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Light-Induced Mutagenicity in *Salmonella* TA102 and Genotoxicity/Cytotoxicity in Human T-cells by 3,3'-Dichlorobenzidine: A Chemical Used in the Manufacture of Dyes and Pigments and in Tattoo Inks

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Abstract

3,3'-Dichlorobenzidine (DCB) is used primarily as an intermediate in the manufacture of diarylide yellow or azo red pigments for printing inks, textiles, paints, and plastics. It is also used in tattoo inks. In this article, we investigate light-induced toxicity of DCB in both bacteria and human Jurkat T-cells. DCB itself is not toxic or mutagenic to *Salmonella typhimurium* TA102, but is photomutagenic at concentrations as low as 2 μM and phototoxic at concentrations $>100 \mu\text{M}$ when the bacteria is exposed to DCB and light at the same time (1.2 J/cm² of UVA and 2.1 J/cm² of visible light). Furthermore, DCB is both photocytotoxic and photogenotoxic to human Jurkat T-cells. Under a constant light irradiation dose of 2.3 J/cm² of UVA and 4.2 J/cm² of visible light, it causes the Jurkat T-cells to become non-viable in a DCB dose-dependent manner and only 40% viable cells remaining at DCB concentrations higher than 50 μM . At the same time, DNA fragmentation is observed for the cells exposed to both DCB and light, determined by single cell gel electrophoresis (Comet assay). As much as 8 % of the cellular DNA is fragmented when exposed to 200 μM DCB and light irradiation. This suggests that DCB can penetrate the cell membrane and enter the cell. Upon light activation, DCB in the cells can cause various cellular damages, including DNA fragmentation, leading to non-viable Jurkat T-cells. It appears, though, non-viable cells may not be caused solely by fragmentation of cellular DNA, but other damages such as to proteins and cell membranes, or other forms of DNA damage such as alkylation that does not cause DNA to fragment, may also be involved. Therefore, persons exposed to DCB through environmental contamination or through tattoo piercing using DCB-contaminated inks must not only concern about its toxicity without exposing to light, but also about its phototoxicity.

Keywords

3,3'-Dichlorobenzidine; *Salmonella typhimurium* TA102; Phototoxicity; Photomutagenicity; Jurkat T-cell; Single cell gel electrophoresis (Comet assay)

Introduction

Tattoos, as a body art, have become popular. According to the "Statue report on the current situation, nature and size of the problem regarding the safety of tattoos, body piercing and of related practices in the EU", 5 to 10% of the general population has tattoo/piercing in Europe and high school students engaged in tattoos are increasing in the United States [Papameletiou *et al.*, 2003]. They predicted that "tattooing/piercing is in a strongly growing trend". There are no strict laws and regulations going together with the rapid increase of

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tattoos/piercing [Zenie *et al.*, 2003]. The European Commission has been trying to develop and implement regulations for the protection of human health associated with tattoo/piercing [Walser, 2003].

Most colorants used for tattoos are pigments and dyes. Pigments are either metallic salts or organic compounds, but dyes are usually organic compounds [Baeumler *et al.*, 2003]. Many synthetic organic colorants are in use in addition to the traditional inorganic pigments. One of the popular colorants is made of azo compounds [Baeumler *et al.*, 2003]. The azo colorants usually contain 3,3'-dichlorobenzidine (DCB), *o*-anisidine, and 4-chloro-toluidine. The Netherland group also pointed out that DCB can be found in the metabolites of azo pigments. In addition, industry used large amounts of benzidine and benzidine congeners to produce commercial azo dyes for textiles, paper, leather and plastics in the past [Haley, 1975; Choudhary, 1996]. DCB is found in commercial pigments such as pigment yellow 87 or pigment orange 16 since it is used as an intermediate in the manufacture of these pigments for printing inks, textiles, paints, and plastics [Claxton *et al.*, 2001; Baeumler *et al.*, 2003]. DCB are also found in tattoo bands, hair dyes, folders of paper, toys, bed clothes, watch straps and ink ("air brush") [IARC, 1981; CFR, 1985; Zeilmaker *et al.*, 2000]. It can also be formed from the reduction of azo dyes by intestinal microbe or environmental microorganism [Claxton *et al.*, 2001].

It has been confirmed that DCB is mutagenic in *Salmonella typhimurium* TA98, TA100, TA1535, TA1537, and TA1538 with the presence of the S9 metabolic enzyme activation system [Garner, 1975; Lazear and Louie, 1978; Lazear *et al.*, 1979; Booth *et al.*, 1980; Vithayathil *et al.*, 1983; Prival *et al.*, 1984; Reid *et al.*, 1984; Gentile *et al.*, 1985; Iba, 1987; Lang and Iba, 1987; Messerly *et al.*, 1987; Chung *et al.*, 2000]. Among all dihalogenated benzidines, it is shown that DCB is the most mutagenic [Savard and Josephy, 1986]. DCB can be absorbed readily through skin [Lee and Shin, 2002]. Although DCB has not been associated with cancer in man, there is sufficient evidences that it is carcinogenic in mice, rats, hamsters, and dogs [IARC, 1981]. As with benzidine, Occupational Safety and Health Administration has strict regulations to avoid workers to get in contact with DCB [CFR, 1985]. The chemical structure of DCB is shown below in Figure 1.

Photochemically DCB is unstable and can dechlorinate under light irradiation [Banerjee *et al.*, 1978; Nyman *et al.*, 2002]. We suspect that during the photochemical transformation or dechlorination of DCB, reactive species may be formed that can cause phototoxicity including photomutagenicity. Therefore, the phototoxicity including photomutagenicity of DCB in *Salmonella typhimurium* bacteria strain TA102 and human Jurkat T-cells is examined in this article.

Methods and Materials

Material

DCB was from Sigma Chemical Company (St. Louis, MO) and used without further purification. *Salmonella typhimurium* TA102 was kindly provided by Dr. Bruce Ames from the University of California (Berkeley, CA). Human Jurkat T-cell (Jurkat TIB152) was from the American Type Culture Collection (Rockville, MD). Most media and chemicals for cell culture were purchased from GIBCO (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Hyclone Laboratory (Logan, Utah). Comet assay kit was purchased from Trevigen Company (Gaithersburg, MD). The Jurkat cells were cultured in a humidified atmosphere with 5% CO₂ at 37°C for 24 h. RPMI 1640 medium was used for these non-adherent cells. Media was made complete by addition of 10% FBS and 1% of 100× antibiotic-antimycotic (containing both fungizone and pen/strep, GIBCO Cat. No. 15240-096).

Light sources

A 300 W Xe/Hg(Xe) lamp from ORIEL Instruments (Stratford, CT) producing a full-spectrum light ranging from 300 to 800 nm was used. A Pyrex glass plate, which filtered out light with wavelengths less than 300 nm, was placed above the light beam at 40 cm from the light bulb. This distance is carefully maintained to obtain an UVA irradiation of 13.8 J/cm²·h and visible light irradiation of 25.3 J/cm²·h. Output energy was measured using a photo-radiometer (Cat. No. 2100) equipped with UVB (280 to 320 nm), UVA (320 to 400 nm), and visible light probes (400 to 750 nm) from Solar Light Co. (Philadelphia, PA).

Photomutagenicity assay

DCB photomutagenicity test was conducted based on the modified bacteria mutagenicity test developed by Maron and Ames [Maron and Ames, 1983]. A detailed procedure was described in two previous publications [Wang *et al.*, 2003; Yan *et al.*, 2004].

Bacteria viability

Bacteria viability was conducted with a modified procedure [Ben-Hur *et al.*, 1980]. A 0.1 mL overnight cultured bacteria suspension ($1\sim 2\times 10^9$ cells/mL, 10 h at 37°C) was mixed with 0.1 mL of various concentrations of DCB solution in dimethyl sulfoxide (DMSO) and 2.5 mL PBS. Before irradiation, a 1.0 mL sample of the bacteria mixture was taken as the dark control. The rest of the sample was irradiated for 5 min (1.2 J/cm² of UVA and 2.1 J/cm² of visible light). After irradiation, another 1.0 mL sample was taken and both 1.0 mL samples were serially diluted with sterilized and de-ionized water and poured onto the nutrient agar plates with the expected bacteria colonies of around 20 to 200 per plate. These plates were then put inversely in an incubator at 37°C for 24 h. The colonies were counted with a colony counter (Bantex, Model 920A). All experiments were in triplicates.

Phototoxicity study of DCB in Jurkat T-cells

After the Jurkat T-cells grew to the expected concentration of no less than 1×10^5 cells/mL, the cells were harvested and centrifuged at 2000 rpm for 5 min. The supernatant was discarded and the pellet was washed twice with $1\times$ PBS. Finally the pellet was resuspended in $1\times$ PBS to reach a cell concentration of approximately 5×10^5 cells/mL. Then the cell suspension was placed in a 96-well plate with 100 μ L in each well. The concentrations of DCB were 2, 10, 50, 100, and 200 μ M, obtained through a serial dilution of the freshly prepared 1 mM DCB stock solution in DMSO with $1\times$ PBS. Then 100 μ L of the desired DCB solution was added into each well containing cell suspension. Two sets of 96-well plates were used with one covered with aluminum foil as the dark control, while the other was irradiated for 10 min with the 300W Xe/Hg(Xe) (UVA 2.3 J/cm² and visible light 4.2 J/cm²). In each 96-well plate, nine-well sets were used for each DCB concentration. Among the nine wells at each DCB concentration, the cells in three wells were taken out for Comet assay and the remaining six for cell viability assay.

Cell viability

The six wells for cell viability test received 100 μ L of fluorescein diacetate (FDA, 10 ng/mL) in each well and incubated at 37°C for 35 min. The plates were then read immediately using a Fluoroscan Ascent FL (Thermo Labsystems) with filters set at an excitation wavelength of 485 nm and emission wavelength of 538 nm. All experiments were in triplicates.

Comet Assay

The cell suspensions taken out from three wells were centrifuged at 2000 rpm for 5 min. The pellets were washed twice with cold $1\times$ PBS at 4°C and resuspended in media to obtain a

cell concentration of 1×10^5 cells/mL. The following are a brief procedure for alkaline Comet assay adopted from the Trevigen instruction manual [Trevigen, 2001]. In a 1 mL test tube, 20 μ L of the cell suspension and 200 μ L of melted agarose was mixed at 40°C, and 75 μ L of the mixed suspension were pipetted onto a pre-warmed slide at about 37°C. The slides were placed in a refrigerator at 4°C for 10 min before being placed in chilled lysis buffer at the same temperature for 45 min. Then the slides were tapped off extra buffer and immersed in freshly prepared alkaline solution (pH > 13, 0.25 M NaOH solution containing 0.1 μ M EDTA) for 45 min at room temperature. Slides were then removed from the alkaline solution and washed twice by immersing in $1 \times$ TBE (tris-borate-EDTA) buffer for 5 min before being placed in a horizontal gel electrophoresis box (25 cm between the two electrodes in $1 \times$ TBE) and ran at 1 V per centimeter for 10 min. Afterwards, slides were placed in 70% ethanol for 5 min, removed, tapped off excess ethanol, and air-dried for 2.5 h at room temperature. Finally, 50 μ L of diluted SYBR Green solution was placed onto each circle of dried agarose and allowed to set for 4 h. The slides were then read with a fluorescence microscope equipped with DNA Damage Analysis Software (Loates Associates, Inc.). Two sets of DNA damage parameters, Comet tail moment and percent of DNA fragmentation, were used for this study. The Comet tail moment is the product of the distance and normalized intensity integrated over the tail length for a damage measure combining the amount of DNA in the tail with the distance of migration. The percent of DNA fragmentation is defined as the percent of DNA in the Comet tail versus total DNA from both Comet tail (DNA fragments) and Comet head (cell nucleus). A total of 70 cells per sample were scored and the tail moment and percent of DNA fragmentation were averaged.

Results

Phototoxicity and photomutagenicity of DCB in *Salmonella typhimurium* TA102

Salmonella typhimurium TA102, in the presence of DCB with concentrations of 0, 0.2, 0.5, 1.0, 2.0, 5.0, 10, 25, 50, and 100 μ M, was irradiated for 5 min under a Xe/Hg(Xe) lamp (1.2 J/cm² of UVA and 2.1 J/cm² of visible light). The bacteria viability is shown in Figure 2. There is no toxic effect observed whether with light or without light irradiation in this concentration range for DCB. However, at a higher concentration of 625 μ M, bacteria colonies decrease >100 times under light irradiation and 10 times without light irradiation (insert in Figure 2). These results indicate that DCB is toxic to TA102 at high concentrations (>100 μ M) with or without light irradiation, but the toxicity is much greater when the bacteria is exposed to DCB and concomitantly irradiated. However, the phototoxicity of DCB is negligible at concentrations lower than 100 μ M with a light dose of 1.2 J/cm² of UVA and 2.1 J/cm² of visible light.

Photomutagenicity of DCB in the concentration range from 0 to 100 μ M was examined with the same light dose as the phototoxicity test and the results are shown in Figure 3. Without light irradiation, DCB is not mutagenic in TA102 (lower line in Figure 3). With light irradiation, DCB is photomutagenic in TA102, exhibiting 4 times higher the number of revertant colonies of the negative control and 2.4 times higher the number of revertant colonies of the light control (first point of the top line in Figure 3). It also shows a 15% decrease of the number of revertant colonies at the DCB concentration of 100 μ M comparing with the number of revertant colonies between 15–50 μ M of DCB and a 43% decrease at an even higher concentration of 625 μ M (insert of Figure 3). These results indicate that DCB is photomutagenic at concentrations of as low as 2 μ M and phototoxic at higher concentrations of >100 μ M. In comparison, the metabolic activation enzyme, liver S9 homogenate (2 mg/plate), increases the number of revertant colonies compared with the negative control, but is 50% lower than using light as the activation system.

Photocytotoxicity and photogenotoxicity of DCB in human Jurkat T-cells

Photocytotoxicity—Human Jurkat T-cells were treated with DCB at concentrations of 0, 2, 10, 50, 100, and 200 μM . Figure 4 shows the cell viability with or without light irradiation. Without light irradiation, a 20% cell death was observed due to exposure to DCB at concentrations $> 50 \mu\text{M}$. There was no significant cell death under 10 μM of DCB. With 10 min of light irradiation (2.3 J/cm^2 of UVA and 4.2 J/cm^2 of visible light), the number of viable cells decreases in a DCB concentration dependent manner from 5 – 50 μM , reaching about 40% of viable cells remaining, and leveling off afterwards.

Photogenotoxicity shown as DNA fragmentation detected by Comet assay—

Jurkat T-cells were treated with DCB and irradiated with light for 10 min. Cells were then treated with alkaline solution followed by electrophoresis. The cells on the slides were viewed with an EP-Fluorescence microscope and photographed with a camera. A normal untreated cell is round in shape with little or no tail (Figure 5a). Cells exposed to either DCB (5b) or light (5c) had very little changes compared with the untreated. Concomitant exposure to DCB and light caused fragmentation to the cellular DNA as shown in the Comet tail (Figure 5d).

Details of the DNA damage were analyzed by the percent of DNA fragmentation (Figure 6A) and the Comet tail moment (Figure 6B). There are very little damages to the cellular DNA if only DCB is present without light irradiation. With light irradiation, both the percent of DNA fragmentation and the tail moment increase with the increase of DCB concentration. An average of about 8% of DNA was fragmented and leaked out of the cell due to exposure to 200 μM DCB and light. Under the same conditions, the Comet tail moment reaches about 5. This implies that DCB can first penetrate the cell membrane into the cell nucleus to be in the proximity of cellular DNA molecules to cause light-induced DNA damage.

Discussion

Although DCB is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity in experimental animals, there is no specific regulation on its use [NTP, 2002]. Human exposure to DCB can be through skin absorption, inhalation, and ingestion. Therefore, it is anticipated that DCB could be distributed in the inner organs as well as in the skin. Since DCB is a component in tattoo inks, tattoo piercing also places DCB in the human skin. In the inner organs, DCB toxicity including carcinogenicity is due to metabolic activation involving oxidation to highly reactive *N*-oxygenated intermediates by the microsomal cytochrome P-450 and flavin monooxygenase enzyme complexes in rodents [Government of Canada, 1993]. Although the 3,3'-dichlorobenzidine-derived *N*-oxygenated intermediates have not been unequivocally identified, they are believed to be responsible for the mutagenic and genotoxic effects (e.g. related to DNA binding) in bacterial and mammalian systems.

Since DCB in the skin cells is subject to light irradiation, understanding of the phototoxicity of DCB must be of human health interests. The results presented here show that DCB is photomutagenic in *Salmonella* bacteria and photocytotoxic, photogenotoxic in human Jurkat T-cells. As is known, light irradiation causes DCB to dechlorinate. During the dechlorination process, reactive chlorine radicals and benzidine radicals are likely formed. These radicals can react with biological molecules nearby and cause cell to lose viability. Although nearly 60% of the Jurkat T-cells become non-viable due to exposure to light and DCB, only 8% of the cellular DNA is fragmented as shown by the Comet assay. In contrast, for the same test on azulene, up to 40% of cellular DNA is fragmented due to the exposure to azulene and light while nearly all the Jurkat T-cells remain viable [Wang *et al.*, 2004].

Therefore, DNA fragmentation caused by exposure to DCB and light should not be the main reason that causes the human Jurkat T-cells to become non-viable. Other damages such as to cell membrane elements and proteins in the cell, or other forms of DNA damage, may be the sources of cytotoxicity for DCB plus light. Nonetheless, DCB is photomutagenic in bacteria and photogenotoxic and photocytotoxic to human cells. Persons exposed to DCB through environmental contamination or through tattoo piercing using DCB-contaminated inks must not only concern about its toxicity without exposing to light, but also about its phototoxicity.

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Figure 1.
The chemical structure of 3,3'-dichlorobenzidine

Figure 2.

Salmonella typhimurium TA102 survival in the presence of 3,3'-dichlorobenzidine with or without light irradiation (1.2 J/cm^2 of UVA and 2.1 J/cm^2 of visible light). Bacteria colonies were grown in Petri dishes with nutrient agar. Insert is the graph for higher DCB concentrations.

Figure 3.

Photomutagenicity of 3,3'-dichlorobenzidine with *Salmonella typhimurium* TA102 with light irradiation or S9 metabolic activation. Samples were irradiated with 1.2 J/cm² of UVA and 2.1 J/cm² of visible light. Insert is at a higher DCB concentration.

Figure 4.

Viability of human Jurkat T-cells after DCB treatment with or without light irradiation (UVA, 2.3 J/cm² and visible light, 4.2 J/cm²). Total cell numbers were 10,000 cells/well. The results shown in this figure were the average from three independent experiments.

Figure 5. Single Cell Gel Electrophoresis of Jurkat T-cells upon treatment with DCB (200 μ M) and light (2.3 J/cm² of UVA and 4.2 J/cm² of visible light). (a) Untreated Jurkat T-cell; (b) Jurkat T-cell exposed to 200 μ M of DCB; (c) Jurkat T-cell exposed to light; (d) Jurkat T-cell exposed to both DCB and light concomitantly.

Figure 6.

DNA damage measured by the percent of the DNA fragmentation (A) and the Comet tail moment (B) for human Jurkat T-cell in the presence of DCB (200 μM) upon light irradiation (2.3 J/cm² of UVA and 4.2 J/cm² of visible light). A total of 70 cells were scored for each point on this graph.