Genetic Rescue of \textit{Leishmania} Deficiency in Porphyrin Biosynthesis Creates Mutants Suitable for Analysis of Cellular Events in Uroporphyria and for Photodynamic Therapy*

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\textit{Leishmania} was found deficient in at least five and most likely seven of the eight enzymes in the heme biosynthesis pathway, accounting for their growth requirement for heme compounds. The xenotransfection of this trypanosomatid protozoan led to their expression of the mammalian genes encoding \(\delta\)-aminolevulinate (ALA) dehydratase and porphobilinogen deaminase, the second and the third enzymes of the pathway, respectively. These transfectants still require hemin or protoporphyrin IX for growth but produce porphyrin when ALA was supplied exogenously. \textit{Leishmania} is thus deficient in all first three enzymes of the pathway. Uroporphyrin I was produced as the sole intermediate by these transfectants, further indicating that they are also deficient in at least two porphyrinogen-metabolizing enzymes downstream of porphobilinogen deaminase, \textit{i.e.} uroporphyrinogen III co-synthase and uroporphyrinogen decarboxylase. Pulsing the transfectants with ALA induced their transition from aporphyria to uroporphyria. Uroporphyrin I emerged in these cells initially as diffused throughout the cytosol, rendering them sensitive to UV irradiation. The porphyrin was subsequently sequestered in cytoplasmic vacuoles followed by its release and accumulation in the extracellular milieu, concomitant with a reduced photosensitivity of the cells. These events may represent cellular mechanisms for disposing soluble toxic waste from the cytosol. Monocytic tumor cells were rendered photosensitive by infection with uroporphoric \textit{Leishmania}, suggestive of their potential application for photodynamic therapy.

\textit{Leishmania}, like other trypanosomatid protozoa, are among the rare examples of aerobic organisms, which depend on oxidative phosphorylation (for \textit{Leishmania mexicana} and \textit{Leishmania amazonensis}, see Refs. 1 and 2), but are defective in the synthesis of heme (3) required for electron transport respiratory complexes. This peculiar defect in tetrapyrrole biosynthesis is manifested as a nutritional requirement for heme by these organisms in chemically defined medium (for review, see Ref. 3). In nature, these parasitic protozoa must acquire protoporphyrin IX or heme exogenously from their hosts as a nutritional factor (3). Exceptional are several entomophilic nonpathogenic \textit{Crithidia} species that harbor \(\beta\)-proteobacteria as endosymbionts presumably to help them complete the heme biosynthetic pathway, thereby sparing their nutritional requirement for heme as an essential growth factor (4).

Earlier biochemical studies of trypanosomatid protozoa have shown that they are deficient in heme biosynthesis (3, 5–8). This was examined according to the following conventional pathway: glycine + succinyl-CoA or 4,5-dioxovalerate + alane \(\rightarrow\) \(\delta\)-aminolevulinate (ALA)\textsuperscript{3} \(\rightarrow\) porphobilinogen \(\rightarrow\) hydroxymethylbilane (by-product = uroporphyrinogen I) \(\rightarrow\) uroporphyrinogen III \(\rightarrow\) co-protoporphyrinogen III \(\rightarrow\) protoporphyrinogen IX \(\rightarrow\) protoporphyrin IX \(\rightarrow\) heme (9). Table I lists the eight enzymes, which are known to catalyze this pathway. The activities of these enzymes are often undetectable or negligible in trypanosomatid protozoa. Reported previously in these organisms were the activities of ALA-synthase/dioxovalerate transaminase and ferrochelatase (7, 8, 10), the first and the last enzymes of the pathway normally present in mitochondria (9). Much less or absent are activities of the second and the third enzymes, \textit{i.e.} \(\delta\)-aminolevulinate dehydratase (ALAD, EC 4.2.1.24) and porphobilinogen deaminase (PBGD, EC 4.3.1.8) (3, 5–7). The pathway thus appears to be incomplete in this group of organisms (3, 6). Endosymbionts are thought to complement this incomplete pathway in their \textit{Crithidia} host by supplying the missing enzymes, \textit{i.e.} PBGD (5).

In the present studies, the heme biosynthetic pathway was found far more defective in trypanosomatids than expected as determined by genetic complementation of naturally symbiont-free \textit{Leishmania}. These organisms normally infec mammalian macrophages as intracellular parasites and acquire heme via the activity of their host cells (3). We report here that transgenic \textit{Leishmania} with \textit{alad} and \textit{pbgd} became highly porphyrpic when supplied with ALA, indicative of their deficiencies in \(\delta\)-aminolevulinate synthase in addition to ALAD and PBGD. The production of uroporphyrin I as the sole intermediate under these conditions also indicates the deficiency of porphyrinogen-modifying enzymes further downstream of the pathway. Supplying the transfectants with exogenous ALA caused cellular accumulation of uroporphyrin I followed by its release. The infection of monocytic tumor cells \textit{in vitro} with these porphyric \textit{Leishmania} followed by UV irradiation resulted in their

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1 The abbreviations used are: ALA, \(\delta\)-aminolevulinate; ALAD, \(\delta\)-aminolevulinate dehydratase; PBGD, porphobilinogen deaminase.
Enzymatic defects of trypanosomatid protozoa in heme biosynthesis

| Enzyme                  | Functional status | Previous (Ref.) | Present |
|-------------------------|-------------------|-----------------|---------|
| 1. ALAS: ALA synthase   | +(6–8)            |                 |         |
| 2. ALAD: ALA dehydratase| +/(3, 5, 7)       |                 |         |
| 3. PBGD: Porphobilinogen deaminase | +/(3, 5, 7) |                 |         |
| 4. UROD: Uroporphyrinogen decarboxylase | ?               |                 |         |
| 5. CPO: Coproporphyrinogen oxidase | ?               |                 |         |
| 6. PPO: Protoporphyrinogen oxidase | ?               |                 |         |
| 7. FeC: Ferrochelatase  | +(3, 6, 8)        |                 |         |

cytosol, suggestive of its potential application for photodynamic therapy of various diseases.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—Wild type L. amazonensis (LV78) promastigotes (clone 12-1) were grown at 25°C in Hapes-buffered Medium199 to pH 7.4 and supplemented with 10% heat-inactivated fetal bovine serum. Transfectants were grown under similar conditions with different concentrations of selective pressure, i.e. G418 and tunicamycin. Cells were also adapted to grow in a chemically defined medium (11). To initiate such cultures, cells were washed twice with the defined medium by centrifugation at 3500 × g for seedling at 2–5 × 10⁶ cells/ml. Cells were counted using a hemacytometer. Macrophages (J774A1) were grown in RPMI 1640 medium-supplemented with 10 or 20% heat-inactivated fetal bovine serum at 37°C. Cultures of all cells rendered porphyric were kept in the dark to avoid cytotoxicity because of photosensitivity.

**Porphyrin Fluorescent Microscopy**—For all microscopic examinations of Leishmania, living cell suspension in 5–10 μl aliquots was placed on a glass slide and then covered with an 18 mm 2 glass coverslip. For routine examinations, the preparations were viewed under phase contrast for cellular structures in conjunction with epifluorescence for porphyrins using a filter set consisting of D405/10X (405 nm exciter), 485/20DC (485 nm dichroic) and R640LP (610 nm emitter) (Chroma Tech Co., Brattleboro, VT) in a Zeiss standard microscope with a pressure mercury lamp (HBO 50 W, Osram). Images were obtained by confocal microscopy using an Olympus Fluoview confocal microscope equipped with a krypton/argon-mixed gas laser. Specimens were illuminated with the 488 nm excitation line. The specific fluorescent emission of the porphyrin was collected by a photomultiplier tube after passing through a 605 nm bandpass emission filter. Differential interference contrast images were simultaneously collected using a transmission field detector coupled to a photomultiplier tube. Detection settings were determined using a negative control by adjusting the gain and offset settings to eliminate background. Images were collected using a ×100 oil immersion objective (NA 1.40) with an electronic zoom of ×3. The confocal aperture was set to 5 mm to maximize the depth of field within the specimen. Digital image acquisition took ~7 s/frame, resulting in movement-induced blurring of the flagella in viable specimens. Images were composed in Adobe Photoshop. Only differential interference contrast images were adjusted for brightness.

**Nucleic Acid Techniques**—The cDNA of rat pbgd (1038 bp) (GenBankTM accession number X06827) (12) was obtained by digesting the plasmids with BamHI. The human alad (993 bp) (GenBankTM accession number M13928) (13) was PCR-amplified from a cDNA cloned in pGEM-T for expansion and then gel-purified after Hl sites digestion for cloning into pX vectors specific for transfection of Leishmania.Selectable markers of Leishmania for G418 and tunicamycin, respectively, are shown. Thin lines, pBluescript with ampicillin resistance gene (AmpR); shaded area, Leishmania DNA containing neomycin phosphotransferase gene (NeoR) and N-acetylglucosamine-1-phosphate transferase (NAGT). Arrows, direction of transcription.

**Expression of PBGD and ALAD in p6.5 and pX vectors for transcription of Leishmania, respectively.** Selectable markers of Leishmania for G418 and tunicamycin, respectively, are shown. Thin lines, pBluescript with ampicillin resistance gene (AmpR); shaded area, Leishmania DNA containing neomycin phosphotransferase gene (NeoR) and N-acetylglucosamine-1-phosphate transferase (NAGT). Arrows, direction of transcription.

**Virus Sensitivity Assays**—For these experiments, transfectants with alad and pbgd and those with pbgd alone were grown in chemically defined medium supplemented with up to 1.6 mM ALA to generate different levels of porphyria. Cell suspensions in 24-well microtiter plates (10⁵ promastigotes/ml/well or 5 × 10⁶ promastigotes + 5 × 10⁵ J774A1 macrophages/ml/well) were irradiated after infection or immediately at room temperature under a long wave UV lamp (254–366 nm multi-bands, Mineralight Lamp, Model UVSL-58, Ultraviolet Products, Inc, San Gabriel, CA) placed ~5 cm above the cell layers. Porphyrin Leishmania were prepared under other conditions and their spent media with different concentrations of released porphyrins were also examined for their effects on J774A1 cells. After illumination for variable time periods, cells were microscopically examined immediately. Cells of the monocyteic tumor line were counted using a hemacytometer 1–2 days after irradiation. All experiments were repeated at least twice.

**RESULTS**

Expression of PBGD and ALAD Only in Leishmania Transfected with the Respective Genes—Western blot analysis of various cell lysates revealed that both enzymes were undetectable in the wild type (Fig. 2, lane 1) and appeared as specific protein bands of the expected size (Fig. 2, lanes 2–5) in the transfectants. Probing the blots with anti-ALAD antiserum alone revealed a single band of ~36 kDa in the transfectants with
range of transfectants with the genes of relevance (Table II). The spe-
taneously in the same one using different vectors.
The results thus indicate that both genes are expressed at the
protein level individually in different transfectants and simul-
taneously only in the defined medium supplemented with
either hemin or protoporphyrin IX (data not shown). Deletion
of the heme compound from this medium resulted in the even-
tual cessation of their growth in all cases after several pas-
sages. Heme biosynthesis pathway thus remains incomplete
in these transgenic Leishmania clearly because of additional en-
zymatic defect(s) downstream of PBGD.

Uroporphyrin I Is the Sole Intermediate Detected in Porphy-
ritic Leishmania—This finding was originally suggested by the
fluorescence emission spectra of porphyrins extracted from por-
phyric Leishmania observed (data not shown) and confirmed by
thin-layer chromatography analysis of these samples (Fig. 3).
Thin-layer chromatography of porphyrins extracted by stand-
ard procedures from porphyric Leishmania and their spent
medium revealed only a single UV-fluorescent species (Fig. 3,
lanes 2 and 5), which co-migrated with uroporphyrin I octa-
methyl esters (lanes 1, 4, and 7). This finding indicates that
only uroporphyrin I was produced by these cells. No porphyrin
bands were visible in samples prepared simultaneously from
cells grown in a chemically defined medium with a modest
selective pressure of 2 × 10^6 to 10^7/mL (Fig. 5). Under all these conditions, cells grew
quantitatively assessed fluorometrically. Initially used were
PM21 products/mg protein/h fall within the range of ~2500 to ~9500 and ~400 to ~1400 for ALAD and
PBGD, respectively. The variations in the specific activities
among different experiments seen may be accounted for by
differences introduced inadvertently in the culture and selec-
tive conditions used. Clearly, both enzymes are fully functional
alone or in combination in the transgenic Leishmania cells.

Both ALAD and PBGD Expressed in the Transfectants Were
Enzymatically Active—Both ALAD and PBGD activities are
absent in wild type cells (data not shown) and present only in
transfectants with the genes of relevance (Table II). The spec-
cific activities in pmol products/mg protein/h fall within the
range of ~2500 to ~9500 and ~400 to ~1400 for ALAD and
PBGD, respectively. The variations in the specific activities
among different experiments seen may be accounted for by
differences introduced inadvertently in the culture and selec-
tive conditions used. Clearly, both enzymes are fully functional
alone or in combination in the transgenic Leishmania cells.

Aminolevulinate-inducible Uroporphyria in Transfectants
with PBGD and alad—Whereas both ALAD and PBGD were
expressed and fully active in Leishmania transfected with the
respective gene, the transfectants produced no detectable por-
phyrins (see Figs. 3, lanes 3 and 6, and 4, panel N, 0 µM ALA)
even ALA was provided to those with both transgenes (Figs.
lanes 2 and 5, and 4, panel N, 125–1000 µM ALA). However,
this porphyric Leishmania along with all other transfectants
resembled nontransfected wild type cells in that they grew
continuously only in the medium supplemented with
either hemin or protoporphyrin IX (data not shown). Deletion
of the heme compound from this medium resulted in the even-
tual cessation of their growth in all cases after several pas-
sages. Heme biosynthesis pathway thus remains incomplete
in these transgenic Leishmania clearly because of additional en-
zymatic defect(s) downstream of PBGD.

Emergence and Cellular Localization of Uroporphyrin I in
Porphyric Leishmania—The porphyric cells emerged only in the
double transfectants after the addition of ALA into their cul-
ture media. Porphyrin-specific signals were followed by epif-
from porphyrin-free defined me-
dium, eliminating the possibility that the porphyrin species
detected may have derived from an exogenous source.

Intracellular Accumulation of Uroporphyrin Followed by Its
Extracellular Release—Porphyric Leishmania released uropor-
phyrin I into the medium, independent of cytolysis. This was
demonstrated under two different conditions to generate mod-
est and high levels of uroporphyria. Cells were handled gently
to avoid inadvertent cytolysis. The kinetics of uroporphyrin
accumulation in and release from porphyric Leishmania was
quantitatively assessed fluorometrically. Initially used were
cells grown in a chemically defined medium with a modest
selective pressure of 2 µg of tunicamycin and 10 µg of G418/ml
in conjunction with increasing but low concentrations of ALA
from 0 to 200 µM (Fig. 5). Under all these conditions, cells grew
from 2.5 × 10^5 to ~10^7/ml in a period of 3 days (Fig. 5, left
panels), except the one with the highest ALA concentration of
200 µM in which case the cell density decreased on day 3 (Fig.
5, bottom left panel). In the absence of ALA, porphyrin was
detected neither in cells nor in their spent media throughout
the period of cell growth (Fig. 5, top middle and right panels).
In the presence of ALA, the cells produced uroporphyrin in an
ALA dose-dependent manner, namely an increase from ~3 to
~8 pmol uroporphyrin/10^6 cells in the presence of 25 to 200 µM
ALA during the first day (Fig. 5, middle panels). The cellular
levels of uroporphyrin declined in these cells from days 2 to 3,
concomitant with its release also in an ALA dose-dependent
manner from 5 to 28 pmol uroporphyrin/ml in the culture
medium (Fig. 5, right panels).
In a separate set of experiments, cells were grown in Medium199 plus heat-inactivated fetal bovine serum under the optimal conditions for uroporphyria, i.e., a 10-fold increase of the selective pressure (20 μg of tunicamycin and 100 μg of G418/ml) and a 5- to 8-fold increase of the substrate (up to 1.0–1.6 mM ALA provided exogenously). Under these conditions, both cellular and released uroporphyrin levels were considerably enhanced (Fig. 4, panels N, 125–1900 μM ALA), the latter reaching a level as much as ~2 μM. Cytolysis was observed in <1% of these cells that did not account for the level of porphyrin release seen.

The results from both sets of the experiments indicate that uroporphyrin is induced in an ALA dose-dependent fashion, which is marked by initial cellular accumulation of uroporphyrin followed by its release and accumulation in the culture medium.

**UV Sensitivity of Porphyric Leishmania and Porphyric Leishmania-infected Monocytic Tumor Cells—**Porphyric Leishmania remained motile and thus viable under all culture and selective conditions used, except when they were subjected to UV irradiation. This sensitivity was indicated by the immediate cessation of the motility of the early porphyrinic cells after exposure to illumination under the setting for epifluorescent microscopy or with the long wave UV lamp. Late porphyrinic cells exposed to ALA 2 days or longer were less sensitive, whereas nonporphyrinic cells were totally insensitive to UV irradiation under these conditions as indicated by their motility.

The monocytic tumor cells, J774A1, were also rendered sensitive to long wave UV irradiation after infection with porphyric Leishmania. Used for these experiments were double transfectants with both alad and pbgd and single transfectants with only pbgd grown under the same conditions. Uroporphyria was generated only in the double transfectants. The results (Fig. 6) showed that UV irradiation lysed only the macrophages infected with porphyric Leishmania and that the cytolysis was proportional to the porphyrinic levels of the latter modulated by prior exposure to different ALA concentrations (Fig. 6, PBDG/ALAD). The nonporphyric Leishmania produced no such effect (Fig. 6, PBDG) regardless of their exposure to ALA and UV irradiation under the same conditions. There was also no cytolysis of the tumor cells when irradiated immediately after mixing them with the porphyric Leishmania or in the presence of their spent media containing uroporphyrin I. The results obtained from these experiments were similar to the control in Fig. 6 (data not shown).

**TABLE II**

| Expt. No. | alad pmol PBG/mg protein/h | pbgd pmol URO/mg protein/h | alad & pbgd pmol PBG/mg protein/h | alad & pbgd pmol URO/mg protein/h |
|-----------|---------------------------|----------------------------|---------------------------------|---------------------------------|
| 1         | 9528                      | 0                          | 8187                            | 698                             |
| 2         | 9830                      | 0                          | 6542                            | 420                             |
| 3         | 2660                      | 0                          | 3910                            | 491                             |

*Transfectants with the pX vector alone in addition to p6.5-pbgd.

1 Grown to stationary phase in a defined medium and harvested for enzyme assays as described under “Experimental Procedures.” See Fig. 1 for the plasmid constructs used for the transfection.

**DISCUSSION**

In this study, both alad and pbgd from mammalian sources were successfully expressed in Leishmania by transfection (Fig. 1), yielding products of expected size (Fig. 2) with enzymatic activities (Table II). Significantly, the episomal transgenes in two different vectors can be selected appropriately to co-express both enzymes with activities. These activities are at least 10 times higher than those normally found in the mammalian cells, e.g., macrophages (3), and more comparable to those in murine Friend virus-transformed leukemia cells induced for erythroid differentiation with a heightened level of heme biosynthesis (23). Both mammalian genes thus appear to express adequately and produce functionally active products in a xenotransgenic system with Leishmania as the recipient.

Despite the fact that functionally active ALAD and PBGD were made available in abundance to these transgenic Leishmania, their nutritional dependence on hemin or protoporphyrin IX was not spared, indicative of more extensive deficiencies of this pathway than previously expected. Transfection of Leishmania with pbgd alone was originally expected to produce the desired phenotype, because PBGD was thought to be the only enzyme that is deficient in this group of protozoa and supplied to several Crithidia species by their endosymbionts (5). Because δ-aminolevulinate synthase activity was detected previously in Leishmania (7, 8) and other trypanosomatids (6), additional transfection of these organisms with alad would be expected to provide the first three enzymes of the pathway sufficient to produce, at the very least, one or more of the porphyrin intermediates if not heme as the final product (see Table I). However, the double transfectants produced porphyrins only when ALA was supplied exogenously (Figs. 3–5). The deficiency of Leishmania in δ-aminolevulinate synthase in addition to ALAD and PBGD is thus apparent. This conclusion is supported by the fact that the substrates for ALA synthesis are not limiting, i.e., glycine and alanine amply supplied in the
culture medium, and succinyl-CoA in abundance (6) from the active TCA cycle in these cells. The loss of alas and alad from *Leishmania* is of interest, because their products are thought to have additional functions in other eukaryotes besides heme biosynthesis, i.e. ALA for the formation of corin ring in cobalamin biosynthesis and ALAD as CF-2 inhibitor of proteasome ATP- and ubiquitin-dependent proteolysis (24). The emergence of uroporphyrin I in the porphyric *Leishmania* entails the occurrence of cellular events in the following order: (a) transport of exogenously supplied ALA into the cytosol where it is converted by ALAD into porphobilinogen, which in turn is changed into hydroxymethylbilane by PBGD, and (b) spontaneous polymerization of hydroxymethylbilanes into uroporphyrinogen I, which undergoes autooxidation to form uroporphyrin I. The

**Fig. 4.** Cellular localization and ALA dose-dependent release of porphyrin from porphyric *L. amazonensis*. Panels A–L, cells transfected with p6.5-pbgd/pX-alad and p6.5-pbgd/pX were examined by confocal microscopy for the presence of cellular porphyrins after exposure to 1 mM ALA for 2 days. See “Experimental Procedures” for the settings used for differential interference (DIC) (panels A, B, G, and J), porphyrin fluorescence (Porphyrin) (panels B, E, H, and K), and merged images of DIC and porphyrin (Merged) (panels C, F, I, and L). Panels A–F, cells transfected with the control P6.5-pbgd/pX; panels G–L, cells transfected with P6.5-pbgd/pX-alad. Note that the porphyrin signals diffused in the cytosol and localized more intensely to cytoplasmic vacuoles (panels H, I, K, and L) in some cells and in the cytosol as a diffused pattern (panel I) in others. Panels M and N, transfectants with P6.5-pbgd/pX-alad (PBGD+ALAD) and the control with P6.5-pbgd/pX (PBGD only) were exposed to 0–1 mM ALA for 4 days. The cultures in 200 µl aliquots were centrifuged to sediment cells for photography with (panel N) and without (panel M) long wave UV illumination. Note: porphyrin fluorescence appears only in the spent medium of PBGD+ALAD increasing with ALA concentrations (125–1000 µM) but not in the controls, i.e. cells with PBGD alone, PBGD+ALAD cells without ALA induction (panel N), and in the absence of UV illumination (panel M).
accumulation of uroporphyrin I as the sole porphyrin species (Fig. 3) indicates the absence or deficiency of the porphyrinogen-modifying enzymes, i.e., uroporphyrinogen co-synthase, uroporphyrinogen decarboxylase, co-proporphyrinogen oxidase, and protoporphyrinogen oxidase (Table I), which would otherwise catalyze the formation of uroporphyrinogen III and co-proporphyrinogen III and protoporphyrinogen IX and proto-porphyrin IX, respectively. Because all of these enzymes downstream of PBGD catalyze a cascade of ring-modifying reactions specific to various porphyrinogens (Table I), their loss may not be unexpected in the absence of the upstream substrate. Curiously, ferrochelatase, the last enzyme of the pathway, remains functional in *Leishmania* for heme biosynthesis. The presence of this enzyme was previously demonstrated biochemically by detecting its catalytic activity (6, 7) as well as nutritionally by the substitution of hemin in the culture medium with protoporphyrin IX for cell growth (5). Nonenzymatic formation of heme from ferric iron and protoporphyrin IX has not been reported, except under nonphysiological conditions (25). It awaits further study to determine whether ferrochelatase may have additional function beyond heme biosynthesis in trypanosomes. The extensive defects of *Leishmania* in this pathway reported here further underscore the importance of heme compounds as the most unique of their essential nutritional requirements. Restricting the availability of this nutrient may thus potentially contribute to the regulation of *Leishmania* virulence in natural infection as well as serve as a potential strategy to design therapeutic drugs for treating leishmaniasis.

Finding the extensive defects of *Leishmania* in heme biosynthesis and their partial rectification by genetic complementation inadvertently provides a novel model suitable for elucidating the cellular response to porphyria. The key feature is the apparent absence of δ-aminolevulinate synthase, which renders this model substrate-inducible with exogenously supplied ALA in an unregulated manner. The accumulation of different porphyrins has been reported in transfectants or mutants of...
for each time point was the average followed by irradiation with long wave UV for 1 h. After further incubation in 24-well microtiter plate to Leishmania.

Porphyrin uptake. The uroporphyrin is not operational in the plasma membrane for the mechanism responsible for vacuolar condensation of the intracellular alad e.g. other lower eukaryotes, levels with (30). However, these mutants develop porphyria of modest pbgd and tested (Leishmania transfectants with both alad and pbgd genes) promastigotes grown in the defined medium supplemented with 400 and 1600 μM ALA. Infection was allowed to proceed overnight followed by irradiation with long wave UV for 1 h. After further incubation overnight, macrophages were stripped and counted. The value for each time point was the average + S.D. from duplicate samples for each of two independent experiments.

Several cellular events were documented for the first time in the development of uroporphyria with the Leishmania model. Uroporphyria was initially found to distribute throughout the cells indicating that it is synthesized in the cytosol and diffused in this compartment, which is consistent with the hydrophilic property of this porphyrin. The cell-wide distribution of uroporphyrin apparently renders these early porphyrin cells more sensitive to UV irradiation presumably as a result of the generation of free radicals via oxidation of this porphyrin (34), accounting for their rapid paralysis. The subsequent condensation of diffused porphyrin into cytoplasmic vacuoles may be carrier-mediated or mediated by a proton pump of the vacuolar membrane. Interestingly, exogenous uroporphyrin is poorly internalized by living Leishmania, suggesting that the mechanism responsible for vacuolar condensation of the intracellular uroporphyrin is not operational in the plasma membrane for porphyrin uptake. The “porphyrinosomes” (Fig. 4, panels H and K) subsequently formed in the porphyrin Leishmania may be the lysosomal or endosomal compartment of these organisms, because uroporphyrin has been reported to accumulate in acidic vacuoles of other cells (35). Of special interest is the disappearance of the cytosolic fluorescence with the vacuolar condensation of uroporphyrin. This cannot be accounted for by the cessation of uroporphyrin synthesis in the system due to substrate limitation or inactivation of the enzymes involved, because there was an abundant supply of the exogenous ALA of up to 2 mM and cells were kept in total darkness to minimize photosensitization of intracellular porphyrin to generate protein-denaturing free radicals. The kinetics of the cellular events suggests that uroporphyrin is removed from the cytosol by an increased efficiency of the vacuolar transport mechanism and/or its efflux out of the cells. Vascular condensation of uroporphyrin appears to occur slightly ahead of its extracellular efflux, suggesting that the former event may produce a signal to trigger the occurrence of the latter. Interestingly, the suspension of Leishmania in uroporphyrin-containing medium does not render them photosensitive, suggesting that the efflux of porphyrins from cells may represent a significant mechanism of toxic waste disposal. A number of membrane proteins have been described in Leishmania as efflux systems related to their drug resistance (36–38), and putative receptors/transporters have also been described for heme (39) and hemoglobin (40). Further investigation is needed to determine whether any of these transport molecules or a novel one may be related to the release of the porphyrins seen. The cellular events of uroporphyrin I emergence, accumulation, and release seen in Leishmania may be relevant to the disposal of “uroporphyria” also found in other conditions, such as porphyria cutanea tarda (41). Further studies of these cellular events in Leishmania may shed light on the mechanisms of cytopathology in and management for this and other types of human porphyria.

The availability of porphyric Leishmania presents the opportunity of considering their use in concept to deliver exogenous porphyrins for photodynamic therapy, still an evolving idea for treating cancers and other malignant diseases (42). We tested this possibility with cells of J774 murine monocytic tumor line of macrophage origin. Uroporphyrinic and aporphyrinic Leishmania infect these cells in vitro equally well, but only the uroporphyrinic Leishmania render them sensitive to cytolyis by UV irradiation (Fig. 6). Intracellular residence of the porphyrin Leishmania is required, because the tumor cells were not photosensitized when mixed with the porphyrinic Leishmania without infection or when suspended in their spent media with abundant uroporphyrin. Thus, the uroporphyrin responsible for photosensitivity of the infected cells must be from “within,” but not from outside the tumor cells, consistent with the observations on the porphyrinic Leishmania themselves (see above). The porphyrinic Leishmania are expected to lodge in the phagolysosomes where these organisms are known to reside normally in the infected cells (43). Thus, cytolsis of the monocytic tumor cells results probably from a combined action of the free radicals from sensitized porphyrins plus the lysosomal enzymes of the target cells and possibly additional lytic factors from disrupted Leishmania in this vacuolar compartment.

Leishmania have evolved tissue-tropism toward the reticuloendothelial system to achieve intralysosomal parasitism of macrophages (43). Especially attractive are the potential use of the nonpathogenic or avirulent species/strains attained via molecular attenuation (44) to deliver pro-drugs for lysosomal activation and antigens for vaccination. When further genetically grafted to express these agents, porphyrinic Leishmania may be used as a “suicidal capsule” for their timely release after pulsing with ALA as a trigger followed by UV irradiation for cytolysis. Along the same vein, cell and tissue specificity of

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K-P. Chang, unpublished observation.
other parasites may be further exploited to design different targeting strategies. The feasibility of this concept to deploy porphyrinic parasites for cell- and tissue-specific photodynamic prophylaxis and therapy must await further experimental evaluation in vivo.

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