UNCONJUGATED BILIRUBIN INHIBITS C1 ESTERASE ACTIVITY

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Abstract

We previously reported an inhibitory effect of unconjugated bilirubin (UB) on complement (C) hemolytic cascade “in vitro” and that the pigment interferes both the C1q-IgM and –IgG interactions. To assess the action of UB on the C1 esterase activity, we evaluated the ability of the pigment to inhibit the C1-mediated hydrolysis of N-acetyl-L-tyrosine ethyl ester. The data demonstrate that UB inhibited the C1 enzymatic activity in a dose-dependent manner. A dot-blot assay showed that [^{14}C]-UB is capable to bind C1r, C1s and human serum albumin to a similar extent. We conclude that UB inhibits the esterase activity of C1, likely as a consequence of a direct pigment-protein interaction. This effect, in addition to that previously observed on C1q, could explain, at least in part, the inhibitory action of UB on C activation by the classical pathway.

Key words: unconjugated bilirubin, C1 esterase activity, inhibitory effect.

1. Introduction

The first component of complement (C), C1, is a macromolecular complex of the three subcomponents, C1q, C1r, and C1s. Serum C1 is in a native or zymogen form that becomes activated when the C1q subcomponent binds to the Fc portion of immune complexes. This binding leads to proteolytic cleavage of the C1r and the C1s subcomponents that renders C1s capable of proteolytically activating the next components, C4 and C2 in the classical pathway of C (Cooper, 1985).

Both in humans and rats, 80% of bilirubin derives from senescent erythrocytes while the remaining 20% comes from degradation of other hemoproteins, such as myoglobin, tissular cytochromes, catalases and peroxidases (Berk et al., 1969). Through two enzymatic steps, the heme group is transformed into bilirubin IXα, the most abundant natural isomer (Blanckaert et al., 1976).

Our previous studies indicated that unconjugated bilirubin (UB) inhibits C-mediated hemolysis “in vitro” and that the inhibitory action of the pigment is mainly exerted on the C1 step. In addition, we showed that UB interferes the interaction of IgM or IgG with C1q, probably as a consequence of a direct binding of the pigment to this subcomponent (Arriaga et al., 1999). Since the action of UB on the enzymatic activity of C1 is yet unknown, it was of interest to evaluate the effect of the pigment on the esterase activity of this component.

2. Materials and Methods

A partially purified preparation of C1 was obtained from fresh human serum and the enzymatic activity of this component, previously activated, was evaluated by its ability to hydrolyze the N-acetyl-L-tyrosine ethyl ester (ATE) (Lachman and Hobart, 1978). The magnitude of hydrolysis of ATE was evaluated by H^+ formation, which was determined by pH measurement with a pocket pH-Meter (ad 110 pH, Wenzel-Heidelberg, Germany). Solutions containing UB (17, 34, 51 or 85 µM final concentration) were incubated at 37°C with 50 µL of 1 M ATE. All mixtures were brought to the same pH (7.9) by addition of adequate volumes of 0.02 N NaOH and to the same volume (1 mL) with esterolytic assay medium (0.9% NaCl solution brought to pH 7.2 with 0.2 M phosphate buffer pH 7.2). Ten µL aliquots of the C1 preparation were added and after each addition 2 min were allowed to elapse to see if any hydrolysis of substrate occurred. The volumes of C1 necessary to bring the solution to pH 7.0 was evaluated in each case. Inhibition percentages were calculated by comparison with those of controls without UB.

[^{14}C]-UB (15.1 Ci/mol) was prepared biosynthetically (Ostrow et al., 1961) from bile of rats infused intravenously with 5-amino [^{14}C]–levulinic acid. The binding of C1r or C1s to UB was evaluated by dot-blot technique (Stott, 1989). HSA was used as positive control since its specific interaction with UB is well known (Brodersen, 1980). Human IgG was used as negative control, since previous experiments showed no binding of UB to IgG (data not shown). Equimolar concentrations of commercial C1r and C1s, human serum albumin (HSA) or human IgG were applied

onto polyvinyldifluoride membranes and incubated with 171 µmol/L UB containing traces of [^{14}C]-UB. At the end of incubation, the membrane was rinsed with phosphate buffered saline pH 7.4, and the spots eluted in 3 mL OptiPhase "Hifase" 3. Radioactivity was measured in a liquid scintillation counter (Rack Beta 1214, Pharmacia, Turku, Finland) and expressed as desintegrations per minute.

3. Results and Discussion

Data in Fig 1 show that UB inhibited the C1 esterase activity at physiopathological concentrations and in a dose-dependent manner ($p<0.05$, Bonferroni test). Maximal inhibitory activity was reached at a 51 µM UB concentration. The data on dot-blot study is shown in Table 1. The highest activities of [^{14}C]-UB were associated to HSA as expected, and also to C1r and to C1s. Far less radioactivity was found to be associated to the negative control IgG ($p<0,05$, Bonferroni test). Whether C1r and C1s compete with HSA for the binding to the common ligand UB "in vivo" is not known, but it is important to note that the concentration of HSA in normal serum is about 250 times higher than those of C1r and C1s (Whicher, 1978). This could tentatively explain why the inhibitory action of UB on C-classical pathway becomes apparent "in vivo" only at high concentrations of the pigment, compatible with hyperbilirubinemic disorders, as demonstrated (Arriaga et al., 2002).

UB presents a dianionic structure conferred by two carboxylic acids. Our findings are in agreement with those of Bureeva et al. (2005) demonstrating that low weight

negative charged compounds are capable to inhibit C1q, C1r and C1s, being both hydrophobic and electrostatic interactions, involved.

In conclusion, we demonstrated that the inhibitory action exerted by UB on the classical pathway is due not only to an interference on the interaction of the C1q subcomponent with IgM or IgG (Arriaga et al., 1999) but also to a diminished enzymatic activity of the C1 esterase.

ACKNOWLEDGEMENTS

This work was supported financially by Research Grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and from the Universidad Nacional de Rosario (UNR).

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FIGURE LEGENDS

FIGURE 1. Inhibitory effect of unconjugated bilirubin (UB) on C1 esterase activity. The effect of UB on the enzymatic activity of C1 was evaluated by the ability of the pigment to inhibit the C1-mediated hydrolysis of N-acetyl-L-tyrosine ethyl ester (ATE). The magnitude of hydrolysis of ATE was evaluated by direct pH measurement. Each value represents the mean ± SD of 3 experiments per group.

*Significantly different from incubations containing 17 and 0 μmol/L UB ($P < 0.05$, Bonferroni).

**Significantly different from incubations containing 34, 17 and 0 μmol/L UB ($P < 0.05$, Bonferroni).

Table 1. Binding of unconjugated bilirubin (UB) to C1r and C1s.

Protein	Radioactivity (DPM)
C1r	4575 ± 686 *
C1s	4646 ± 697 *
HSA	5016 ± 822 *
IgG	668 ± 87

[a] Equimolar concentrations of commercial C1r and C1s, human serum albumin (HSA) or human IgG were applied onto polyvinyldifluoride membranes and incubated with 171 µmol/L UB containing traces of [^{14}C]-UB. Radioactivity was then assessed, and expressed as desintegrations per minute (DPM). Values are means ± SD of 3 experiments per group. *Significantly different from IgG ($P<0.05$, Bonferroni).