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The molecular basis of bilirubin encephalopathy and toxicity: Report of an EASL Single Topic Conference, Trieste, Italy, 1–2 October, 2004☆

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1. General considerations

1.1. Introduction

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The incidence of bilirubin-induced neurological dysfunction (BIND) in jaundiced newborns is likely increasing because of the early hospital discharge of the infants and the lack of defined criteria for, and mandatory reporting of, the diagnosis of BIND [1]. On the other hand, there have been recent advances in understanding the molecular mechanisms by which unconjugated bilirubin (UCB) enters and damages central nervous system (CNS) cells [2,3], suggesting new approaches for the early diagnosis, prevention and treatment of BIND. We, therefore, under the scientific sponsorship of EASL, organized an interactive workshop of experts in the fields of bilirubin metabolism, transport and toxicity, CNS structure and function, and the clinical management of jaundiced newborns. The presentations at the EASL Single Topic Conference held in Trieste on 1 and 2 October, 2004, summarized in this report, review what is known about BIND and provide guidance for future research.

1.2. Bilirubin-induced neurological dysfunction (BIND) and kernicterus: the clinical approach

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The new American Academy of Pediatrics Clinical Practice Guideline for the 'Management of Hyperbilirubinemia in the Newborn Infant 35 or More Weeks of Gestation' [1] encourages use of the term 'acute bilirubin encephalopathy' to describe the acute manifestations of UCB toxicity seen in the first weeks after birth, and the term 'kernicterus' to describe the chronic, permanent clinical sequelae of UCB toxicity. Specific recommendations include: identification of hemolysis using corrected end-tidal carbon monoxide measurements [4]; use of an hour-specific total serum bilirubin (TSB) nomogram [5]; improvement in the accuracy and precision of TSB measurements and correlation with transcutaneous bilirubin measurements [6] in newborns with differing risk factors for hyperbilirubinemia; evaluation of the safety and efficacy of pharmacologic therapies, such as the competitive inhibition of heme oxygenase (HO) with metalloporphyrins [7]; dissemination of the information in the Guideline; and monitoring Guideline compliance.

The Guideline does not address infants <35 weeks gestation, in particular, very low birth weight infants, who may be especially vulnerable to hearing loss and poor neurodevelopmental outcome, even with peak TSB levels as low as 10 mg/dL (171 μmol/L) [8]. Although neonatal jaundice is normally a benign condition, increased bilirubin production (e.g. hemolysis) or decreased bilirubin elimination

☆ The abstract of oral and abstract presentations together with a copy of the slides presented during each speech are available for consultation at the address, www.fegato.it/meeting.

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(e.g. conjugation defects) may engender dangerously high TSB levels, causing acute bilirubin encephalopathy and/or kernicterus in some infants. African-American infants have a high incidence of hemolysis due to glucose-6-phosphate dehydrogenase deficiency and deserve special attention [9]. Early recognition of these complications would be facilitated by development of a multifactorial index of risk for selection of neonates who need chemoprevention or phototherapy. Measurement of unbound (free) UCB in plasma (B_f), which is the key to bilirubin neurotoxicity, is fundamental to proper assessment of the risk of BIND in jaundiced neonates.

1.3. Bilirubin: physical chemistry, protein-binding and redox properties

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1.3.1. Physical chemistry

Internal hydrogen bonding of the fully protonated UCB diacid creates a folded, biplanar structure with both hydrophobic and polar regions arrayed radially [10]. This precludes stable insertion into membranes [11], limits aqueous solubility (70 nM) [10] and retards ionization of the –COOH groups, so that pK'a values are 8.1 and 8.4 [10]. At pH 7.4, over 80% of unbound UCB (B_f) is the toxic diacid species [10], that readily diffuses across membranes [12], including the blood–brain barrier [13].

1.3.2. Protein-binding

Binding to plasma albumin limits passage of UCB into the CNS [14,15]. The affinity of human serum albumin (HSA) for UCB is much lower than thought previously and decreases markedly with increasing [HSA] and [Cl^-] [16,17], the presence of competitive inhibitors, and probably acidosis. Applying the new affinity constants reveals that toxicity to CNS cells in vitro occurs only at $B_f > 70$ nM [18], but that UCB is neuroprotective at lower B_f values [19]. Toxicity thus is associated with formation of small UCB aggregates above aqueous saturation, and short exposure to high B_f is not a valid model for the clinically relevant condition of prolonged exposure at lower B_f [20]. Data are needed on the cerebrospinal fluid (CSF) and brain tissue concentrations of total and unbound UCB when toxicity occurs.

1.3.3. Redox properties

Conjugated and unconjugated bilirubin and biliverdin are potent antioxidants [21,22], even when bound to HSA [23]. They act by being themselves oxidized, consuming reactive oxygen species (ROS). The ability of very low [UCB] to protect cells against vastly higher concentrations of ROS is due to rapid enzymatic regeneration of UCB from biliverdin [24], new formation of UCB by heme oxygenase [25], and uptake of UCB dissociating from the very large reservoir of UCB bound to HSA in plasma.

1.4. How bilirubin crosses membranes and enters cells

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It remains unknown which, if any, transporter is responsible for the uptake of UCB by the hepatocyte [26,27]. The diffuse yellow staining of many tissues in jaundiced neonates suggests that the entry of UCB into non-hepatic tissues occurs via passive diffusion. The physicochemical characteristics of the amphiphilic UCB molecule facilitate its interaction with the surface of phospholipid bilayers [11] and there is minimal steric constraint to passive diffusion of UCB through cellular membranes [28]. Stopped-flow fluorescence techniques show that UCB, but not conjugated bilirubin, rapidly diffuses through model membranes and rat hepatocyte membranes, with a first-order rate constant of $5.3\ s^{-1}$ ($t_{1/2}$ 130 ms) [12]. As confirmed by data presented by **Richard A. Wennberg (University of Washington, Seattle, WA, USA)**, the uncharged UCB diacid diffuses rapidly and spontaneously across the bilayer of phospholipid vesicles, subsequently releasing its protons and acidifying the internal milieu of the vesicles [12].

Cellular uptake by simple diffusion can exhibit saturation kinetics, depending on the rate-limiting step [29]. Hepatocellular uptake of UCB is more rapid for UCB bound to HSA than to BSA (bovine serum albumin), despite a threefold higher binding affinity of HSA for UCB. This is consistent with 9× higher solvation (off) rates from HSA than from BSA and indicates dissociation-limited diffusion, in which the rates of UCB transfer are determined mainly by the relative concentrations of donor and acceptor molecules [15,28] (albumin and apolipoprotein D in plasma and ligandin in the cytosol).

Thus, membranes, including those comprising the blood–brain barrier, do not appear to be a significant barrier to the entry of UCB into cells. The passive diffusion of UCB across the blood–brain barrier is countered by the metabolism of UCB and active secretion of UCB (and/or its metabolites) into the plasma by the brain capillary endothelium [3,30]. Inferior function of these systems, rather than enhanced passive permeability of the blood–brain barrier to UCB, probably contributes to the enhanced risk of neurotoxicity in the newborn compared with adults [30], and in some strains of jaundiced Gunn rats as compared with others [31].

2. Mechanisms of UCB neurotoxicity

2.1. Role of UCB in altering the cytokine network and inducing apoptosis and cell death (Fig. 1)

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CNS cells exposed to UCB in vitro show mixed features of necrosis and apoptosis [32,33], with oxidative stress,