>Ringer solution alone</td>
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<tr>
<td>Diluted leukaemic plasma</td>
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<td>1:200</td>
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<tr>
<td>1:10</td>
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<tr>
<td>Diluted normal plasma</td>
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<td>1:200</td>
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<tr>
<th>Table 2. Effects of ‘untreated’ and ‘treated’ normal and leukaemic plasma on sodium efflux rate constant (O(^{K_{Na}}))</th>
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<tbody>
<tr>
<td>Plasmas were ‘treated’ by incubation with group O leukaemic erythrocytes to encourage the binding of the erythrocyte of the ouabain-like inhibitory factor in the plasma. Results are means ± SEM of seven experiments. Plasmas were diluted 1:10 in Ringer solution. Significance of difference: *P &lt; 0.05.</td>
</tr>
<tr>
<td><strong>K_{Na}</strong></td>
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<tr>
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</tr>
<tr>
<td>Normal plasma</td>
</tr>
<tr>
<td>Untreated</td>
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<tr>
<td>Treated</td>
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<tr>
<td>Leukaemic plasma</td>
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<tr>
<td>Untreated</td>
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<tr>
<td>Treated</td>
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TABLE 3. Effects of normal and leukaemic plasma 'catches' on sodium efflux rate constant (\(^{\circ}K_{Na}\))

'Catches' were obtained by washing the leukaemic cells after incubation with normal or leukaemic plasma. Results are means ± SEM of seven experiments. Significance of differences: *P < 0.05; **P < 0.02.

<table>
<thead>
<tr>
<th>'Catch'</th>
<th>Total</th>
<th>Ouabain-sensitive</th>
<th>Ouabain-insensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal plasma</td>
<td>0.496 ± 0.03</td>
<td>0.280 ± 0.02**</td>
<td>0.216 ± 0.02</td>
</tr>
<tr>
<td>Leukaemic plasma</td>
<td>0.467 ± 0.01</td>
<td>0.197 ± 0.02</td>
<td>0.270 ± 0.01*</td>
</tr>
</tbody>
</table>

TABLE 4. Effects of normal plasma, leukaemic plasma and Ringer 'catches' on sodium efflux rate constant (\(^{\circ}K_{Na}\))

'Catches' were prepared and used as described in the Materials and methods section. Results are means ± SEM of 17 experiments. Significance of differences: *P < 0.05; **P < 0.01.

<table>
<thead>
<tr>
<th>'Catches'</th>
<th>Total</th>
<th>Ouabain-sensitive</th>
<th>Ouabain-insensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ringer</td>
<td>0.445 ± 0.05</td>
<td>0.262 ± 0.04</td>
<td>0.183 ± 0.03</td>
</tr>
<tr>
<td>Normal plasma</td>
<td>0.455 ± 0.03</td>
<td>0.286 ± 0.03</td>
<td>0.169 ± 0.03</td>
</tr>
<tr>
<td>Leukaemic plasma</td>
<td>0.392 ± 0.02*</td>
<td>0.192 ± 0.01**</td>
<td>0.200 ± 0.02</td>
</tr>
</tbody>
</table>

Effects of leukaemic and normal plasma 'catches' on sodium efflux

Experiments conducted with leukaemic plasma 'catch' showed a slight and statistically insignificant fall in the total sodium efflux rate constant. The ouabain-sensitive component was significantly lower (P < 0.02) and the ouabain-insensitive component was significantly higher (P < 0.05) than with normal plasma 'catch' (Table 3).

The leukaemic plasma 'catch' therefore contained an anti-ouabain factor, but not an inhibitory substance, which presumably remained bound to leukaemic erythrocytes during the wash.

'Baiting' experiments with normal and leukaemic plasmas and Ringer 'catches'

To explore the effects of 'catches' on sodium efflux, 17 more experiments were carried out with normal and leukaemic plasmas and Ringer solution. 'Catches' were prepared after incubation with leukaemic group O erythrocytes and used in sodium efflux experiments on the day the 'baiting' experiments were performed. Table 4 shows the results of these experiments. Neither normal plasma 'catch' nor Ringer 'catch' had any significant effect on sodium efflux, but leukaemic plasma 'catch' caused a significant reduction (P < 0.05) in the total sodium efflux rate constant and in the ouabain-sensitive component (P < 0.01). There was no significant change in the ouabain-insensitive component. The results of these 17 experiments differ very little from the results of the seven earlier experiments summarized in Table 3.

Effects of leukaemic erythrocyte 'wash'

Leukaemic erythrocytes were washed three times with Ringer solution which was pooled. This 'wash' was divided into two and efflux experiments were carried out with and without ouabain. This 'wash' was expected to contain the inhibitory factor. The washed leukaemic erythrocytes (3 ml) were incubated with 4.5 ml of leukaemic plasma. The wash from these cells after incubation was expected to contain anti-ouabain-like factor. Seven of these experiments were performed and the results are shown in Fig. 1. As compared with the normal erythrocyte efflux in Ringer (Fig. 1, step 3), leukaemic erythrocyte 'wash' caused a significant fall in the ouabain-insensitive component (P < 0.05), a slight fall in the total sodium efflux rate constant, and a slight increase in the ouabain-sensitive component about as much as it increased its effect on the ouabain-insensitive component, but these differences were not significant. Treatment of leukaemic plasma caused a significant decrease on the ouabain-sensitive efflux rate constant from 0.320 ± 0.02 to 0.258 ± 0.02 (P < 0.05) and the ouabain-insensitive component increased slightly but insignificantly from 0.149 ± 0.02 to 0.173 ± 0.02 (Table 2).
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FIG. 1. Multistage sodium efflux experiments showing the evidence for the existence of two distinct transport modifying factors in leukaemic blood. The data are expressed as total (T), ouabain-sensitive (OS), and ouabain-insensitive (OI) efflux rate constants. This protocol was designed (see the Materials and methods section) to separate the inhibitory and anti-ouabain-like factors. The effects of ‘wash/catch’ on sodium efflux are interpreted as inhibitory (reduction in OI component) and as anti-ouabain-like (reduction in OS component). (1) Sodium efflux from unwashed leukaemic erythrocytes. (2) Leukaemic erythrocytes washed three times with Ringer solution. (3) Efflux from normal erythrocytes in Ringer solution. (4) Efflux from normal erythrocytes as in (3) with leukaemic erythrocyte ‘wash’. (5) Efflux from normal erythrocytes with untreated leukaemic plasma in Ringer solution (1:10). (6) Leukaemic plasma and washed leukaemic erythrocytes incubated at 37°C for 2 h. (7) Efflux from normal erythrocytes as in (5) with postincubation leukaemic plasma in Ringer solution (1:10). (8) Leukaemic erythrocytes washed in Ringer solution. (9) Efflux from normal erythrocytes in Ringer solution. (10) Efflux from normal erythrocytes as in (9) with leukaemic erythrocyte wash. (11) Efflux from washed leukaemic erythrocytes.

These effects are similar to those shown by leukaemic plasma at 1:10 dilution (see Table 1). The leukaemic plasma ‘catch’ (steps 9 and 10) caused a significant fall in the ouabain-sensitive component from 0.280 to 0.197 (P < 0.02). This has the effect produced by leukaemic plasma at a high dilution (Table 1). The sodium efflux rate constant of unwashed leukaemic erythrocytes was 0.441 ± 0.01 with an ouabain-sensitive component of 0.301 ± 0.02 (step 1). After various washes the total sodium efflux rate constant had increased to 0.541 ± 0.01 (P < 0.05) (step 11), due to an increase in the ouabain-insensitive component.

Sodium efflux in the presence of ouabain (1 mmol/l)

The effect of the leukaemic erythrocyte ‘wash’ (containing the inhibitory factor) and the postincubation ‘catch’ (containing the anti-ouabain factor) on sodium efflux were also measured in the presence of ouabain (1 mmol/l). The inhibitory factor exerted an inhibitory effect in
addition to ouabain and the anti-ouabain factor reduced slightly the ouabain-induced inhibition from 1.84 ± 0.05 (inhibitory factor and ouabain) to 1.58 ± 0.07 mmol h⁻¹ l⁻¹. Although a statistical significance was not achieved, the results of these six experiments suggest that the inhibitory factor exerts most of its effect on ouabain-uninhibited component and anti-ouabain-like factor decreases ouabain-induced inhibition in spite of a high ouabain concentration.

Net fluxes

In four experiments normal erythrocytes were incubated in duplicate for 6 h with ouabain, inhibitory factor and anti-ouabain-like factor. Neither the inhibitory factor nor the anti-ouabain-like factor showed any significant influence on net sodium efflux transport.

Discussion

The present study confirms our previous finding that leukaemic plasma exerts an inhibitory effect on sodium efflux from erythrocytes. The effect of leukaemic plasma on the ouabain-sensitive and insensitive components varied according to the extent of dilution of the plasma in Ringer solution; at a high dilution (i.e. 1:200) the ouabain-inhibitable component was reduced (anti-ouabain effect), whereas at a low dilution (1:10) the ouabain-insensitive component was reduced. The possibility that there might be two distinct factors in leukaemic plasma was explored by 'baiting' studies.

The multistage 'bait' and 'wash' studies confirmed the presence of the two factors with some distinct features. The factor capable of inhibiting sodium efflux (the inhibitory factor) has its main effect on the ouabain-insensitive component. It inhibits total sodium efflux rate constant even in the presence of a high concentration of ouabain (1 mmol/l). Experiments on erythrocyte ghosts have shown that, unlike ouabain, this factor does not inhibit the Na⁺, K⁺-activated ATPase (M. A. Mir, unpublished work). The erythrocytes gained no more sodium when incubated with the inhibitory factor and ouabain, than when incubated with ouabain alone. All the evidence therefore suggests that this factor has a mechanism of action different from and unrelated to that of ouabain.

There are two features which suggest that the inhibitory factor has a high affinity for the erythrocyte membrane. First, leukaemic erythrocytes before washing show a lower than normal efflux rate constant and it improves after washing (Fig. 1, steps 1 and 11) and the 'wash' contains the inhibitory factor; secondly, erythrocytes have a higher than normal number of binding sites or a higher affinity for ouabain as reported previously [1]. Because of a high affinity of the inhibitory factor for the erythrocyte membrane it was mostly attached to the erythrocyte membrane and leukaemic plasma seemed to contain only a small amount of it. Washed leukaemic erythrocytes picked up a factor which was mostly in the plasma, was obtainable in the subsequent wash and showed an anti-ouabain effect (Fig. 1, steps 6, 8–10).

The factor capable of exerting an anti-ouabain effect reduced the ouabain-inhibitable component of sodium efflux from normal erythrocytes (Table 2; Fig. 1, steps 8, 10). This factor reduces the inhibitory effect of ouabain presumably by preventing ouabain from reaching the binding sites, but unlike ouabain it does not inhibit net sodium transport. The factor seems to be capable of binding to the erythrocyte membrane, since the anti-ouabain effect is present in the erythrocyte wash ('catch') after these cells pick up this factor during incubation with leukaemic plasma. The facts that the anti-ouabain factor can antagonize the effect of ouabain on efflux and can also be separated by incubation of leukaemic erythrocytes with leukaemic plasma, suggest that this factor also binds to the erythrocyte membrane. Once bound to the cell membrane, it either reduces the number of sites available to ouabain or reduces the effect of ouabain.

From the results of the present studies it is not possible to be certain whether the inhibitory and anti-ouabain-like factors bind to separate sites or to the same site. The inhibitory factor does not appear to act on a site different from ouabain, and the anti-ouabain factor either prevents the accessibility of the binding sites to ouabain or reduces the ouabain effect. These facts suggest, but do not prove, that the two factors bind to two different sites with negative co-operativity between them; once the inhibitory factor binds to its site it reduces the binding of the anti-ouabain factor. The present methods of 'baiting' have identified the two effects, but the washes are crude and the effects not completely separable; the factors need to be purified for more definitive studies.

The present study demonstrates that leukaemic blood contains two factors which influence sodium transport in erythrocytes. These factors may have similar effects on sodium transport in other cells. Patients with acute myeloid leukaemia
are known to develop widespread electrolyte disturbances [6–9]. Hypokalaemia occurs in 59% of the patients [9] and hyponatraemia in as many as 75% [8]. Other metabolic disturbances such as metabolic alkalosis, hypocalcaemia, hyperphosphaturia, hypo-uricaemia and hypomagnesaemia, have been widely reported to occur in acute myeloid leukaemia [10, 11]. The development of these widespread disorders suggests that there may be a generalized membrane disorder or that there may be circulating substances which act on various cell systems (i.e. kidney, muscle, heart etc.) and interfere with their membrane function.

Acknowledgments

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References