PLASMA INHIBITORS OF THE ERYTHROCYTE HEXOSE MONOPHOSPHATE SHUNT IN URAEMIA

J Zingraff, P Kamoun, P Lebreton, T Drüke, N K Man, P Jungers

Hôpital Necker, Paris, France

Summary

Sulphaemoglobin production, induced by an oxidative stress (ascorbate and cyanide) has been studied in uraemic patients. Results are expressed as the ratio of optic density of sulphaemoglobin (620nm) to optic density of total haemoglobin (540nm). The mean (± SEM) ratio found was 0.35 ± 0.03 in 28 controls and 0.56 ± 0.03 in 51 uraemic subjects (p<0.001). Cross incubation tests demonstrated that the anomaly was caused by a plasma factor. In vitro studies — guanidinic compounds added to control erythrocyte suspensions before incubation — suggest that this factor might be guanidinic propionic acid.

Introduction

Increased susceptibility to oxidant-induced haemolysis was reported in haemodialysis patients more than five years ago [1]. Contaminants (chloramines) of dialysis water were identified [2,3] and incriminated in the oxidative denaturation of haemoglobin but the underlying defect was believed to be of endogenous origin [4]. The anomaly can be transmitted to normal, non-uraemic erythrocytes by cross incubation of these cells in plasma of affected patients suggesting that the defect is due to a circulating substance accumulating in the plasma of some uraemic patients. Influence of dialysis against oxidant-free dialysate has been reported highly efficient in removing the substance in vitro but without any effect in vivo [4,5]. A toxic role has been attributed to guanidinic compounds to explain haemolysis in some patients with end-stage renal failure [6]. The purpose of this study was to demonstrate the presence of a plasma ‘toxin’ inducing in red blood cells a defect in oxidant defence and to find out if this factor possibly could be a guanidinic metabolite.
Patients and Methods

Blood from 51 end-stage renal failure patients has been studied. All but 5 were treated by long-term haemodialysis performed by means of a disposable plate kidney, thrice (34 patients) or twice (12 patients) weekly. Dialysate water was softened or deionised but detectable chloramine concentrations were never found. None of the patients took medications currently known to be oxidant drugs.

The control group consisted of 28 non-uraemic, non-anaemic subjects.

Erythrocyte oxidant sensitivity has been studied by a modified ascorbate cyanide test [1,7]. Ten ml of heparinised blood was centrifuged and haematocrit was adjusted to 32% by removal of either plasma or red blood cells. Two ml were then mixed with 50 micromoles of sodium ascorbate and 28 micromoles of glucose and 100 micromoles of the following solution (pH 7.0) was added:

- 1 volume of monopotassium disodium phosphate buffer (30mmol/L)
- 1 volume of sodium cyanide (0.5mmol/L)
- 3 volumes of 1/6 N HCl

After incubation for 3 hours at 37°C with linear stirring, 0.2ml of the erythrocyte suspension was added to 10ml of a phosphate buffer, pH 6.6 (67mmol/L) and centrifuged at 3000 rpm. To the supernatant was first added 50 micromoles of a freshly prepared solution containing equal volumes of 2mol/L sodium cyanide and 2.1 N acetic acid and finally 50μl of 15 N NH4(OH) prior to spectrophotometric reading at 620nm (‘sulphaemoglobin’). For the second reading at 540nm (haemoglobin) 2ml of this solution was mixed with 8ml of the phosphate buffer (pH 6.6) containing 50μl of freshly dissolved potassium ferricyanide (0.6mol/L). Prior to the reading 50μl of the cyanide, acetic acid solution was added. The results, expressed as the ratio of the optic densities OD 620nm, does not represent the percentage of produced ‘sulphaemoglobin’.

Cross incubations have been performed on washed erythrocytes suspended in non autologous plasma without preincubation. The influence of plasma dilutions has been studied by mixing variable amounts of uraemic plasma with control plasma. Guanidinic compounds have been tested in control red blood cell suspensions added before incubation to a final concentration of 20 micromoles/L.

Results

The mean value (± SEM) of the OD 620nm/OD 540 nm ratio found in the uraemic patients was 0.56 ± 0.03, significantly different from the mean ratio in the control group: 0.35 ± 0.03 (p<0.001). The values are indicated in Figure 1. No influence on the mean results of the ascorbate cyanide test was noted for dialysis frequency (twice versus thrice weekly), dialysis membranes (polyacrylonitrile RP 5 versus Cuprophan RP5) nor for the quality of water treatment: softened or deionised water, nor did we find different mean results in the 5 patients not yet on dialysis whose values varied widely (from 0.21 to 0.90) as compared with the haemodialysis treated group. No correlation was found between the results of the test and haematocrit of the subjects studied. Kinetic studies demonstrated that the differences observed between uraemics and controls were significant.
Figure 1. Ascorbate guanide test in 51 uraemic subjects (B) as compared with 28 controls (A). The erythrocytes are all tested in autologous plasma after the first hour of incubation. The test was repeated 5 times during one year in one patient and in one control subject, showing a wide variability of the results. However, the mean value of the control was significantly lower (0.26 ± 0.05, range 0.13 — 0.43) than that of the patient (0.55 ± 0.05, range 0.43 — 0.69), n = 5, p<0.05.

Results of cross incubations are indicated in Table 1. They demonstrate clearly that the disorder of uraemic erythrocyte metabolism is induced by a plasma factor and that after washing red blood cells — even from affected patients — they

<table>
<thead>
<tr>
<th>Cross Incubation: 1 hour</th>
<th>Uraemic RBC</th>
<th>Control RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uraemic plasma</td>
<td>0.30 ± 0.05</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>n = 8</td>
<td>p &lt; 0.02</td>
<td>p &lt; 0.02</td>
</tr>
<tr>
<td>Control plasma</td>
<td>0.18 ± 0.01</td>
<td>0.18 ± 0.02</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cross Incubation: 3 hours</th>
<th>Uraemic RBC</th>
<th>Control RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uraemic plasma</td>
<td>0.47 ± 0.07</td>
<td>0.46 ± 0.06</td>
</tr>
<tr>
<td>n = 8</td>
<td>p &lt; 0.01</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>Control plasma</td>
<td>0.32 ± 0.05</td>
<td>0.32 ± 0.05</td>
</tr>
</tbody>
</table>

Ratio OD 620/540nm Mean ± SEM
exhibit normal results using the ascorbate cyanide test when they are incubated in normal plasma. Moreover progressive diluting of 'toxic' plasma with normal plasma showed a striking dose related effect when tested on control red blood cells. The results of addition of guanidinonic compounds to red blood cell suspensions of 4 control subjects are indicated in Figure 2. Guanidinonic propionic acid appeared to be toxic in these in vitro experiments at a final concentration of 20 micromoles/L.

Discussion

The modified ascorbate cyanide test [7] has been proposed to explore the activity of the erythrocyte hexose monophosphate shunt (HMP shunt). The products accumulating during the test are cyanmethaemoglobin and several products resulting from haemoglobin oxidation. They are grouped under the name of sulphhaemoglobin'. The determination of the ratio of optical densities at 620nm and 540nm makes the test semi-quantitative although this expression does not indicate the percentage of produced sulphhaemoglobin. Increased values of the ratio

![Graph showing OD 620nm/OD 540nm for different conditions]
indicate a deficient erythrocyte oxidant defence of affected patients, exposing them to increased haemolysis [5,8]. The present study showed a significant increase in erythrocyte oxidant susceptibility in an important number of uraemic patients. These test results agree with the finding of increased peroxide induced haemolysis in uraemic red blood cells [9] as ascorbate, in the presence of oxy-haemoglobin generated H₂O₂.

In our series we found — in contrast to Yawata et al [1,4] — no correlation between the results of the test and the patients' haematocrit but the group studied was very heterogenous, including patients with polycystic kidneys, anephrics and iron deficient patients.

Purification of urban water by chlorinated products is unusual in Paris and on several occasions we found no chloramine contamination in the tap water. This may easily explain why the quality of water treatment did not influence sulph-haemoglobin formation in our experience. Based on the results of cross incubation tests and on the evidence of a dose related effect we conclude that the anomaly is conferred upon erythrocytes by one or more plasma toxins present in excess in some uraemic patients. We thus confirm the results of previously reported studies [1] and suggest that one of these toxins might possibly be guanidinic propionic acid.

Acknowledgment

This work was supported in part by Grant No. 78 5205 5 from the INSERM.

References

2. Yawata, Y, Kjellstrand, CM, Buselmeier, TJ, Howe, R and Jacob, HS (1972) *Trans. ASAIO*, 18, 301
4. Yawata, Y and Jacob, HS (1975) *Blood*, 45, 231

Open Discussion

LEBER (Giessen) Cyanide and ascorbate when added to erythrocytes inhibit the catalase system and increase the generation of oxygen radicals. I cannot see why your results are related to the HMP-shunt, because the glutamine peroxidase system functions normally under uraemic conditions indicating that there is enough MADPH to detoxify the created oxygen radicals.

ZINGRAFF We did no other biochemical studies, just the ascorbate cyanide test but the Minneapolis group studied hexosemonophosphate shunt and established
the relation between abnormal test results and inhibition of the HMP shunt, so I believed the link was clear to everybody.

VAN DER HEM (Chairman) What is the molecular weight of the substances you suspect are guilty?

ZINGRAFF I don’t remember the exact molecular weight but I believe it is in the range between 200 and 300 daltons.