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Short communication

In vivo anti-complement effect of bilirubin-IXα

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Abstract

The effect of the IXα isomer of unconjugated bilirubin (UB) on complement-mediated intravascular hemolysis was evaluated in rats carrying naturally occurring heteroantibodies against sheep erythrocytes. Several doses of UB were administered i.v. to these animals in order to induce different levels of hyperbilirubinemia. Intravascular hemolysis was promoted by transfusion with a sheep red cell suspension. Hemoglobin in urine was assessed as a marker of intravascular hemolysis. The urinary excretion of hemoglobin was attenuated by UB in a dose-dependent manner. To establish whether complement was involved in the hemolytic reaction, we evaluated the hemolytic activity of complement in these same animals, before and after sheep erythrocyte transfusion. The significant consumption of complement, which was partially prevented by UB, corroborated its participation in the intravascular hemolytic reaction in the current experimental conditions. The data suggest an inhibitory action of UB on complement-mediated hemolysis *in vivo*.

Keywords: Unconjugated bilirubin; Complement; Intravascular hemolysis; Hemoglobinuria

1. Introduction

Complement is a major effector of the immune response, and is involved in many tissue-damaging processes [1]. The complement system can be activated through either of two distinct enzymatic cascades, the so-called CP [2] and the alternative pathway [3]. The proteins of the CP have been grouped into three functional units, namely (a) the recognition unit, C1, constituted by C1q, C1r, and C1s; (b) the activation unit, represented by C4, C2, and C3; and (c) the membrane-attack unit represented by C5–C9. The CP is generally initiated by the interaction of C1q with complexes constituted of IgM or IgG and antigens [2]. When the antigen is present on the surface of red blood cells, *in vivo* activation of complement may lead to intravascular hemolysis [4].

In a previous study, it was demonstrated that the IXα isomer of UB, the major bile pigment produced in the catabolism of hemoproteins [5], inhibits the complement hemolytic cascade *in vitro*, by acting particularly on the C1 component [6]. We have shown that UB interferes with the interaction of IgG or IgM with C1q, probably as a consequence of a direct binding of the pigment to this subcomponent. To evaluate whether the anti-complement property of UB may ameliorate complement-mediated hemolysis *in vivo*, we studied the ability of the pigment to prevent an acute hemolytic reaction induced by transfusion of sheep red cells into rats carrying naturally occurring heteroantibodies capable of complement fixation. UB levels were increased artificially in these animals through i.v. infusion of the pigment. The data indicate that increased concentrations of the pigment in serum attenuated the hemolytic capacity of complement in a dose-dependent manner.

2. Materials and methods

2.1. Chemical reagents

UB (predominantly the IXα isomer) was purchased from the Sigma Chemical Co. All other chemicals were of analytical grade purity, and used as supplied.

2.2. Buffers

The following buffer was used: veronal-buffered saline (VBS) containing 142 mM NaCl, 5 mM sodium veronal,

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Abbreviations: UB, unconjugated bilirubin; CP, classical pathway.

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0.15 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.4 (VBS–Ca^{2+}–Mg^{2+}).

2.3. Erythrocytes

Sheep erythrocytes, collected in citrate–NaCl–dextrose solution [0.8, 0.42, and 2.05% (w/v), respectively], pH 6.1, were washed twice in 150 mM NaCl, followed by another wash in VBS–Ca^{2+}–Mg^{2+}. Finally, sheep erythrocytes were resuspended in this buffer.

2.4. Animals and treatment

Outbred female adult Wistar rats (National University of Rosario) with xenoreactive natural antibodies against sheep erythrocytes were used in these experiments. The *in vitro* complement fixation capacity of these antibodies was demonstrated previously by titration (data not shown). To establish whether UB can inhibit the hemolysis mediated by complement *in vivo*, the animals, previously made hyperbilirubinemic by UB infusion, received a single injection of a sheep erythrocyte suspension. All procedures were conducted in accordance with the NIH guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the National University of Rosario. Briefly, rats were anesthetized with sodium pentobarbital (10 mg/kg body weight), and the femoral vein was cannulated with polyethylene tubing (PE 50). A sample of blood was obtained from the tail vein, and used for the determination of complement hemolytic activity. The animals were then injected via the femoral vein with an initial UB dose of 0.5, 1.0, 1.5, or 3.5 μmol per 100 g body weight, dissolved in 1 mL of an NaOH–saline solution (pH 7.8–7.9), immediately followed by an infusion of the pigment of 0.03, 0.06, 0.10, or 0.25 μmol per 100 g body weight per min, respectively (UB-infused groups), or with the UB solvent (control group); these infusions were maintained throughout the experiment. Ten minutes later, 0.5 mL of a 40% suspension of sheep erythrocytes in VBS–Ca^{2+}–Mg^{2+} was administered i.v. to the same animals. Thirty minutes later, the rats were killed by cardiac puncture, and blood samples were collected. An aliquot of blood was allowed to clot at 4° for 4 hr, and was centrifuged (1500 *g*, 10 min, 4°) to obtain serum, while another aliquot was collected in a tube containing a 3.8% sodium citrate solution, and centrifuged (1500 *g*, 10 min, 4°) to obtain plasma. The hemolytic activity of complement was determined in samples of serum taken both before transfusion with sheep erythrocytes and at the end of the experiment. The UB concentration was measured in serum obtained at the end of the experiment by the method of Jendrassik and Grof [7]. Plasma samples were used for hemoglobin determination. Urine samples were collected at the end of the experiment from the urinary bladder by puncture, and the volume was determined. Urine samples were used for protein analysis and hemoglobin determination. An additional group of rats were injected with the sheep erythrocyte suspension solution (vehicle). Plasma and urine samples were collected, as described above, for hemoglobin determination.

All experimental procedures were carried out under subdued light, to minimize photoconversion of bilirubin to its photoisomers.

2.5. Determination of hemoglobin in plasma and urine

The content of plasma hemoglobin was determined by its capacity to catalyze the reaction between benzidine and hydrogen peroxide in an acidic medium, as previously described [8]. To analyze the presence of hemoglobin in urine, samples from the different groups were subjected to electrophoresis on a 1% agarose gel [9]. Protein bands were detected by staining with amido black, and identified using appropriate standards. The hemoglobin concentration was determined in the same samples [8]. Excretion of hemoglobin in urine was calculated as a product of hemoglobin concentration and the volume of urine.

2.6. Measurement of total hemolytic activity of complement

Measurement of the total hemolytic activity of complement in samples of serum was performed as previously described [10]. Hemolytic activity of the complement was expressed as CH50 units/mL, and the percent decrease after sheep erythrocyte transfusion was calculated.

2.7. Statistical analysis

Data are presented as means ± SD. Statistical analysis on plasma hemoglobin concentration and urinary excretion of hemoglobin was performed using one-way analysis of variance, followed by the Bonferroni test. Statistical analysis on the percent decrease in complement hemolytic activity between control and UB-infused rats was performed using the Mann–Whitney *U*-test. Values of $P < 0.05$ were considered to be statistically significant.

3. Results and discussion

Traditionally, complement has been thought to play an important role in the pathophysiology of immune intravascular hemolysis. Generally, antibodies that produce intravascular lysis of the majority of the red cells transfused are those that are lytic *in vitro*. When erythrocyte destruction is very rapid, most of the cells are lysed within 10 min and, as a result, hemoglobin is released into the plasma. If the production of hemoglobin exceeds the binding capacity of haptoglobin, free hemoglobin can be found in plasma and urine. As a consequence, excretion of hemoglobin in urine may be considered indicative of

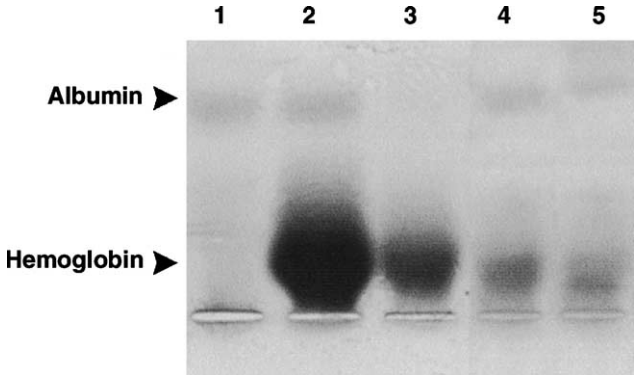

Fig. 1. Urine electrophoresis of sheep erythrocyte-transfused rats. Electrophoresis of urine samples was performed on a 1% agarose gel. Protein bands, stained with amido black, were identified as serum albumin and hemoglobin. Lane 1, normal rat urine; lane 2, urine from rats receiving the solvent of UB; lanes 3–5, urine from rats receiving UB, and exhibiting serum levels of 15, 46 and 304 μM UB on the average, respectively. Rats corresponding to lanes 2–5 were transfused with sheep erythrocytes.

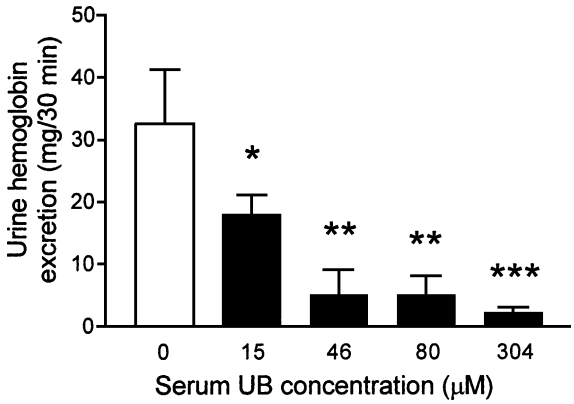

Fig. 2. Effect of sheep erythrocyte transfusion on hemoglobin excretion in urine. Rats were infused with increasing doses of UB (black bars) or with the solvent of UB (white bar). All animals were transfused with sheep erythrocytes. UB was not detectable in serum from animals receiving only the pigment vehicle (0 μM). Data represent means ± SD of 3–4 animals per group. Key: (*) significantly different from 0 μM UB, $P < 0.05$; (**) significantly different from 0 and 15 μM UB, $P < 0.05$; and (***) significantly different from 0, 15, 46, and 80 μM UB, $P < 0.05$.

intravascular hemolysis [4]. In this work, we promoted an acute hemolytic reaction mediated by complement in rats previously receiving an overload of UB, and determined both the level of hemoglobin in plasma and its urinary excretion. Thirty minutes after transfusion with sheep erythrocytes, we measured the hemoglobin concentration in plasma of control and UB-infused rats. The level of hemoglobinemia in controls was 1.77 ± 0.51 mg/mL (mean ± SD, N = 4). This value was not affected by UB overload (data not shown). Plasma hemoglobin in rats receiving the sheep erythrocyte vehicle solution (normal group) was lower than 0.01 mg/mL. Fig. 1 shows that urine from rats transfused with sheep erythrocytes exhibited a major protein band, not present in normal rat urine, that was identified as hemoglobin, which was decreased in intensity in the hyperbilirubinemic animals. The electrophoresis study also indicated that no additional proteins appeared in the urine, except for albumin, which was also present in normal urine. This suggests that hemoglobinuria resulted from glomerular filtration of free hemoglobin, rather than by indiscriminate passage of red cells through glomerular structures, due to an alteration of its barrier properties.

Data in Fig. 2 confirmed the protective effect of UB on intravascular hemolysis following sheep erythrocyte administration, and revealed an inverse relationship between the mass of hemoglobin excreted in urine and plasma UB levels. The reason why there was no difference in the plasma level of hemoglobin between control and UB-infused rats, despite the fact that hemoglobin urinary excretion was decreased in a dose-dependent fashion, is uncertain. It is possible that the peak of hemoglobin production occurred prior to the 30-min period we had chosen for blood sampling and/or that renal depuration is a very efficient process for free hemoglobin.

To confirm that complement was involved in the hemolytic reaction, we evaluated the hemolytic activity of complement in samples of serum from control rats and from rats infused with UB. The hemolytic activity of complement was determined before and after sheep erythrocyte transfusion in both groups, and the percentages of diminution were calculated. Complement hemolytic activity in serum from both groups decreased after red cell transfusion (see Fig. 3), indicating participation of complement in the intravascular hemolytic reaction under the current experimental conditions. The calculated percent decrease in complement activity was significantly lower ($P < 0.01$) in UB-infused rats [30 (23–42), median (range), N = 6] than in control rats [51 (46–56), median (range), N = 6]. The partial prevention by UB of complement activation and hemolysis agrees well with our previous findings *in vitro* [6].

We demonstrated here for the first time that increased levels of pigment in serum may attenuate the complement-

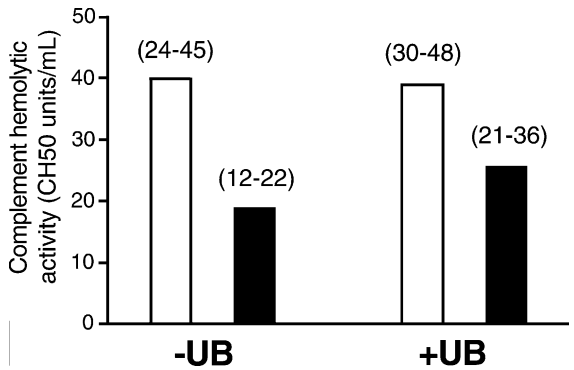

Fig. 3. Complement hemolytic activity in serum before and after sheep erythrocyte transfusion. The change in complement hemolytic activity induced by transfusion with sheep red cells was studied in rats infused with UB, exhibiting a pigment serum concentration of 80 μM, on the average, by the end of the experiment. The figure shows the CH50 in serum from rats receiving solvent (−UB) or UB (+UB), before (white bars) and after (black bars) transfusion with sheep erythrocytes. Data represent the median from 6 experiments per group. The range is shown in parentheses.

mediated hemolytic reactions *in vivo*. In a previous study, we speculated that UB binding to the C1q molecule, either at or near the site of interaction with IgM or IgG, may be involved in the inhibitory effect of UB on complement activity *in vitro* [6]. It is possible that this interaction between UB and C1q explains the inhibitory effect of the pigment on intravascular hemolysis. Considering that complement is involved in host defense against bacterial and viral invasion, the current data suggest that hyperbilirubinemia may contribute to increased susceptibility to infections.

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