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Bilirubin Toxicity and Differentiation of Cultured Astrocytes

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OBJECTIVE:
To study the toxicity of bilirubin in primary cultures of newborn rat cerebral cortical astrocytes.

STUDY DESIGN:
Primary cultures of newborn rat astrocytes were incubated at bilirubin concentrations of 0, 1, 5, 10, 25, 50, 100, 200, and 2000 μM, at a bilirubin:albumin molar ratio of 1.7. Bilirubin toxicity was determined by changes in cellular morphology, trypan blue staining, and lactate dehydrogenase (LDH) release into the culture medium at various times of incubation. To determine if differentiation of astrocytes affects bilirubin toxicity, cultures were treated with dibutyryl cyclic adenosine monophosphate.

RESULTS:
All three indices of toxicity showed a bilirubin concentration dependence. LDH release in experimental cultures was significantly elevated ($p < 0.05$) above that of control cultures by 24 hours at bilirubin concentrations of $\geq$100 μM. The absolute amount of LDH release differed significantly between the 200 and 2000 μM cultures from 1.5 to 24 hours, after which duration of exposure appeared to take over and all cultures approached maximum. LDH release for the lower concentrations all reached maximum by 120 hours, except for the 1 μM cultures, which showed no significant elevation above control throughout the study period. At 100 and 200 μM bilirubin, LDH release by untreated cells was significantly higher ($p < 0.05$) than release by treated cells by 36 hours.

CONCLUSION:
Undifferentiated astrocytes appeared to be more sensitive to bilirubin toxicity, which may correlate with the greater susceptibility of newborns to kernicteric injury. Studies with primary astrocyte culture may provide insight into how bilirubin sensitivity changes with brain development as well as the cellular and biochemical mechanisms of bilirubin encephalopathy.

Hyperbilirubinemia-induced encephalopathy, i.e., kernicterus, has been well described.[1–4] However, the principal underlying mechanisms of bilirubin neurotoxicity have been much more difficult to determine despite extensive study. Currently, bilirubin is postulated to have a multifactorial role in cellular damage. Exposure to bilirubin has been associated with changes in energy metabolism; changes in carbohydrate, amino acid, and lipid metabolism; alteration in membrane function; decreased membrane potential; alteration in enzymatic function; and inhibition of protein and DNA synthesis.[1–4] Models that have been used to study these mechanisms include in vivo models such as hyperosmolar opening of the blood–brain barrier[5] and congenitally jaundiced rats,[3] or in vitro models, e.g., perfused brain sections,[6] synaptosome preparations,[7,8] neural-derived transformed cell lines,[9] and glial cell cultures.[10–12] We chose to study bilirubin toxicity in primary culture of astrocytes.

Investigation with primary culture of astrocytes has several advantages. It involves normal diploid cells with biochemical and morphologic features close to those found in vivo.[13] It is a simple system, consisting of a highly enriched preparation of astrocytes,[14] which eliminates interaction with other cell types and systemic factors that may confound delineation of the primary insults caused by bilirubin. The role of astrocytes in bilirubin encephalopathy has largely been neglected. Astrocytes comprise approximately one-third of the total cellular volume of the brain cortex in humans.[15] They are far more than a simple scaffolding on which neuronal cells grow. The biochemical properties of astrocytes have been fairly well established: they possess high metabolic activity, remove neuroactive compounds from the extracellular space, and are the exclusive or predominant site of glutamine synthesis in the brain.[15–17] Thus, astrocyte culture provides another model for examining the biochemical insults caused by bilirubin. Furthermore, morphologic and biochemical differentiation of cultured astrocytes can be induced by exposure to dibutyryl cyclic adenosine monophosphate (dBcAMP).[17,18] These morphologic changes include the projection of microfilament rings into newly radiating cell processes; these cultured cells then appear more like in

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vivo mature astrocytes. Besides morphologic changes, dBcAMP exposure also induces changes in receptor expression, protein synthesis, phosphorylation, and enzyme activity. If such differentiation alters sensitivity to bilirubin injury, further insight may be gained into the cellular mechanisms of bilirubin toxicity.

METHODS

Chemicals

Bilirubin (Lot 18F-0962, manufacturer's minimum purity 99%), human serum albumin (HSA) (fraction V, essentially free fatty acid (FFA)-free), and dBcAMP were obtained from Sigma Chemical Co. (St. Louis, MO). Stock solutions of 20 mM bilirubin and 1.33 mM HSA were prepared using 0.1 N NaOH and Dulbecco's modified Eagle's medium (DMEM), respectively. Bilirubin stock was then added to HSA stock in a 1:9 ratio, resulting in a bilirubin/HSA ratio of 1.7. This solution of 2000 μM bilirubin was titrated to a pH of 7.4 with HCl and subsequently diluted with DMEM to the desired bilirubin concentrations (2000, 200, 100, 50, 25, 10, 5, and 1 μM; equivalent to 117, 11.7, 5.8, 2.9, 1.8, 0.58, 0.29, and 0.58 mg/dl, respectively). Cultures were then incubated in these solutions and 100-μl samples were drawn at the desired times for lactate dehydrogenase (LDH) determination. Controls were also done, using DMEM and HSA solutions as incubation media. All procedures involving bilirubin were performed in a dimly lit room to prevent photoisomerization.

Cultures

The preparation of primary cultures of rat cerebral cortical astrocytes from newborn Sprague-Dawley rats (Veterans Affairs Medical Center animal facility; Palo Alto, CA) was described previously.[14,19] The neopallium, i.e., the portion of cortex dorsolateral to the lateral ventricles, was obtained aseptically from the brain. The neopallia were freed of meninges and cut into small cubes (<1 mm^3) in DMEM (Applied Scientific; San Francisco, CA). The tissue was disrupted by vortex mixing for 1 minute, and the suspension was passed through two sterile nylon sieves (Nitex; L. and S. H. Thompson, Scarborough, Ontario, Canada) with pore sizes of 80 and 10 μm. A volume of cell suspension equivalent to approximately one thirtieth of the cerebral hemispheres from one brain was placed in a 35-mm tissue culture dish (Falcon; Becton Dickinson, Oxnard, CA). Fresh DMEM supplemented with 20% fetal calf serum (HyClone Laboratories, Inc., Logan, UT) was added to yield a final volume of 2 ml. All cultures were incubated at 37° C in a 95%:5% (vol:vol) mixture of atmospheric air and CO_2 with 95% humidity. The culture medium was changed after 3 days of seeding and subsequently two times per week with DMEM containing 10% fetal calf serum. After forming a confluent monolayer, some cultures were treated with 0.25 mM dBcAMP for at least 1 to 2 weeks to produce morphologic and biochemical differentiation.[17] Cultures were used for experiments after they were at least 4 weeks old.

Trypan Blue Staining

The viability of the cells at 20 hours was evaluated by incubating them with trypan blue.[20] Nuclear uptake of stain was taken as an indicator of cell damage.

Lactate Dehydrogenase Determination

LDH activity in incubation medium was measured using the a reagent (LDH Reagent; Sigma Diagnostics, St. Louis, MO). Media obtained from cultures (90 μl) after various periods of bilirubin exposure were mixed with 0.5 ml of reagent. Changes in absorbance per minute at 340 nm were measured using a spectrophotometer (DU Series 70; Beckman Instruments, Inc., Fullerton, CA) at room temperature. One unit of LDH activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADH per minute under the conditions of this assay.[14] Maximal LDH release in a culture was determined by terminal extraction with distilled water.

RESULTS

Morphology and Staining

Figure 1 shows phase-contrast micrographs of cultures exposed to 0,100, 200, and 2000 μM bilirubin (Figure 1, *A*, *C*, *E*, and *G*, respectively) and light micrographs of the same fields (Figure 1, *B*, *D*, *F*, and *H*, respectively), all at 20 hours of exposure. Bilirubin exposure induced morphologic changes consistent with cellular injury. These include a shift from epithelium-like cells to process-bearing cells with dense cytoplasmic extensions, structural dissociation, cytoplasmic darkening and granulation, loss of cells, and cellular contraction, as evidenced by the increased space between adjacent cells. The degree of morphologic change depended on bilirubin concentration, with the most marked change occurring in the cultures exposed to 2000 μM bilirubin (Figure 1, *G*). In Figure 1, parts *B*, *D*, *F*, and *H* show that the degree of brown staining of the cytoplasm, cell membrane, and possibly the nucleus by bilirubin also increased with increasing bilirubin concentration. Cellular silhouettes can be seen in increasing degree, from no staining (Figure 1, *B*) to all cells being stained (Figure 1, *H*).

The same fields also show viability staining with trypan blue (Figure 1, *B*, *D*, *F*, and *H*). As with morphologic changes, trypan blue staining indicates bilirubin dose-dependent toxicity, with the greatest number of nuclei staining observed at the highest bilirubin concentrations.

Lactate Dehydrogenase Measurement

A control astrocytic culture contained approximately a maximum of 280 mU of LDH per μg of protein. Figure 2 shows the time course of LDH release into the medium by astrocytes in response to different bilirubin concentrations. LDH levels became significantly (two-tailed *t*-test, $p < 0.05$) elevated above control at 20.5 hours in cultures exposed to 200 and 2000 μM bilirubin, at 24 hours in cultures exposed to 100 μM bilirubin and at 48 hours in cultures exposed to 10 μM. Cultures given 2000 μM bilirubin had significantly ($p < 0.05$) greater LDH release than those given 100 μM from 1.5 hours on and those given 200 μM bilirubin from 1.5 to 24 hours. At 36 hours and later, however, LDH release in the 200 and 2000 μM cultures approached maximum (approximately 200 mU/culture), and no significant difference could be detected. LDH measurements for the 100 and

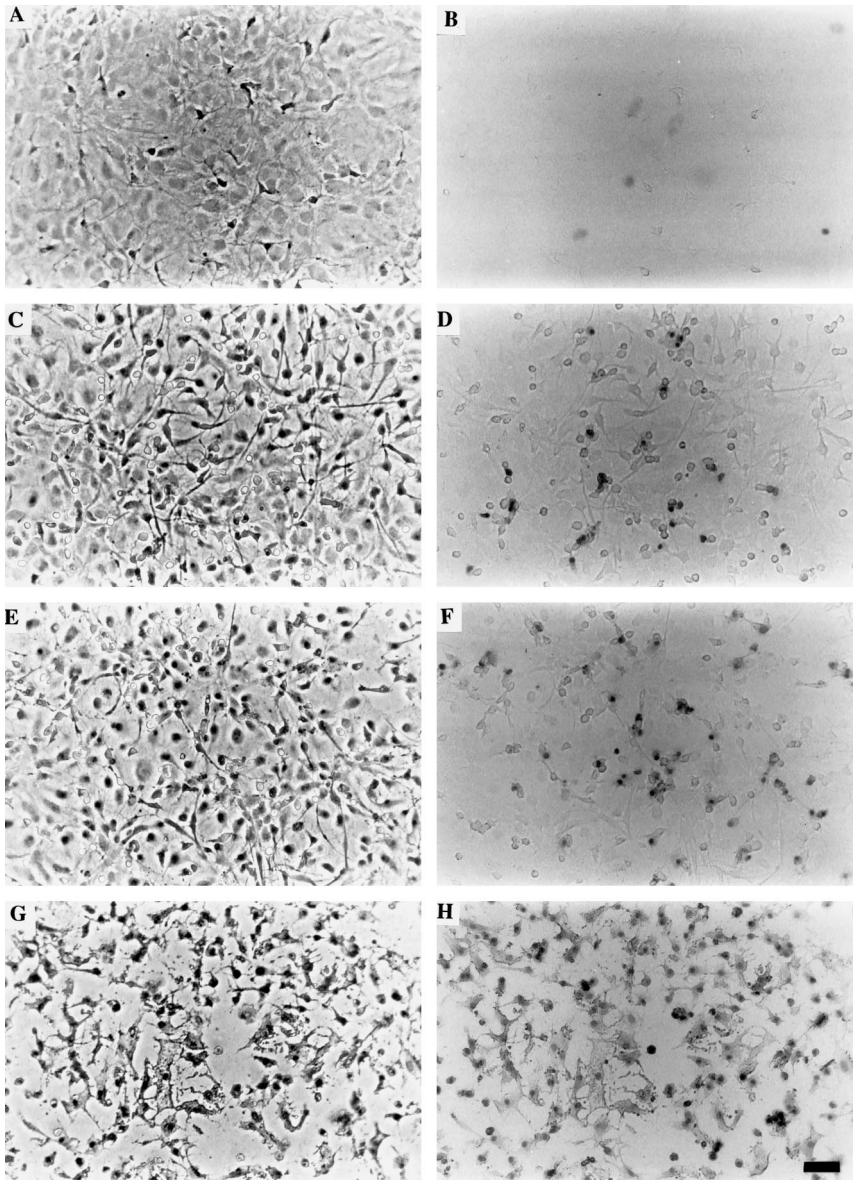

Figure 1. Morphology and staining of undifferentiated primary cultures of cerebral cortical astrocytes. Shown are phase-contrast micrographs of cultures incubated in DMEM **(A),** 100 μM bilirubin **(C),** 200 μM bilirubin **(E),** and 2000 μM bilirubin **(G)** at 20 hours. Note increased cytoplasmic darkening, granulation, and loss of cells (particularly in **G**) with increasing bilirubin concentration. Light micrographs of the same fields following viability staining with trypan blue, at 20 hours, are shown next to corresponding phase-contrast micrographs [DMEM **(B),** 100 μM bilirubin **(D),** 200 μM bilirubin **(F),** 2000 μM bilirubin **(H)**]. Note increased cytoplasmic and/or membrane staining by bilirubin with increasing bilirubin concentration. Trypan blue staining of astrocyte nuclei also increases with bilirubin concentration. The bar represents 5 μm.

200 μM cultures differed significantly ($p < 0.05$) at 20.5, 24, and 48 hours. LDH for the 100 and 200 μM cultures and the 2000 μM cultures was significantly ($p < 0.05$) elevated above that for the 10 μM cultures from at least 24 hours until the end of the study period (48 hours).

At bilirubin concentrations of 5, 10, 25, and 50 μM, LDH release was also concentration-dependent, and maximum levels were reached for these concentrations by 120 hours. These LDH levels were less than those at bilirubin concentrations of ≥100 μM (results not shown). Cultures exposed to 1 μM bilirubin showed no significant elevation in LDH release over control up to 96 hours.

Figure 3 shows the time course of LDH release into the medium by dBcAMP-treated and untreated astrocytes exposed to 100 (Figure 3, *A*), 200 (Figure 3, *B*), and 2000 μM (Figure 3, *C*) bilirubin. At 24 hours and later for 200 μM and at 36 hours and later for 100 μM bilirubin, LDH release per culture was significantly greater ($p < 0.05$ at 24 hours and $p < 0.01$ at 36 and 48 hours) in untreated cultures. For cultures exposed to 2000 μM bilirubin, LDH release was also reduced in dBCAMP-treated cultures versus controls, but these differences were not statistically significant. From 0 to 4 hours for the three concentrations pictured, dBcAMP-treated cultures actually had significantly ($p < 0.05$) higher LDH levels than untreated cultures.

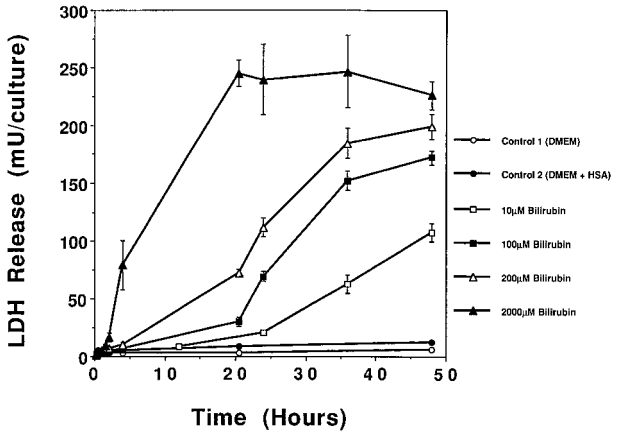

Figure 2. LDH release into culture medium as a function of time of incubation is shown. The data represent the pooling of several experiments and thus each point may represent three, five, or eight cultures. SEM values are shown by vertical bars. See text for multiple statistical comparisons of different concentrations and timepoints.

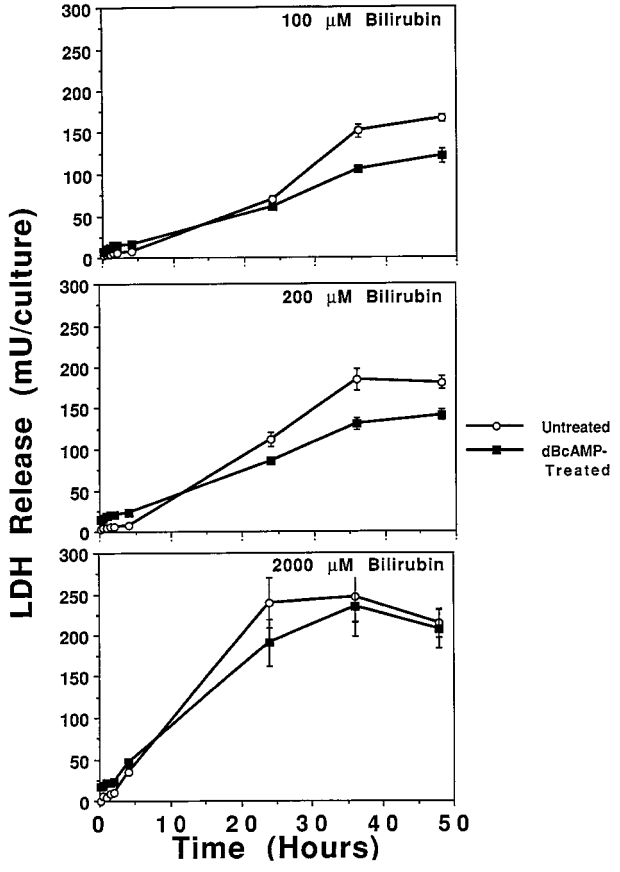

Figure 3. dBcAMP-treated and untreated astrocytes. LDH release into culture medium as a function of time of incubation in dBcAMP-treated and untreated astrocytes is shown. Each point represents five or eight cultures, and SEM values are indicated by vertical bars.

DISCUSSION

Bilirubin induces injury in primary cultures of astrocytes, as defined by our morphology, viability, and LDH release studies. Furthermore, the degree of injury induced is dependent on bilirubin concentration. The amount of morphologic change, staining, and LDH release increased with greater bilirubin concentrations.

Morphologically, bilirubin induces changes similar to those seen with other mechanisms of cellular injury, including cellular dissociation and darkening/granulation of the cytoplasm.[14] Whether actual volume change exists as the result of bilirubin administration or simply due to increased osmolarity in the medium remains to be investigated. However, cells incubated in HSA stock solution did not show marked volume change. The increased staining of cells by bilirubin noted on light microscopy (Figure 1, *B*, *D*, *F*, and *H*) probably reflects binding of bilirubin to membrane elements and is likely to be one of the modes of its toxicity.[1,3,21]

With measurement of LDH release, in addition to bilirubin concentration, duration of exposure appeared to be a factor. From 36 hours on for 200 and 2000 μM bilirubin, and by 120 hours for 5, 10, 25, and 50 μM bilirubin, LDH measurements approached maximum and did not differ significantly. LDH release in cultures exposed to 1 μM bilirubin showed no significant elevation over control and thus probably reflects a concentration that can be tolerated by astrocytes.

dBcAMP induces morphologic and biochemical differentiation of cultured astrocytes.[17,18] This allows the comparison of undifferentiated (untreated) cells with differentiated (treated) cells. Based on LDH release, from 24 and 36 hours of incubation on, at 200 and 100 μM bilirubin, respectively, immature astrocytes are more sensitive to bilirubin than are differentiated astrocytes. The fact that no significant difference between dBcAMP-treated and untreated cells exposed to 2000 μM bilirubin could be found probably reflects the overwhelming toxicity of such a high concentration. The higher LDH levels for dBcAMP-treated cells from 0 to 4 hours may be due to an increased background rate of senescence in differentiated (dBcAMP-treated) cells or due to an increase in LDH activity within cells caused by dBcAMP.[17]

The amount of LDH in a culture was similar to the amounts (per microgram of protein) reported previously.[14] The peak LDH release we measured (approximately 200 mU/culture) did not reach the theoretical maximal level (approximately 280 mU/culture), probably due to the sampling techniques used in experimental versus control cultures. In the experimental cultures, some LDH was probably retained by the cells after cell death and not released into the medium, explaining the failure to reach maximum LDH level for a culture.

Bilirubin concentrations of 100 and 200 μM are roughly the 50th and 90th percentiles of maximum serum bilirubin levels in white newborn infants of $>$2500 gm.[22] The amount of bilirubin getting past the blood–brain barrier is probably considerably lower. However, these levels were used to simulate the longer period of exposure to elevated bilirubin levels in newborns. Furthermore, the blood–brain barrier may not be as competent in newborns, especially if other conditions, such as hypoxemia, hypercarbia, or hyperosmolarity exist.[20] Thus, portions of the newborn brain may be exposed to concentrations approximating serum levels. In addition, our experiments using lower concentrations showed significant toxicity of bilirubin in

concentrations as low as 5 μM. We used 2000 μM concentrations to examine the extreme case.

The presence of serum albumin has been shown to influence LDH quantification.[23,24] In our experience, HSA in the medium led to elevated measurement of LDH. Correction for this, however, did not interfere with the interpretation of our results.

The biochemical mechanisms of bilirubin toxicity in astrocytes remain to be further studied. As stated previously, bilirubin appears to exert its cellular toxicity on several targets, including energy, carbohydrate, lipid, and amino acid metabolism; DNA and protein synthesis; and membrane function.[1–3] Cellular staining seen on light microscopy, supports a role in alteration of membrane function. Staining by bilirubin may, however, represent uptake rather than membrane binding. Which of these occurs is a topic for future study. Other prospects for study include the influence of bilirubin on glucose and glutamate uptake, second messenger systems, and apoptosis, possibly in primary culture of neurons and co-cultures of astrocytes and neurons.

Despite extensive study into the mechanism of bilirubin encephalopathy, limited work has involved primary cultures of neural cells. Amit and Brenner[10] described the increasing resistance to bilirubin toxicity in fetal rat glial cells (primarily astrocytes) as they aged from 2 to 12 days. They also reported on the dependence of bilirubin toxicity on the bilirubin/albumin (B/A) ratio. Other experiments, using a fixed bilirubin concentration and variable B/A ratio, have shown that rat astrocytes were less sensitive to bilirubin toxicity than human fibroblasts.[11] Our high B/A ratio was chosen to optimize the amount of free bilirubin within a stable medium. Keeping the B/A ratio fixed while adjusting our total bilirubin concentration was our approach to altering the free bilirubin available to affect cellular toxicity. It has been hypothesized that other clinical factors such as acidosis and hypoxia may affect the availability and toxicity of bilirubin, thereby making the clinical correlation of bilirubin encephalopathy dependent on more than the bilirubin level or the B/A ratio.[25]

Primary cultures of astrocytes offer a convenient means of investigation. Astrocytes are sensitive to bilirubin injury. As suggested in clinical kernicterus, bilirubin toxicity in astrocytes appears to be dependent on dose and duration of exposure. It also appears that immature cells are more sensitive to bilirubin than differentiated cells. This greater sensitivity may correlate with the observation that newborns are more susceptible to kernicteric injury. In another model of injury, mercury-based toxins have been shown to induce an increase in apoptosis in astrocytes that is more profound in the immature rat brain compared with a more mature one.[26] The role of astrocytes as an integral part of the blood–brain barrier suggests that bilirubin toxicity to astrocytes could play an important role in blood–brain barrier integrity and bilirubin transport that leads to deeper neuronal injury and kernicterus.[27] It has also been shown that apoptosis can be induced in cultured neurons by removal of the neurotrophic support of astroglial cells.[28] Further studies are needed to better understand the effects of bilirubin on the interplay of maturation and injury in astrocytes and neurons.

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