Unconjugated bilirubin inhibits in vitro cytotoxic T lymphocyte activity of human lymphocytes

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Abstract

Septic complications have been major problems in the management of patients with obstructive jaundice and neonatal jaundice. This study investigates effects of bilirubin on human T lymphocyte responses against allogeneic mixed lymphocyte reaction. In vitro exposure of human peripheral blood mononuclear cells (PBMC) with unconjugated bilirubin at pathological levels (6 to 12 mg/dl) did not alter the subsets of CD3, CD4, CD8, CD14, CD19 and CD56 positive populations, or expression of costimulatory surface molecules CD2, CD3, CD4 and CD8. Further incubation of bilirubin-treated PBMC with irradiated B lymphoid Raji cells after removal of the extracellular bilirubin resulted in a dose-dependent decrease of cytotoxic T lymphocyte (CTL) activity, DNA synthesis, and expression of Tac antigen (CD25) and transferrin receptor (CD71). However, no significant change of interleukin-2 (IL-2) production was observed after this incubation between bilirubin-treated and -untreated PBMC. These results suggest that bilirubin inhibits the induction of CTL activity, and this defect may result from the impaired responsiveness against IL-2. These observations may help explain the increased infection observed in hyperbilirubinemic patients.

Keywords: T lymphocyte; Bilirubin; Cytotoxicity; Major histocompatibility complex

1. Introduction

Sepsis is a major complication leading to the high mortality in patients with obstructive jaundice and neonatal jaundice [1,2]. However, the mechanisms for this increased susceptibility to infection have not been fully understood. We previously demonstrated that mitogen-induced production of interleukin-1 and interleukin-2 (IL-2) by peripheral blood mononuclear cells (PBMC) was impaired in patients with obstructive jaundice and that this defect improved after external biliary drainage [3]. Unconjugated bilirubin is known to inhibit key enzymes in various cellular functions [4]. Desmet et al. [5] demonstrated that both unconjugated and conjugated bilirubin were deposited in the cytoplasm of infiltrating mononuclear leukocytes and Kupffer cells, as well as hepatocytes of the liver tissue in patients with obstructive jaundice. We have also demonstrated that unconjugated bilirubin may be incorporated into human lymphocytes and monocytes via different energy-dependent mechanisms [6]. These findings suggest that bilirubin compounds may, at least in part, be responsible for the decreased immune responses in jaundiced patients.

T lymphocytes play an important role in host defense system against microorganisms [7]. It is known that immunized T cells of intestines can migrate to the lung and respond to eliminate bacteria [8]. CD4-positive T cells respond to class II major histocompatibility (MHC) antigen, while CD8-positive T cells to class I antigen. MHC class II antigen is mainly implicated in the proliferative allogeneic stimulation and lymphokine release. Class I antigens activate cytotoxic T lymphocyte (CTL) precursors to a state of responsiveness to soluble mediators [9]. Cell surface antigens, CD2, CD3, CD4, CD8, LFA-1 and Tp 103 expressed on T cells, are involved in T cell-target cell interaction or T cell activation. After exposure to foreign...
MHC determinants, T cells proliferate and acquire cytolytic capacity toward cells bearing the specific sensitizing alloantigen. IL-2 plays a major role in the growth and differentiation of CTL precursors and in CTL proliferation [10].

This study was undertaken to investigate in vitro effects of unconjugated bilirubin on allogeneic T lymphocyte responses against human B lymphoid cell line 'Raji' cells which express both class I and class II MHC molecules [11].

2. Materials and methods

All immunological experiments were performed with viability of PBMNC greater than 95% as determined by trypan blue dye exclusion.

2.1. Medium and buffers

1. BSA/PBS: 3.0 g/dl bovine serum albumin (BSA) (Fraction V, Sigma, St. Louis, MO, USA) dissolved in 10 mmol/1 sodium phosphate (pH 7.4), 0.15 mol/1 NaCl (PBS).

2. PBS/BCS/NaN3: PBS containing 2% (v/v) bovine calf serum supplemented (BCS) (Hyclone Laboratories, Logan, UT, USA) and 1.0 g/l sodium azide.

3. CM-10 medium: RPMI-1640 medium supplemented with 10% (v/v) BCS, 50 μg/ml gentamycin (Sigma), 25 mmol/1 Hepes (Sigma), 1 × minimal essential medium amino acids (Hazleton Biologics, Lenexa, KS, USA).

2.2. Bilirubin treatment of human PBMNC

Human PBMNC were separated from venous blood of healthy donors by the density gradient of lymphocyte separating media as described before [6]. Bilirubin treatment of freshly-isolated human PBMNC was carried out at 37°C for 60 min at 5 × 10^6 cells/ml in the presence or absence of bilirubin IXα, a major isoform of unconjugated bilirubin (Sigma) at 6 to 12 mg/dl in BSA/PBS as described before [6]. The levels of bilirubin and albumin used in the current experiments were comparable to the serum levels observed in hyperbilirubinemic patients.

2.3. CTL generated from allogeneic MLR

Allogeneic mixed lymphocyte reaction (MLR) was performed as described before [12]. Bilirubin-untreated or -treated PBMNC were resuspended at 5 × 10^6 cells/ml in CM-10 (Responder cells). Raji cells, human B cell Burkitt lymphoma cell line, were γ-irradiated (5 kRad) and resuspended at 7.5 × 10^5 cells/ml in CM-10 (stimulator cells). 1.0 ml of the responder cell suspension and 1.0 ml of the stimulator cell suspension were added into a well in duplication of a 24-well microplate (Corning Glass, Corning, NY, USA). The plates were incubated at 37°C for 96 h in a humidified atmosphere of 5% CO₂. In some experiments, mouse anti-human IL-2R β chain monoclonal antibody, TU27 [13], was added in the culture to measure the real production of IL-2 in supernatant by avoiding the possible effect of decreased IL-2 utilization in bilirubin-treated PBMNC with MLR. After the incubation, the culture supernatant and the non-adherent cells were collected after the centrifugation at 250 × g for 15 min. The IL-2 activity in the supernatant (IL-2 production) was measured by a CTL-L2 assay. The cells were washed once with PBS, and used for effector cells of CTL assay, or analyzed by a direct immunofluorescence assay. The viability of bilirubin-untreated and -treated PBMNC was greater than 95% after the 96 h MLR culture.

2.4. CTL cytotoxic assay

CTL assay was performed by a 4 h chromium-release assay as described before [12]. Briefly, non-adherent cells recovered from MLR were incubated at 37°C for 4 h with 5 × 10^3 cells of ^51Cr-labeled Raji cells at effector/target ratio 50, 25, 12 and 6 in 300 μl of CM-10 in a well of 96-well round bottom microplate (Corning) in quadruplicate. Control wells, for spontaneous release and maximal release from target cells, contained CM-10 medium and dilute detergent, respectively. After the incubation, 200 μl of cell free supernatants were harvested from each well, and the radioactivity was measured by a gamma-counter. Specific chromium release (%) was calculated as (experimental release - spontaneous release) - (maximal release - spontaneous release) × 100. The lytic unit at 20% killing (LU_{20}) conversion was done by an exponential-fit method of analysis, which allows comparison of the lytic activities of different effector populations [12].

2.5. Measurement of lymphocyte proliferation in MLR

DNA synthesis of PBMNC was estimated by [³H]thymidine uptake as described before [12]. Briefly, following to the bilirubin-treatment, 2 × 10⁵ cells of bilirubin-untreated or -treated PBMNC were incubated at 37°C for 96 h in a 5% CO₂ incubator with 3 × 10⁴ cells of γ-irradiated Raji cells in 200 μl of CM-10 in a well of 96-well flat-bottom plates (Corning) in quadruplicate. The cells were pulsed with 0.5 μCi/well of [³H]thymidine for the final 4 h of the incubation and harvested onto a glass fiber filter sheet by a semiautomatic cell harvester. The radioactivity trapped on the harvesting filters was measured in a liquid scintillation counter.

2.6. IL-2 assay

IL-2 activity was measured by the CTL-L2 proliferation assay according to Gillis et al. [14]. CTL-L2 cells in
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growing phase were harvested, incubated at 37°C for 60 min in RPMI-1640 medium to remove endogenous IL-2, washed once with RPMI-1640 medium and resuspended at 5 · 10^5 cells/ml in CM-10. 100 µl of the CTLL-2 suspension were added to each well of 96-well flat-bottom microplates in quadruplicate with 100 µl of serial dilutions of test IL-2 samples, standard IL-2 (human recombinant IL-2, Cetus, Emeryville, CA, USA), or media only for the negative control. The cells were incubated at 37°C for 18 h in a 5% CO₂ incubator, pulsed with 0.5 µCi/well of [³H]thymidine for an additional 4 h, and harvested. The uptake of [³H]thymidine by IL-2-boosted cells was quantified by a scintillation counter and the titer of IL-2 was expressed by probit analysis. The activity of IL-2 (IU/ml) in test samples was determined by comparing their activities with the activity of the standard IL-2 sample run in every assay. Even after the extensive washes of bilirubin-treated PBMC, these cells release intracellular bilirubin into the culture supernate during the 96 h MLR. The concentrations of bilirubin remaining in the supernatants were proportional to the bilirubin doses to treat cells, but the levels were quite low (less than 0.6 mg/dl, Fig. 1A). These modest levels of bilirubin do not interfere with the biologic response of CTLL-2 cells (Fig. 1B).

2.7. Analysis of expression of cell surface antigens

Expression of CD2, CD3, CD4, CD8, CD14, CD19, CD56, Tac antigen (CD25) and transferrin receptor (TfR; CD71) on lymphocyte fraction was analyzed by a direct immunofluorescence method using a flow cytometry FACScan (Becton-Dickinson, Immunocytochemistry systems, Mountainview, CA, USA) as described before [15]. All mouse monoclonal antibodies (FITC-labeled) against these antigens and control antibodies were purchased from Becton-Dickinson. Amounts of the antigen expressed on a cell were estimated by the difference between the mean intensities of the FL1 fluorescence for the antibody directed and for the control antibody, as described before [16].

2.8. Statistical analysis

Statistical significance was analyzed by the Student’s t-test for paired samples.

3. Results

3.1. Effect of bilirubin on expression of cell surface antigen

Our previous study [6] demonstrated that in vitro exposure of human PBMC to bilirubin at 0 to 12 mg/dl resulted in a dose-dependent increase of intracellular bilirubin (0 to 4 or 6 · 10⁻¹⁵ mol/cell). The subsets of cell surface markers in bilirubin-treated PBMC were unchanged when compared with bilirubin-untreated PBMC (normal values of CD14 for monocytes, 11.3 ± 5.7%; CD3 for whole T cells, 65.3 ± 8.1%; CD4 helper T cells, 38.5 ± 6.5%; CD8 cytotoxic T cells, 27.6 ± 7.5%; CD19 for B cells, 9.8 ± 3.8%; CD56 for NK cells, 10.7 ± 5.2%). Subsequently, we studied the amounts of cell surface antigens CD2, CD3, CD4, and CD8, which are involved in CTL-target cell interaction and T cell activation [9]. Similarly, the amounts of these antigens expressed on the bilirubin-treated PBMC were also unchanged as compared to bilirubin-free control cells (normal values (arbitrary units) of CD2, 59.0 ± 7.9; CD3, 66.1 ± 10.1; CD4, 11.5 ± 1.0; CD8, 242 ± 54.5).

![Fig. 1. Effect of bilirubin on biological CTLL-2 proliferation assay. (A) Concentrations of bilirubin released into the culture supernatants of MLR. Freshly-isolated PBMC were treated at 37°C for 60 min with 0 to 12 mg/dl of bilirubin, washed three times, and incubated at 37°C for 96 h with irradiated Raji cells (MLR) as described in Section 2. The levels of bilirubin in the supernatants were measured. (B) Effect of bilirubin on CTLL-2 assay. IL-2 dependent cell line, CTLL-2, was cultured at 37°C for 22 h with serially diluted human recombinant IL-2 (rIL-2) in the presence or absence of the above final concentrations of bilirubin as described in Section 2. [³H]Thymidine uptake was quantified by a liquid scintillation counter.](image-url)
Table 1
Effect of bilirubin on CTL activity, DNA synthesis and IL-2 production of PBMNC after allogeneic MLR

<table>
<thead>
<tr>
<th>Bilirubin dose (mg/dl)</th>
<th>Raji cells</th>
<th>CTL activity (LU20)</th>
<th>[3H]Thymidine uptake (cpm)</th>
<th>IL-2 production (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (-)</td>
<td>0.83 ± 0.33</td>
<td>1246 ± 702</td>
<td>5.1 ± 8.3</td>
<td></td>
</tr>
<tr>
<td>0 (+)</td>
<td>14.3 ± 8.0</td>
<td>16730 ± 8976</td>
<td>175 ± 175</td>
<td></td>
</tr>
<tr>
<td>6 (+)</td>
<td>12.7 ± 9.8</td>
<td>12460 ± 8206</td>
<td>200 ± 118</td>
<td></td>
</tr>
<tr>
<td>8 (+)</td>
<td>7.6 ± 8.3</td>
<td>6560 ± 4146</td>
<td>219 ± 123</td>
<td></td>
</tr>
<tr>
<td>10 (+)</td>
<td>2.1 ± 2.2</td>
<td>2790 ± 812</td>
<td>246 ± 140</td>
<td></td>
</tr>
<tr>
<td>12 (+)</td>
<td>0.85 ± 0.72</td>
<td>1508 ± 596</td>
<td>173 ± 113</td>
<td></td>
</tr>
</tbody>
</table>

Freshly-isolated PBMNC were treated with bilirubin at 0 to 12 mg/dl in BSA solution. The cells were washed and further incubated for 96 h in the absence or presence of irradiated B lymphoid Raji cells (MLR). The cells were evaluated for CTL activity against Raji cells by a chromium-release assay (n = 8). IL-2 activity of the MLR supernatant was measured by a CTLL-2 proliferation assay (n = 8). In another set of experiments, the cells were pulsed with [3H]thymidine for the final 4 h of the MLR, and the radioactivity was estimated by a liquid scintillation counter (n = 9). Data represent a mean ± S.D. of healthy donors. Statistical significance was evaluated by comparing each data point of bilirubin-untreated cells with irradiated Raji cells. * P < 0.05, ** P < 0.01, *** P < 0.001.

3.2. CTL activity, proliferation and IL-2 production of bilirubin-treated PBMNC in allogeneic MLR

Bilirubin-untreated and -treated PBMNC were cultured for 96 h with irradiated B lymphoid Raji cells and assayed for cytotoxic activity against Raji cells (CTL activity), DNA synthesis, and IL-2 production (Table 1). T cells are primarily responsible for these responses [12]. Bilirubin-treated PBMNC in the MLR showed a dose-dependent decrease of CTL activity. Similarly, bilirubin-treated PBMNC after the MLR showed a dose-dependent decrease of DNA synthesis. However, bilirubin-treated PBMNC in the MLR produced equal or even enhanced levels of IL-2 as compared to bilirubin-untreated cells. To avoid the possible effect of decreased IL-2 utilization in bilirubin-treated PBMNC, IL-2 levels in the supernatants were measured after the MLR co-cultured with or without anti-IL-2R β chain: TU27 (Fig. 2). TU27 increased the IL-2 level of supernatant in bilirubin-untreated PBMNC by 43%. IL-2 levels in bilirubin-treated PBMNC with TU27 were unchanged to the bilirubin-free control level. In contrast, a slight increase of IL-2 was observed in PBMNC treated at 8 or 10 mg/dl of bilirubin without TU27. Therefore, the slight increase (about 30%) of IL-2 in the supernatants in bilirubin-treated PBMNC with MLR may result from the decrease of IL-2 utilization.

3.3. Expression of Tac Ag and TfR in bilirubin-treated PBMNC in allogeneic MLR

Expression of activation antigens Tac and TfR was studied on bilirubin-treated PBMNC after allogeneic MLR (Table 2). In bilirubin-untreated PBMNC, the MLR enhanced the expression of both antigens (4.6-fold for Tac and 3.3-fold for TfR as compared to the cells without the MLR). In bilirubin-treated PBMNC, expression of both
Table 3
Summary of in vitro bilirubin studies

<table>
<thead>
<tr>
<th>Donor</th>
<th>Cell population</th>
<th>Bilirubin treatment</th>
<th>Stimulus</th>
<th>DNA synthesis</th>
<th>IL-2 production</th>
<th>Tac or TfR expression</th>
<th>Cytotoxic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Jaundiced patients</td>
<td>PBMNC</td>
<td>(−)</td>
<td>Con A</td>
<td>N.D.</td>
<td>decreased</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>2. Healthy</td>
<td>PBMNC</td>
<td>(+)</td>
<td>PHA</td>
<td>decreased</td>
<td>decreased</td>
<td>unchanged</td>
<td>N.D.</td>
</tr>
<tr>
<td>3. Healthy</td>
<td>PBL</td>
<td>(+)</td>
<td>(−)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>NK, ADCC decreased</td>
</tr>
<tr>
<td>4. Healthy</td>
<td>PBMNC</td>
<td>(+)</td>
<td>IL-2</td>
<td>decreased</td>
<td>N.D.</td>
<td>unchanged</td>
<td>LAK, ADCC decreased</td>
</tr>
</tbody>
</table>

1. Ref. [3]; 2. Ref. [17]; 3. Ref. [18]; 4. Present study. Abbreviations: TfR, transferrin receptor; PBMNC, peripheral blood mononuclear cells; Con A, concanavalin A; N.D., not done; PBL, peripheral blood lymphocytes; NK, natural killer; ADCC, antibody dependent cellular cytotoxicity; LAK, lymphokine activated killer; MLR, mixed lymphocyte reaction; CTL, cytotoxic T lymphocyte.

antigens after the MLR decreased in a dose-dependent manner.

4. Discussion

It is now widely accepted that T cell-dependent immune responses may be polarized by the activation of different CD4-positive helper T cells. Th1 cells secrete IL-2 and interferon-γ, and predominate in cell-mediated immunity, particularly during intracellular infections (e.g. protozoa, bacteria, viruses). Conversely, Th2 cells produce IL-4, IL-5, IL-6, IL-9 and IL-10, which help antibody production, and play a prominent role in anti-helminth and allergic responses [5]. IL-2 plays a major role in the growth and differentiation of CTL precursors and proliferation of CTL [10]. The present study clearly demonstrates that unconjugated bilirubin at pathological levels inhibits in vitro CTL activity and proliferative responses of human PBMNC in allogeneic MLR. This inhibition did not result from the alteration of cell populations or expression of costimulatory molecules, CD2, CD3, CD4 and CD8. Although bilirubin inhibits CTL or proliferative response in the MLR, IL-2 production was not inhibited in these cells. Furthermore, the expression of Tac antigen and TfR was impaired in bilirubin-treated lymphocytes after the MLR, induction of which was dependent on the binding of IL-2 to its high affinity receptors. These results suggest that expression of high affinity IL-2 receptors, binding of IL-2 to its high affinity receptors, or the subsequent signal transduction may be impaired in bilirubin-treated lymphocytes.

Our previous data revealed that incubation of bilirubin-treated PBMNC with phytohemagglutinin A (PHA) resulted in a decrease of DNA synthesis and IL-2 production without significant change of expression of Tac antigen and TfR [17]. The discrepancy between the current data and those experiments may be due to the difference of stimulation methods. PHA primarily induces T cell proliferation but not any cytolytic activity. Although the [3H]thymidine uptake by the PHA-induced PBMNC was higher than that of the current study, the IL-2 production was much lower than the current study. In another experiment, we have demonstrated that unconjugated bilirubin also inhibits in vitro MHC-unrestricted cytotoxicity and proliferative response against IL-2 in human lymphocytes [18]. These data are summarized in Table 3. Among these different stimulations, proliferative responses and cytolytic activities were usually inhibited in bilirubin-treated lymphocytes. Further studies are now under way regarding the mechanistic view of effects of unconjugated bilirubin on lymphocyte function.

In summary, unconjugated bilirubin inhibits CTL activity and proliferative response in allogeneic MLR at the levels observed in patients with obstructive jaundice and neonatal jaundice. These results may help explain the poor prognosis of these patients when complicated with infections.

References