Transgenic *Leishmania* Model for Delta-Aminolevulinate-Inducible Monospecific Uroporphyria: Cytolytic Phototoxicity Initiated by Singlet Oxygen-Mediated Inactivation of Proteins and Its Ablation by Endosomal Mobilization of Cytosolic Uroporphyrin

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Inherent deficiencies of *Leishmania* in heme biosynthesis were genetically complemented for delta-aminolevulinate-inducible biosynthesis and accumulation of light-excitable uroporphyrin. The phototoxic flagellar immobilization and cytolyis phenotypes and porphyrin mobilization noted previously were further analyzed biochemically and cytologically to delineate the mechanism of phototoxicity and detoxification in this monoporphyrinic model. Under optimal conditions of induction for approximately 3 days, cells remained viable but became increasingly uroporphyrinic, peaking at ≥90% of the population by approximately day 2; thereafter, a small population of less porphyric or aporphyric cells emerged. On exposure to light, the flagella of porphyrinic cells were immobilized in milliseconds, and singlet oxygen became detectable in their lysates. Both photosensitive phenotypes increased proportionally with the cellular uroporphyrinic levels and were susceptible to inhibition by azide, but not by d-mannitol. Brief irradiation of the uroporphyrinic cells produced no appreciable protein degradation but inactivated cytosolic neomycin phosphotransferase and significantly bleached cytosolic green fluorescent protein, which was azide reversible. These cells were irreparably photodamaged, as indicated by their subsequent loss of membrane permeability and viability. This is the first in situ demonstration that early inactivation of functional proteins by singlet oxygen initiates the cytolytic phototoxicity in uroporphyria. Detoxification appears to involve endocytic/exocytic mobilization of uroporphyrin from cytosol to “porphyrinosomes” for its eventual extracellular expulsion. This is proposed as the sole mechanism of detoxification, since it is attributable to the reversion of porphyrinic to aporphyric cells during uroporphyrinogenesis and repeated cycles of this event plus photolysis selected no resistant mutants, only aporphyric clones of the parental phenotypes. Further characterization of the transport system for uroporphyrin in this model is expected to benefit not only our understanding of the cellular mechanism for disposal of toxic soluble wastes but also potentially the effective management of human uroporphyria and the use of uroporphyrinic *Leishmania* for vaccine/drug delivery.

Uro-, copro-, and protoporphyrins (URO, COPRO, and PROTO, respectively) are ubiquitous intermediates in tetrapyrole/heme biosynthesis of all aerobic cells (41). Porphyrins are photactivatable to generate cytotoxic singlet oxygen (1O₂) and other reactive oxygen species (ROS) secondarily (2, 5, 7, 38). Singlet oxygen rapidly oxidizes biomolecules and is thus biologically very destructive, although the range of its effectiveness is extremely short (20 Å) (27, 28). Porphyrins and their derivatives have been used as photosensitizers clinically for photodynamic therapy of tumors (15, 35) and experimentally as photodynamic microbicides and pesticides (3, 13). Biosynthesis of all natural porphyrins is tightly regulated by necessity, since their accumulation often results in phototoxicity as seen in congenital human porphyria (41).

For effective use of porphyrins in practical applications and to elucidate cytophototoxicity in porphyria, cellular models are required to study their photodynamic interactions with biomolecules in a living cell environment. Conventional models include exposure of cells to delta-aminolevulinate (ALA) for transient porphyria (42), chemical induction of hepatic porphyria (14), and random genetic mutations for producing porphyric mice (1, 46), *Saccharomyces cerevisiae* (50), and zebrafish (49). Porphyrinia developed in these models is either of low level or not monospecific, thereby lacking sensitivity and specificity. Specific porphyrinic mutants of *Escherichia coli* (30) and mice (34) produced by gene knockouts are difficult to maintain or nonviable, resulting from the indispensability of the heme biosynthetic pathway due to the lack of efficient mechanisms for the uptake of exogenous heme.

The trypanosomatid protozoa, such as *Leishmania*, present an ideal model, since they have evolved a mechanism to take up hemoglobin, readily available in their habitats, as a nutritional source of heme (33, 43) that presumably resulted in the loss of their genes encoding heme biosynthetic enzymes (16, 32, 37). Evaluation of these genetic defects has led us to transfet *Leishmania* with mammalian cDNAs encoding the second and third enzymes in the heme biosynthetic pathway. These transgenic *Leishmania* protozoa are uroporphyrinogenic, as
they develop uroporphyrin when exposed to ALA—the product of the first enzyme in heme biosynthesis. ALA is nontoxic to *Leishmania* spp., which neither synthesize nor metabolize this compound (16). However, the transfectants are able to take up ALA for its conversion by the transgene products into URO, which accumulates as uroporphyrin I (16, 37) in the absence of downstream enzymes necessary for its further conversion into COPRO and PROTO.

In the present study, the inducible neogenesis of URO in *Leishmania* model is exploited to study phototoxicity and detoxification of cellular uroporphyrin. We demonstrated the production of \( \mathrm{O}_2 \) on exposure of the uroporphyrin *Leishmania* to light, apparently responsible for initiating the phototoxic phenotype observed during ALA-induced uroporphyrinogen-esis. The \( \mathrm{O}_2 \) generated appears to immobilize the flagella of these cells by inactivating functional proteins, as both were inhibited by azide, a \( \mathrm{O}_2 \)-specific scavenger. Escalation of the oxidative damages to cytosolic proteins by the \( \mathrm{O}_2 \) is thus suggested to culminate in the profound phototoxic phenotypes of cytosis. Disposal of phototoxic URO via its vacuolar condensation for extracellular exit, noted previously (37), was underscored here by finding the association of “porphyrinomes” with endocytic/exocytic vesicles. Exposure of cells to repeated cycles of uroporphyrinogenesis/photolysis selected an aporphyrin population of parental phenotypes, but no resistant clones. Vacular and extracellular transport of uroporphyrin is thus proposed as the sole mechanism of detoxification.

**MATERIALS AND METHODS**

**Cell culture.** Promastigotes of *Leishmania amazonensis* (LV 78 clone 12-1) were maintained in medium 199 (Sigma) with 10% heat-inactivated fetal bovine serum (HBFS), pH 7.4 (37). Transfection of promastigotes with pX- were maintained in medium 199 (Sigma) with 10% heat-inactivated fetal bovine serum (HIFBS), pH 7.4 (37). Transfection of promastigotes with pX-

**Transfectants were maintained in medium containing 100 g/ml of hygromycin.**

**Hygromycin (LV 78 clone 12-1)** were repeatedly plated for cloning at 10⁴ to 10⁵ cells/well in 24-well culture plates with a long-wavelength UV source (maximum wavelength of 366 nm) at a distance of 5 cm (37).

**Cell viability assays.** The viability of UV-exposed porphyric and aporphyrnic cells was assessed by incubation at 25°C in the dark for 10 h at 2 x 10⁵/ml in complete culture medium. Additionally, 100-g/ml aliquots of cells in BSA-HBSS were each mixed with 50 g/ml of a tetrazolium salt [2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-(2H)-tetrazolium-5-carboxanilide (XTT)] (cell viability kit from Roche). After incubation at 25°C for 2 h, soluble formazan derivatives were read according to the manufacturer’s protocol at 450 nm and 660 nm in a GENios plate reader (Texas Instruments) and analyzed by using XFLUOR4 software, version V 4.11.

**Flow cytometry.** Cells were induced to undergo uroporphyrinogenesis as described above. Aliquots of 100 g/ml each were collected daily and resuspended in BSA-HBSS to 10⁷ cells/ml for flow cytometry (BD-LS II). Fluorescence of URO (excitation wavelength [\( \lambda_e \]) of 405 nm and emission wavelength [\( \lambda_m \]) of 610 nm) and GFP (\( \lambda_e \) of 488 nm and \( \lambda_m \) of 510 nm) was determined in each sample at a flow rate of <500 events/second. The specificity of the fluorescence settings was verified and defined by using nonfluorescent and single-fluorescent cells. A total of 10,000 events were recorded and analyzed by BD Bioscience software FACS Diva (RFUMS Flow Cytometry Core Facility).

**Fluorimetry of porphyrins.** Aliquots (200 g/ml) of cell suspensions (2 x 10⁶ cells) were periodically withdrawn and centrifuged to sediment cells for porphyrin extraction and estimation by fluorimetry ([\( \lambda_e \]) of 405 nm and \( \lambda_m \) of 615 nm) (Pershkin-Elmer model LS50B) as described previously (16). Fluorimetric assay for singlet oxygen. Singlet oxygen produced by UV excitation of uroporphyrin was determined fluorometrically as oxidized singlet oxygen sensor green (SOSG) according to the manufacturer’s protocol (Molecular Probes) (10). Optimal conditions were first determined by using chemically pure URO (Porphyrin Products) serially diluted from 1 pm to 1 nM for reactions with individual samples. Each sample was subsequently assayed under the optimal conditions determined. For each assay, 10⁷ cells were lysed in 200 g/ml of HBSS containing 10 g M SOSG by three cycles of freeze-thawing in liquid nitrogen. After long-wavelength UV illumination for 5 min, lysates were each diluted to 3 ml and blanked with nonirradiated samples for fluorimetry of oxidized SOSG (\( \lambda_e \) of 488 nm and \( \lambda_m \) of 525 nm). The specificity of the fluorescence was indicated by its absence when read under the conditions for URO (see above). Readings obtained from the samples fell within the linear range of fluorescence intensities in the standard curve for URO.

**Western blot analysis.** Rabbit antiserum raised against porphobilinogen deaminase (40), neomycin phosphotransferase (NEO) (5’-3’ Inc., CO), a *Leishmania* cytotoxic protein (p36) (26) and a surface glycoprotein (gp63) (8), and a mouse monoclonal antibody clone B2 for GFP (Santa Cruz) were used. Immunoblots were reacted with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:10,000) or goat anti-mouse immunoglobulin G (1:4,000) and developed by using the Pierce SuperWest Pico reagent.

**Neomycin phosphotransferase activity.** A total of 10⁶ cells in ALA-free HBSS were lysed by three cycles of freeze-thawing in the presence of a protease inhibitor cocktail (Sigma). Lysates were centrifuged at 50,000 x g for 1 h to obtain supernatants, which were used as the enzyme source (10 g/ml aliquots) for reaction at 37°C for 15 min with neomycin sulfate (5 mM) and [32P]ATP (20 Ci/mmol). Phosphotransferase activity of ~3.0 pmol per assay as the substrates under the conditions described previously (36). Reaction products were extracted and subjected to liquid scintillation counting to determine activity (36).

All experiments described above were repeated at least three times, each in triplicate, and data from a representative experiment are presented. The error
bars in all figures were calculated as standard errors of the means from triplicate samples for each time point.

RESULTS

Population dynamics and heterogeneity of porphyric cells in ALA-induced uroporphyrinogenesis. The triple transfectants expressing GFP were exposed to ALA for up to 3 days in the dark. Flow cytometric analysis of these cells collected at different times for URO and GFP double fluorescence revealed population heterogeneity of porphyria (URO fluorescence) without loss of cell viability (GFP fluorescence) (Fig. 1). Samples collected at time zero of ALA exposure showed that cells were positive for GFP and completely negative for URO, as expected (Fig. 1A). After exposure to ALA for 16 h, URO fluorescence emerged in 15 to 20% of the GFP-bright and thus metabolically active population (Fig. 1B). The URO-positive population, consisting of cells with variable fluorescent intensities, expanded significantly to 90% of the GFP-positive cells after exposure to ALA for approximately 2 days (Fig. 1C). URO- and GFP-positive populations largely superimposed. Cells negative for either or both URO- and GFP-positive populations are negligible in number, although the fluorescence of GFP-positive population decreased slightly. By approximately day 3 after ALA exposure, the bulk of the double-fluorescent population remained intact, although a small subpopulation of URO-less positive and URO-negative cells appeared (<10%) (Fig. 1D). However, all these cells were GFP positive and motile when directly observed by microscopy. Taken together, the results demonstrated that cells induced to develop uroporphyrinaemia remained viable throughout the entire period of the study and a small population of these porphyric cells became aporphyrinic beyond day 2. When transferred to culture medium for further incubation, the entire population became aporphyrinic (not shown).

Light-induced SOSG fluorescence by O$_2$ increases with URO level in the cell lysates, which is sensitive to inhibition by azide during uroporphyrinogenesis. Cells were collected periodically under conditions identical to those described in the legend to Fig. 1 for lysate preparation. This was necessary for this assay, since intact Leishmania cells were impervious to SOSG. Fluorescence intensities of SOSG oxidized by O$_2$ produced from light-excited URO in the lysates were assessed
Flagellar immobilization of uroporphyrinogenesis as shown in Fig. 1. Freeze-thawed lysates were assayed in the presence of aporphyric cell lysates (not shown). Observed when equivalent amounts of URO were similarly treated with ALA for 16 h) by sodium azide, but not by mannitol, was found to decrease from ~50% to ~15% with increasing concentrations of sodium azide, but not with D-mannitol up to 100 mM (Fig. 4B). D-Mannitol remained ineffective even when used to treat cells together with ALA in the dark for up to 1 day. Sodium azide thus not only abolished the SOSG fluorescence for $^{1}O_2$ but also inhibited flagellar immobilization of light-exposed uroporphyrin cells.

Flagellar immobilization of porphyric cells by light invariably followed by cellular permeabilization and loss of viability. For phototoxicity assays, suspensions of uroporphyrin cells optimally induced with ALA for 36 h were used for long-wave-length UV illumination for up to 30 min in microtiter wells (Fig. 5). Flagellar immobilization under these conditions showed biphasic kinetics: an initial rapid rise almost to the maximum in <5 min followed by a very slow increase in the next ~25 min, reaching a maximum of ~90% (Fig. 5A). No flagellar immobilization occurred in any of the controls, as expected (Fig. 5A, DT-ALA+UV, DT-ALA-UV, and DT-ALA-UV treatments). Although this and the fluorescent microscopic assays (Fig. 4A) produced different kinetics of flagellar immobilization for porphyric cells due to the different experimental conditions used, the values obtained at the end point are comparable, i.e., ~90% immobilized. Cells so treated were found to be nonviable when inoculated into culture medium and incubated overnight. Microscopy for their motility in combination with permeability to PI showed that while control nonporphyric cells remained 100% viable (Fig. 5B, DT-ALA+UV and DT-ALA-UV), porphyric cells lost their viability progressively with time of illumination (Fig. 5B, DT-ALA+UV), the kinetics being similar to that of the flagellar immobilization evaluated 16 h earlier (Fig. 5A). Thus, porphyric cells, once immobilized, can no longer recover their

Fluorimetrically (Fig. 2A). Clearly, the fluorescence was detected after illumination of porphyric cell samples (with ALA [Fig. 2A]), but not those from aporphyric cells (without ALA [Fig. 2A]). Its intensity initially increased exponentially with time for up to ~2 days and decreased thereafter in parallel with the decreasing cellular URO levels (Fig. 2A, cellular URO bars). The fluorescence of SOSG for $^{1}O_2$ became essentially undetectable in the presence of sodium azide, but not D-mannitol (Fig. 2B). The same level of inhibition by azide was observed when equivalent amounts of URO were similarly assayed in the presence of aporphyric cell lysates (not shown). Illumination of uroporphyrin cells thus generates $^{1}O_2$ proportionally to the URO accumulated therein.

Flagellar immobilization of uroporphyrin *Leishmania* by microscopic illumination and its azide sensitivity. Fluorescent microscopic illumination of uroporphyrinic *Leishmania* instantly immobilized their flagella. We documented this event as “blurry” versus “sharp” images of motile and nonmotile flagella by phase-contrast microscopy captured 10 milliseconds before and after illumination at 405 nm (Fig. 3A and B, panels 1 and 2, versus Fig. 3D, panels 1 and 2). Flagellar motility was paralyzed by this brief illumination of porphyric cells (Fig. 3A and B, panels 3 and 4), but not aporphyric cells (Fig. 3D, panels 3 and 4; Fig. 4A, without ALA). Immobilized cells increased in number exponentially to a maximum of ~95% of the population in the first 2 days under the standard conditions (Fig. 4A, cells treated with ALA) or the alternative conditions (see Fig. S1 in the supplemental material). This increase was in parallel with the increasing levels of cellular URO in both cases (Fig. 4A) (see Fig. S1 in the supplemental material). Flagellar motility of cells was more light sensitive during the first 2 days of ALA exposure (up to ~95% immobilized) when URO fluorescence was cytosolically more diffused or diffused plus vacuolar (Fig. 3A and B, panels 3 and 4), but it became much less light sensitive beyond day 2 (~75% immobilized) when URO was mobilized to vacuoles or “porphyrinosomes” (Fig. 3C, panels 3 and 4). The last population was estimated to represent ~20% of the porphyric cells, which constituted the bulk of cells insensitive to light for flagellar immobilization, the remainder being a very small number of aporphyric cells seen beyond ~day 2 (cf. Fig. 1D).

Light-sensitive flagellar immobilization was inhibited by sodium azide, but not by D-mannitol, as observed by their effects on SOSG fluorescence intensities for $^{1}O_2$. We examined light-sensitive flagellar immobilization inhibition by using cells that had been ALA induced in the dark for 1 day when ~50% of them were susceptible to light for flagellar immobilization (Fig. 4A). When microscopically assessed, flagellar immobilization was found to decrease from ~50% to ~15% with increasing concentrations of sodium azide, but not with D-mannitol up to 100 mM (Fig. 4B). D-Mannitol remained ineffective even when used to treat cells together with ALA in the dark for up to 1 day. Sodium azide thus not only abolished the SOSG fluorescence for $^{1}O_2$ but also inhibited flagellar immobilization of light-exposed uroporphyrin cells.

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viability. Flagellum-immobilized cells indeed were leaky and non-viable when assessed immediately after illumination. Uroporphyrinic cells were centrifuged with and without prior UV irradiation for 30 min (Fig. 5C). URO sedimented with nonirradiated cells, leaving the supernatant nonfluorescent, as expected (Fig. 5C, left bottom panel). In contrast, URO fluorescence appeared in the supernatant of irradiated cells, indicative of their leakiness (Fig. 5C, right bottom panel). In addition, permeability of these uro-

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**FIG. 3.** Rapid flagellar immobilization of live uroporphyrinic cells by microscopic illumination. Microscopic illumination of the porphyric cells for 10 milliseconds at 405 nm (see arrow) immobilizes their flagella, giving sharp instead of blurry images when acquired under the same exposure conditions (for panels A and B, compare panels 1 and 2). Aliquots of 5 μL were observed in triplicate samples by phase-contrast microscopy to count motile/stiff flagellum of >100 cells before and after illumination. The cellular distribution of URO is shown in panels 3A to C. Flagellar immobilization became less light sensitive at the late phase of uroporphyrinaemia, e.g., treatment with ALA (+ ALA) for 64 h (C), and was never observed with non-ALA-treated (− ALA) and thus aporphyric cells (D). Phase-contrast and fluorescence images were merged to show cellular localization of uroporphyrin at specific time points (panels 4, Merged). Motility is shown to the right of the figure as follows: −, not motile; +, motile.

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**FIG. 4.** Uroporphyrinic level-dependent and azide-reversible flagellar immobilization of cells during delta-aminolevulinate-induced uroporphyrinogenesis. (A) Flagellar immobilization as a function of cytosolic uroporphyrin accumulation. See the legend to Fig. 1 for experimental conditions with ALA (+ALAL) and without ALA (− ALA). At the time points indicated, aliquots of cells were withdrawn to count the cells and determine the percentage of porphyric promastigotes (+ALAL/mL) immobilized by fluorescent microscopic illumination at 405 nm (+mL) and to determine the level of URO by fluorimetry of cell lysates (in picomoles/10⁸ cells) (see Materials and Methods for details). Note that flagellar immobilization is observed only in light-exposed porphyric cells, not in the controls without ALA exposure. (B) Differential inhibition of light-induced flagellar immobilization by ROS scavengers. Cells exposed to 1 mM ALA in the dark for ~24 h were analyzed for flagellar immobilization by fluorescence microscopy (ALA+mL) in the presence and absence of different ROS scavengers. See Materials and Methods for additional details. Note the dose-dependent inhibition of flagellar immobilization by sodium azide, but not by d-mannitol.
FIG. 5. Kinetics of flagellar immobilization and cytology of porphyric cells in suspension during the course of UV illumination. Cells (DT) were rendered optimally uroporphyrin (with ALA [+ALA]) for 36 h under the conditions described (see the legend to Fig. 4) and then illuminated by long-wavelength UV in ALA-free HBSS at $5 \times 10^7$ cells/ml for up to 30 min. Controls included single and double omissions of ALA exposure and UV illumination, i.e., cells without ALA (DT−ALA) or without UV (DT−UV) or both (DT−ALA−UV). (A) Kinetics of flagellar immobilization with time after UV illumination. Immobilized cells were determined microscopically as described above. Note the absence of flagellar immobilization in all control cells (DT−ALA+UV, DT+ALA−UV, and DT−ALA−UV). (B) Coincidence in the kinetics of flagellar immobilization and irreversible loss of cell viability. Samples after UV illumination for 30 min in panel A (1-ml aliquots) were resuspended in medium 199 containing 10% FBS at 25°C, and cell viability was evaluated by microscopy for both cellular integrity and permeability to propidium iodide. (C and D) Permeabilization of the flagellum-immobilized uroporphyrin cells after long-wavelength UV illumination for 30 min. (C) Retention and leakage of uroporphyrin from these cells before (left panel) and after (right panel) UV illumination. After centrifugation, URO sedimented with the nonilluminated cells to the pellet (left bottom panel) but remained in the supernatants of the UV-illuminated cells (right bottom panel). In the left bottom panel, the white arrow points to the URO-fluorescent pellet, while in the right bottom panel, the white arrow points to the less fluorescent pellet. (D) Permeability of the porphyric cells to PI for staining nuclei without UV illumination (−Light) and with UV illumination (+Light). Fluorescent nuclei greatly increased in number after UV illumination (right bottom panel versus top panel), corresponding to “ghost” cells instead of intact cells (bottom panel, white and red arrowheads versus black arrowhead). PI+ve, PI positive. (E) Loss of tetrazolium reduction activities of uroporphyrin cells after UV illumination. See Materials and Methods for measuring absorbance of reduced XTT. Note the ~10-fold reduction of these activities in the experimental group (exposure to ALA for 36 h in dark and UV illumination for 30 min [+ALA+UV]) than all the control groups (−ALA−UV, +ALA−UV, and −ALA+UV).
porphyrinogenesis followed by photolysis (ALA plus UV). After irradiation of porphyric cells for as long as 30 min (Fig. 8, cells treated with ALA and UV, 0 to 5 min). In contrast, irradiation of aporphyric cells for as long as 30 min had no effect on NEO activity (Fig. 8, no ALA but treated with UV for 30 min). The results presented provide unequivocal evidence for functional inactivation of cytosolic proteins, independent of their degradation.

**Exposure of cells to repeated cycles of treatments for uroporphyrinogenesis and photolysis selected aporphytic clones of parental phenotypes, but not resistant mutants.** Cells were consecutively subjected to six cycles of ALA-induced uroporphyrinogenesis followed by photolysis (ALA plus UV). After radiation in uroporphyrinogenic cells after exposure to light at least in the first several minutes (Fig. 6A). Some protein bands appeared to shift or lose intensity with time of irradiation in these samples (Fig. 6A, lanes 5 to 8) compared with those of the nonirradiated samples and other controls (Fig. 6A, lanes 2 to 4). Western blot analysis of specific proteins showed variable changes in their intensities during the course of UV irradiation (Fig. 6B). The difference in the intensity of these proteins between nonirradiated or aporphyric samples (Fig. 6B, lanes 1 to 3) and irradiated porphyric samples (Fig. 6B, lanes 4 to 7) was either unapparent, i.e., the endogenous surface zinc metalloproteinase (gp63), or modest up to 5 min, i.e., those abundantly expressed by the transgenes, i.e., porphobilinogen deaminase, NEO, and GFP. There was clear evidence of significant changes only for one endogenous cytosolic protein (p36), as indicated by its diminished intensity from 2 to 15 min (Fig. 6B, lanes 4 to 7). It is not known if this may be related to the NADPH Rossman fold found in p36 (26). The presence of NADPH together with URO was reported to mediate partial conversion of 1O2 by light into the hydroxyl radical (47) that is known to hydroxylate preferentially valine and leucine residues (20), which are exceptionally abundant in the p36 sequence (23%) (GenBank accession no. L11705). Thus, p36 may be more extensively modified than the others, rendering it either more susceptible to aggregation/degradation or less recognizable by the antibodies used, accounting for its decrease in intensity seen. Overall, changes are very limited in the bulk of the cellular proteins in uroporphyrinogenic cells after brief irradiation.

However, cytosolic GFP and NEO were functionally compromised within the time frame of irradiation for up to 5 min when no apparent degradation of these proteins was evident in the uroporphyrinogenic cells. Fluorescence of cytosolic GFP visibly lost intensity by microscopic illumination of uroporphyrinogenic cells for 500 ms (Fig. 7A, compare the three panels 2 and panels 4). No loss of GFP fluorescence was noted in these cells under aporphyric conditions (Fig. 7A, bottom row). Fluorescence bleaching of GFP was unapparent when assayed under similar experimental conditions in the presence of sodium azide (Fig. 7B, compare top and bottom panels) at the same concentration (1.5 mM) that removed 1O2 (Fig. 2B) and inhibited flagellar immobilization (Fig. 4B). There was no spectral shift associated with fluorescence bleaching and its reversal by azide for any of these samples when scanned fluorimetrically (17) (not shown).

Irradiation of porphyric cells inactivated their NEO catalytic activities dramatically (Fig. 8). The enzyme activities decreased almost exponentially from the highest level to essentially zero after irradiation of porphyric cells for a short duration, i.e., <5 min (Fig. 8, cells treated with ALA and UV, 0 to 5 min). In contrast, irradiation of aporphyric cells for as long as 30 min had no effect on NEO activity (Fig. 8, no ALA but treated with UV for 30 min). The results presented provide unequivocal evidence for functional inactivation of cytosolic proteins, independent of their degradation.

**Cellular protein integrity and functional inactivation following brief irradiation of uroporphyrinogenic cells.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the total cellular proteins showed no evidence of their significant deg-
each of the six successive combination treatments, a small percentage of cells survived, none of which was found to contain URO when examined by fluorescence microscopy. However, they are not mutants selected for resistance to the treatments. First, the transgenes of these survivors apparently remained intact, since they grew as well as their parental aporphyrinic cells under the same selective conditions used for their routine maintenance, i.e., 20 μg of tunicamycin and 100 μg of G418 per ml. Second, cells so selected after each cycle of the combination treatments were found as susceptible as their parental transfectants to ALA for developing uroporphyrina and photolysis (not shown). Finally, four of the eight clones obtained by plating the survivors after the last cycle of the combination treatments on LIT agar were found to undergo uroporphyrinogenesis (see Fig. S2 in the supplemental material) and photolysis (not shown), just like the parental cells never exposed to ALA plus UV. Thus, there is a transition of photosensitive porphyria to aporphyrinia during uroporphyrinogenesis in a small number of these cells (cf. Fig. 1D), independent of selection for their mutations.

Colocalization of “uroporphyrinosomes” and endocytic/exocytic vesicles. Porphyrinogenic and control cells were exposed

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to ALA under optimal conditions for the former to develop URO-containing vacuoles or “porphyrinosomes” (Fig. 9, panels 3, Uroporphyrin). All markers used fluoresced in the expected cellular targets in both porphyric and aporphyric cells, except for the presence of “porphyrinosomes” in the former. Exposure of the porphyrinic cells (Fig. 9, panels 1) to dextran-FITC (Fig. 9A and B, Dextran) or FM 4-64 (Fig. 9D, FM 4-64) as markers for endocytic (or exocytic) vesicles revealed colocalization of the fluorescent signals for both URO and the respective marker in the same vacuoles (Fig. 9A, B, and D, panels 4 [Merged]), suggestive of their identity or continuity. FM 4-64 labeled additional structure (Fig. 9D, panel 2), which colocalized substantially with dextran-FITC in aporphyric cells (Fig. 9C, panel 4 [Merged]), but not with URO in porphyric cells (Fig. 9D, panel 4 [Merged]). This suggests that “porphyrinosomes” may be a specific section or in continuum with this section of the endocytic/exocytic pathway. The specificity of this localization is underscored by the apparent lack of colocalization of fluorescence for URO and rhodamine 123 for mitochondria (Fig. 9E) or LysoTracker for acidic compartments, e.g., acidocalcisomes (Fig. 9F). “Filamentous” structures lit up by both FM 4-64 and dextran-FITC might be part of multivesicular tubular lysosomes, although they were not as striking as those reported by others (29). Whether LysoTracker labels *Leishmania* lysosomes is a matter of some controversy (4, 33). Our negative finding with this marker for colocalization with “porphyrinosomes” thus does not necessarily contradict the report that URO is present in the lysosomes of liver cells in hepatic uroporphyria (39).

**DISCUSSION**

As far as we know, the transgenic *Leishmania* model is the only model available that can be induced by ALA to become highly porphyric from a totally aporphyrinic state. Neogenesis of URO and uniporphyrin specificity of this model offer the unique opportunity, not previously available, to study the mechanisms of phototoxicity and detoxification in uroporphyria. Several phenotypes noted previously in passing were further studied quantitatively to facilitate such investigation, e.g., light-sensitive flagellar immobilization followed by cytolsis of the porphyric cells on the one hand, and vacuolar condensation of URO preceding its extracellular exit on the other hand (16, 37).

We first demonstrated here by flow cytometry of GFP and URO doubly fluorescent cells that they maintain viability (GFP fluorescence) throughout the entire course of ALA-induced uroporphyrinogenesis, independent of the rise and fall in their cellular levels of URO. Both flow cytometry of live cells for the URO-fluorescent population (Fig. 1A to C) and fluorimetry of URO in their cell lysates (Fig. 2A and 4A, cellular URO) showed that the overwhelming majority of the population (≥90%) develops uroporphyria exponentially up to approximately day 2. Beyond day 2, a small population of viable cells shifted from porphyric to aporphyrinic (Fig. 1D) in parallel to a decrease, albeit variable in extent, of URO in the cells (16, 37) (Fig. 2A and 4A, 64-h exposure to ALA). The viability of the porphyric cells indicates that dark toxicity of uroporphyria, if present, is very marginal, pointing to the necessity of light for manifestation of the cytototoxic phenotypes seen.

Exposure of uroporphyrinic cells to light indeed excites URO therein to generate cytotoxic 1O2, as shown in the cell lysates by the sensitive and specific assay for the fluorescence of oxidized SOSG (Fig. 2). Significantly, exposure of the live but cytosolically porphyric cells to light immobilized their flagella (Fig. 3) instantly—a time frame expected for 1O2 production by photoexcited URO (27). The 1O2 produced is likely directly responsible for this early phototoxic phenotype, since both increase and decrease with the cellular level of URO during uroporphyrinogenesis (Fig. 2A and 4A), and since both are abolished by azide, a 1O2-specific scavenger (45), but not by D-mannitol, a scavenger for other ROS (23) (Fig. 2B and 4B). Flagellar immobilization is a prelude to cell death (Fig. 5) and thus an early sensor for the irreversible phototoxicity apparently initiated by 1O2 in uroporphyria.

In this study, evidence is presented suggesting that 1O2 generated inactivates protein functions. The time frame of illumination for flagellum immobilization (in milliseconds) is too short to expect any appreciable protein degradation as the possible cause of this phenotype. There are indeed no significant changes in the total or specific proteins after irradiation of uroporphyrinic cells for as long as 2 min when cytosolic GFP fluorescence was bleached (Fig. 7A) and neomycin phosphotransferase activity was significantly inhibited (Fig. 8). A major role of 1O2 in this is indicated by the inhibition of GFP fluorescence bleaching by azide (22). Crucial residues for fluorescence of GFP (9, 31) and catalytic function of NEO (19) may be modified by this ROS in the uroporphyric cells, accounting for their loss of these activities, as reported for several functional proteins in solution (11, 18). By inference, 1O2 may cause flagellar immobilization by inactivating a protein(s) crucial for motility and/or energy supply, e.g., oxidative damage of dynein in the axonemes, as found in ROS-mediated immobilization of human (12) and sea urchin sperm (21).

The cumulative effects of protein inactivation by 1O2 and the
oxidative activities of other ROSs generated secondarily are the principal cause of membrane permeabilization, loss of viability, and cytolysis observed during the later stage of phototoxicity (Fig. 5). Hydrophilic URO has no membrane affinity, unlike hydrophobic PROTO. The latter is known to accumulate in mitochondrial membranes (24), resulting in apoptosis of protoporphyric cells upon illumination (44, 48). Exposure of our mono- or aporphyrinic cells to light is likely to result in necrotic cytolysis, as we noted neither chromosomal DNA ladder nor morphological changes in the mitochondria of irradiated cells (not shown). Thus, although 1O2 is produced by light excitation of all porphyrins (5, 35), the targets susceptible to the attacks by this ROS depends critically on their membrane association or cytosolic location, thereby producing very different phototoxic cytolysis.

The finding of “porphyrinosomes” in association with endocytic/exocytic vesicles (Fig. 9) supports our previous proposal that vacuolar mobilization of URO is related to its extracellular exit for alleviation of phototoxicity (37). Endosomal origin or continuity of “porphyrinosomes” raises the possibility that URO may be imported into endocytic vesicles and evacuated via the exocytic pathway, thereby contributing to its extracellular exit. It would seem feasible to speculate that some endosomes when formed from the flagellar pocket membrane might...
include an efflux pump for unidirectional transport of URO, giving rise to the “porphyrinosomes” observed. Specific ABC transporters have been reported as responsible for porphyrin trafficking in mammalian cells (25). Whether such Leishmania homologues exist awaits further investigation. Significantly, vacuolar mobilization of URO coincides with the loss of photosensitivity of porphric cells, suggestive of its role in detoxification by itself and by facilitating exocytic exit of URO. Indeed, phototoxicity is totally absent when cells were exposed to extracellular URO released in abundance by uroporphric cells (16, 37) or added exogenously to aporphyric cells at a very high concentration (up to 100 μM) (37). Cells either do not take up URO or do so but expel it rapidly, perhaps via the putative efflux pump proposed. In either case, the vacuolar and plasma membranes are obviously not susceptible to the attacks by the light-generated 1O2. It is unknown whether this is due to the presence of membranous sinks for this ROS (6), which dampens its oxidative activities, and/or to the very short range of its effectiveness (27, 28). In any case, 1O2 is apparently incapable of crossing membrane barriers, consonant with the proposal that cytolytic phototoxicity is initiated by inactivation of cytosolic proteins instead of membrane disruption. Further characterization of the URO transport is warranted, since it may well be an important efflux system of general importance for cellular disposal of unwanted soluble molecules, e.g., endogenously generated metabolic wastes and xenogenically derived toxic compounds, e.g., antileishmanial drugs.

Vacular and extracellular mobilization of URO is likely the only mechanism for uroporphric cells to escape from irreversible phototoxicity. No known detoxification mechanism has been reported so far in any biological system for direct neuronal uptake of 1O2. Indeed, there is little or no evidence for the development of resistance in tumor cells to photodynamic therapy (35), as observed here. Namely, exposure of cells to repeated cycles of uroporphyrinogen/photolysis selected no transgenes for URO-negative phenotypes and/or of the endogenous genes for the phenotypes altered in ALA uptake, URO transport, and/or antioxidant enzymes. Instead, survivors so selected were invariably found to be of the parental phenotypes (see Fig. S2 in the supplemental material). Thus, the only mechanism of detoxification left for consideration is URO mobilization, accounting for the appearance of aporphyic cells during urorophyrinogenesis (Fig. 1D) and their unfailing emergence after each successive cycle of the treatments and after the last cycle in the cloned populations (see Fig. S2 in the supplemental material).

In summary, an ALA-inducible monoporphric model was examined to elucidate the phototoxic and detoxification mechanisms in uroporphria for the first time in the absence of hydrophobic COPRO and PROTO. The results suggest that cytolytic phototoxicity is irreversible and initiated by functional inactivation of proteins mainly by 1O2 generated via light excitation of cytosolic URO. An endocytic transport system is proposed for effective evacuation of cytosolic URO as an efficient mechanism of detoxification. Further characterization of the putative transport system is of interest in considering its potential as a constitutive mechanism of cells for soluble waste disposal (37). Also of considerable interest is its potential manipulation for upregulation to alleviate cellular pathology in human uroporphria, i.e., congenital erythropoietic porphyria (41), and for downregulation to enhance suicidal cytolysis of uroporphriic Leishmania proposed for vaccine/drug delivery (16, 37).

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REFERENCES

1. Abitbol, M., F. Bernex, H. Puy, H. Jouault, J. C. Drybach, J. L. Guenet, and X. Montaugutelli. 2005. A mouse model provides evidence that genetic background modulates anemia and liver injury in erythropoietic porphyria. Am. J. Physiol. Gastrointest. Liver Physiol. 288:R205–R216.
2. de Lamirande, E., and C. Gagnon. 1992. Microbial glycolipids: possible virulence factors that scavenge oxygen radicals. Proc. Natl. Acad. Sci. USA 89:2453–2457.
3. Chen, D. Q., B. K. Kolli, N. Yadava, H. G. Lu, A. Gilman-Sachs, D. A. Peterson, and K. P. Chang. 2000. Epidermal expression of specific sense and antisense miRNAs in Leishmania amazonensis: modulation of gp63 level in promastigotes and their infection of macrophages in vitro. Infect. Immun. 68:58–86.
4. Codi, C. L., D. Prasher, W. M. Westler, F. G. Prendergast, and W. G. Ward. 1993. Chemical structure of the hexapetate chromophore of the Aequorea green-fluorescent protein. Biochemistry 32:1212–1218.
5. Dougherty, T. J., C. J. Gomer, B. W. Henderson, G. Jori, D. Kessel, M. Blum, A., and L. I. Grossweiner. 1985. Hydrogen peroxide, superoxide, and hydroxyl radicals are involved in the phototoxic action of hematoporphyrin derivative at 546 nm in phosphate buffer and in the presence of egg phosphatidylcholine liposomes. Photochem. Photobiol. 41:27–32.
6. Chan, J., T. Fujiwara, P. Brennan, M. McNeil, S. J. Turco, J. C. Sible, M. Snapper, P. Aisen, and B. R. Bloom. 1989. Microbial glycolipids: possible virulence factors that scavenge oxygen radicals. Proc. Natl. Acad. Sci. USA 86:2453–2457.
7. Chekalayeva, L. V., I. N. Shevchuk, V. A. Chekalayeva, and K. Ilmarinen. 2006. Hydrogen peroxide, superoxide, and hydroxyl radicals are involved in the phototoxic action of hematoporphyrin derivative against tumor cells. J. Environ. Pathol. Toxicol. Oncol. 25:51–77.
8. Chen, D. Q., B. K. Kolli, N. Yadava, H. G. Lu, A. Gilman-Sachs, D. A. Peterson, and K. P. Chang. 2000. Epidermal expression of specific sense and antisense miRNAs in Leishmania amazonensis: modulation of gp63 level in promastigotes and their infection of macrophages in vitro. Infect. Immun. 68:58–86.
9. Codi, C. L., D. Prasher, W. M. Westler, F. G. Prendergast, and W. G. Ward. 1993. Chemical structure of the hexapetate chromophore of the Aequorea green-fluorescent protein. Biochemistry 32:1212–1218.
10. Danon, A., N. S. Coll, and K. Apel. 2006. Cytochrome-c-dependent execution of programmed cell death induced by singlet oxygen in Arabidopsis thaliana. Proc. Natl. Acad. Sci. USA 103:17066–17041.
11. Davies, M. J. 2005. Photodynamic therapy targeted to pathogens. Int. J. Immunopharmac. Pharmacol. 17:245–254.
12. De Verneuil, H., S. Sassa, and A. Kappas. 1983. Effects of polychlorinated biphenyl compounds, 2,3,7,8-tetrachlorodibenzo-p-dioxin, phenobarbital and iron on hepatic uroporphyrinogen decarboxylase. Implications for the pathogenesis of porphyria. Biochem. Biophys. Res. Commun. 120:578–582.
13. De Lamirande, E., and C. Gagnon. 1992. Reactive oxygen species and human spermatozoa. I. Effects on the motility of intact spermatozoa and on sperm acrosomes. J. Androl. 13:368–379.
14. Demidova, T. N., and M. R. Hamblin. 2004. Photodynamic therapy targeted to pathogens. Int. J. Immunopharmac. Pharmacol. 17:245–254.
15. De Verneuil, H., S. Sassa, and A. Kappas. 1983. Effects of polychlorinated biphenyl compounds, 2,3,7,8-tetrachlorodibenzo-p-dioxin, phenobarbital and iron on hepatic uroporphyrinogen decarboxylase. Implications for the pathogenesis of porphyria. Biochem. J. 214:145–151.
16. Dougherty, T. J., C. J. Gomer, B. W. Henderson, G. Jori, D. Kessel, M. Korbelik, J. Moon, and Q. Peng. 1998. Photodynamic therapy. J. Natl. Cancer Inst. 90:889–905.
17. Dutta, S., K. Furuyama, S. Sassa, and K. P. Chang. 2008. Leishmania spp.: delta-aminolevulinate-inducible neogenesis of porphyria by genetic complementation of incomplete heme biosynthesis pathway. Exp. Parasitol. 118:629–636.
18. Dutta, S., D. Ray, B. K. Kolli, and K. P. Chang. 2005. Photodynamic sensitization of Leishmania amazonensis in both extracellular and intracellular stages with aluminum phthalocyanine chloride for photolysis in vitro. Anti-microb. Agents Chemother. 49:4474–4484.
19. Finley, E. L., J. Dillon, R. K. Crouch, and K. L. Schey. 1998. Identification of trypanphot oxidation products in bovine alpha-crystallin. Protein Sci. 7:1239–1247.
20. Fong, D. H., and A. M. Berghuis. 2002. Substrate promiscuity of an amino-glycoside antibiotic resistance enzyme via target mimicry. EMBO J. 21:2323–2331.
