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Unconjugated Bilirubin Effect on ^{3}H-Ouabain Binding to Human Fetal Red Cells

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Human fetal red cells show heterogeneity of ^{3}H-ouabain binding sites. These cells were chosen as a model to look into unconjugated bilirubin effects on the primary active Na^+–K^+ transport mechanism. Evidences are presented suggesting that unconjugated bilirubin affects ^{3}H-ouabain binding but not through a direct effect. This is supported by the fact that the "low affinity" subgroup sites of the last mentioned ligand persists after unconjugated bilirubin treatment of cells, whereas the "high-affinity" subgroup disappears.

Key words: Ouabain Binding, Fetal red Cells, Bilirubin.

Red cells from the late fetal (neonatal) haemopoietic stage differ in structural and functional characteristics from adult red cells (7). The first mentioned cells, "natural targets" of circulating ligands, e.g. unconjugated bilirubin (ub), during the perinatal period influence their distribution into tissues (1, 12, 17).

Multiple cellular effects have been reported induced by ub, such as metabolic (22) and ionic transport function (5, 6, 24) impairments. Among the latter mentioned effects, an inhibition of the ouabain-sensitive potassium flux fraction is well substantiated (16).

^{3}H-ouabain has been widely used as a marker of Na^+–K^+ primary active transport sites due to its capacity to bind selectively to the polypeptidic chains of this polymeric transport mechanism (9). On account of the similarity of effect between ouabain and ub on the primary active Na^+–K^+ transport mechanism, a probable interaction between both ligands on these transport sites could be suggested.

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Unconjugated bilirubin could impair ^{3}H-ouabain binding by a direct (competitive) or by an indirect action. This last effect being related through acting on the phospholipidic environment of the Na-K primary active transport proteins (2, 19).

Fetal red cells, that present heterogeneity of ^{3}H-ouabain binding sites (present paper) like "ghosts" from adult red cells (3, 13) could be used as an experimental model to elucidate this problem.

This paper presents evidences that point out to the indirect mechanism of interference on ^{3}H-ouabain binding, as the more probable one.

Materials and Methods

Origin and general handling of samples. — Erythrocytes from umbilical cord blood were used within 12 hours after delivery. The red cells, collected in heparinized tubes, were freed from plasma and buffy coat after centrifugation.

Three alternate procedures (resuspension in isotonic saline media-centrifugation) were undertaken to free the cells from the remaining plasma.

Resuspension in incubation media. — The erythrocytes resuspended at a 0.5 (v/v) haematocrit in media of composition (mM): NaCl, 140; KCl, 0.1; $CaCl_2$, 0.05; Tris-HCl, 10 (pH 7.4, 37 °C); glucose, 10.

To this medium, variable amounts of ^{3}H-ouabain with or without ub were added to achieve the appropriate concentration of these ligands. Albumin was also added in order to give a 10:1 (ub:alb) concentration ratio.

Purity of unlabelled and labelled ouabain. — Unlabelled "cold" ouabain: Commercially available ouabain (Sigma) was dissolved in ethanol/benzene (9/1, v/v). Then it was desiccated under a nitrogen atmosphere. The powder thus obtained was diluted in isotonic, phosphate buffered, saline solution to give 10-100 µM ouabain concentrations.

The optical density (220 nm) was determined (DU-2 Beckman Spectrophotometer) in these solutions. The molar extinction coefficient is given by

$$\Sigma_M^{1cm} = d(OD)/d[OB]$$

where OD: optical density and [OB]: Ouabain concentration. The mean value obtained for this coefficient was 15422 ± 480 (n=5) (media ± SEM, number of determinations).

Tritiated ouabain: Commercially available ^{3}H-ouabain (New England Nuclear - USA), dissolved in chloroform:methanol: water (65:30:5, v/v) was checked by thin layer chromatography on silica gel.

A single patch of radioactivity was detected in all the radiochromatographic assays done. The radiochemical purity was within the specification claimed by the supplier.

The Σ_M^{1cm} values obtained were similar to those above mentioned for "cold" ouabain.

Specific activity of labelled ouabain.— Displacement experiments between "cold" and "labelled" ligands were undertaken using red cells (10). To cellular suspension (hematocrit v/v value lesser than 0.05) in saline buffered medium a constant aliquot of ^{3}H-OB was added plus variable aliquots of a concentrated (unlabelled) solution of ouabain to obtain variable concentrations (M) of this last mentioned ligand ($1x10^{-3}$–0.628×10^{-7}). After 90 min of incubation (37 °C) the amount of radioactivity bound to cells was measured in cell lysates.

The concentration of labelled ouabain was estimated from the value of unlabelled ouabain that reduced the labelled ouabain bound to cells to one half of its control (no "cold" ouabain added) value. The calculated labelled ouabain concentration amounted to 80 % of that claimed by the supplier.

Labelled ouabain bound to cells. — To 0.05 (v/v) hematocrit cellular suspensions, ^{3}H-OB were added in order to give concentrations within the 0.1-10 (10^{-7} M) range. These cellular suspensions were incubated (37 °C) for 60 min. Aliquots (100 µl) were then withdrawn, centrifuged (12000 x g, 15 s) and the supernatant was removed. Then the cells were freed from the unbound label by three consecutive procedures: resuspension (in ten times its volume with isotonic saline without label) –centrifugation-supernatant removal. The final washing solution did not differ from the background value when counted for tritium.

The labelled ligand bound to cells was counted by liquid scintillation spectrometry (LS-8000 Beckman) (18) in supernates (after centrifugation) of cell lysates treated with perchloric acid (10 %).

Specific ouabain binding to cells was determined from the total label bound by subtracting the unspecific binding (measured in cells incubated with ^{3}H-OB and 10^{-3} M non-labelled ligand). Unspecific /total binding amounted to less than 0.05 in all the experiments accomplished. The number of labelled ouabain molecules bound per cell was estimated according to GARDNER and CONLON (10).

Analysis of data. — Scatchard's plots analysis corresponding to one or two classes of binding sites (23) were done.

Labelled ouabain as well as unconjugated bilirubin concentrations were varied but at a constant 10:1 ([^{3}H-OB]: [ub]) ratio. ^{3}H-OB concentrations varied between 0.1 and 10 (10^{-7}) M.

The mean ± SE for duplicate determinations attained in samples from different individuals are presented.

A personal computer (AT IBM compatible) was used for solving data analysis with a computer program (ENZFITTER).

Chemicals. — Unconjugated bilirubin, albumin, ouabain, Tris were purchased from Sigma; all the other reagents were also of analytical grade.

Results

Binding of ^{3}H-OB (fixed concentration). Effect of ub.— The number of ^{3}H-OB molecules bound per cells at 0.1 • 10^{-7} M

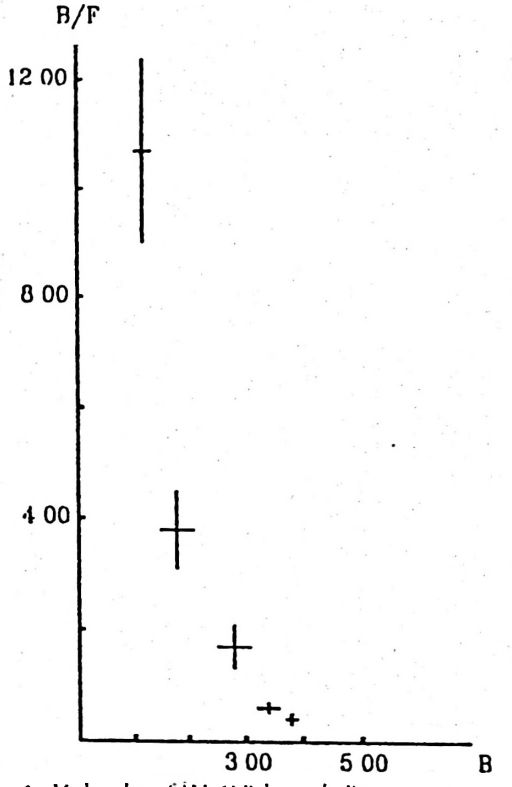

Fig. 1. *Molecules of ^{3}H-OB bound (B) per unit concentration of free (F) ^{3}H-OB (B/F) plotted as a function of B.*

B/F, B data: mean (intersection of vertical with horizontal lines and ± SEM (length of vertical and horizontal lines) are presented. Analytical expression assayed: $r = F \cdot Cap_1/(F+Kd_1) + F \cdot Cap_2/(F+Kd_2)$ r: number of molecules bound per cell; Cap_1, Cap_2: capacity of classes of sites 1 and 2; Kd_1, Kd_2: dissociation constant of sites 1 and 2. Two classes of sites with maximal number of bound ligand molecules per cell (capacity) of 330 and 74 were individualized with association constants (Kd^{-1}) values of 1.0 and 10.0 (10^{-7}, M^{-1}), respectively.

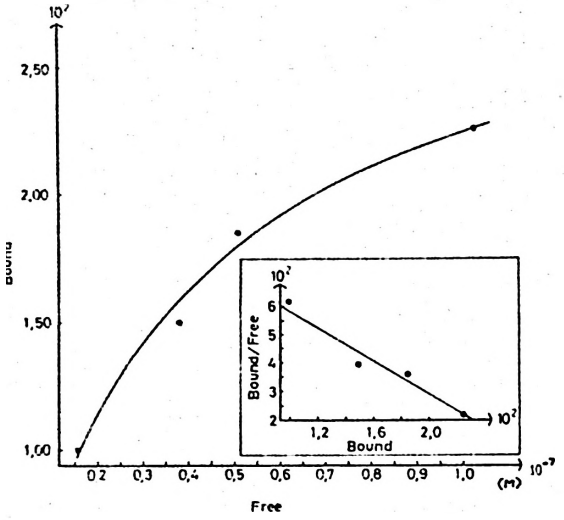

Fig. 2. *Effect of unconjugated bilirubin on ouabain binding.*
A Scatchard's plot analysis for one class of sites was assayed (r = F • Cap1/ (F+ Kd1) (significance of r; F • Cap1 and Kd1 as referred in legend to fig. 1). The corresponding values for Cap1 and 1/kd1 were 300 ± 20 (sites/cell) and 1.7 ± 0.1 (10^{-7}, M^{-1}), respectively. A representative experiment is presented.

ligand concentration (371 ± 28, n = 9), was described by 0.1 µM ub 235 ± 43, n=8 and with 50 µM ub, no further decrease was found.

Binding of ^{3}H-OB (variable concentrations). Effect of ub. — Two site groups of ouabain binding (in the absence of ub) here identified (high capacity -"low affinity" and "low capacity"- high affinity"). Binding capacities differed by a factor of three. Binding affinities differed by a factor of ten (fig. 1).

In the presence of ub, a lineal Scatchard analysis (one site) adequately fitted the data (fig. 2). The binding parameters of this remaining group of sites in the presence of ub was similar to that of the "high capacity - low affinity" type (in the absence of ub) above mentioned. The affinity constant amounted to 1.7±0.1 (10^{-7} M^{-1}) for experiments done under similar conditions in samples from four different individuals.

Discussion

The number of ^{3}H-OB binding sites in these cells were found to lie within the quantity previously referred to for adult cells (9).

^{3}H-OB binding to cells, analysed trough a Scatchard's plot, showed a concave-up curvature. Several causes for this non-linear Scatchar's plot (4, 20, 25) could reasonably be discarded: unspecific binding (it amounted to less than 10 % of the total binding and was discounted); chemical ("cold" –OB) or radiochemical (tritiated –OB) impurities of both ligand species that could give rise to a competence phenomenon; several sites of ^{3}H-OB binding on the transport mechanism, (a single site has been identified (per α-subunit) for ^{3}H-OB binding on transport ATPase (14); the low density of binding sites that make the existence of cooperating phenomena among sites very unlikely (9, 10).

Thus, it can be concluded that the concave-up Scatchard's plot could be related to heterogeneity of binding sites.

Within the limitations imposed by the experimental (and analytical) conditions here established, two classes of sites could be distinguished.

The very low density of ^{3}H-OB binding sites per unit of cellular surface makes the distribution of both classes of sites more feasible in different sub populations of cells.

It should be noticed that a "compact" life-span distribution (among different subpopulations) of cord blood red cells with reference to that from adult's blood has been previously reported (8, 21).

^{3}H-OB binds to the α polypeptidic chain of the Na^+–K^+ pump (14).

The hydrophobic character of ub explains its choice for lipidic phases. This, plus the fact that the Km value of ATPase

is not changed by ub would explain an indirect effect (alteration of the phospholipid environment) on the ^{3}H-OB binding to Na^+–K^+ "pump" sites (11, 15).

These facts could explain the finding that the decrease, in neonatal red cells-ub treated, of the number of ^{3}H-OB binding sites to two/thirds of its total value is due to the loss of the "high affinity-low capacity" class.

J. L. CORCHS, M. J. CORCHS y R. E. SERRANI. *Efecto de la bilirrubina no conjugada sobre la fijación de ^{3}H-ouabaina a eritrocitos fetales humanos.* Rev. esp. Fisiol. (J. Physiol. Biochem.), 50 (1), 5-10, 1994.

Los eritrocitos fetales humanos presentan heterogeneidad de los sitios de fijación de ^{3}H-ouabaína. Estas células se seleccionan como modelo experimental para el análisis de los efectos de la bilirrubina no conjugada sobre el mecanismo del transporte activo primario Na^+-K^+. Los resultados sugieren que la bilirrubina no conjugada afecta la fijación de ^{3}H-ouabaína, aunque no a través de un mecanismo de competencia directa. Después del tratamiento con bilirrubina persiste el grupo de sitios de unión de ouabaína referido como de "baja afinidad", mientras que el grupo de "alta afinidad" desaparece.

Palabras clave: Fijación de ouabaína, Eritrocitos fetales, Bilirrubina.

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