Metronidazole Resistance in the Protozoan Parasite Entamoeba histolytica Is Associated with Increased Expression of Iron-containing Superoxide Dismutase and Peroxiredoxin and Decreased Expression of Ferredoxin 1 and Flavin Reductase*

(Received for publication, December 17, 1998, and in revised form, March 29, 1999)

Claudia Wassmann‡, Andrea Hellberg, Egbert Tannich, and Iris Bruchhaus§

From the Bernhard Nocht Institute for Tropical Medicine, Bernhard-Nocht-Strasse 74, 20359 Hamburg, Germany

To obtain insight into the mechanism of metronidazole resistance in the protozoan parasite Entamoeba histolytica, amoeba trophozoites were selected in vitro by stepwise exposures to increasing amounts of metronidazole, starting with sublethal doses of 4 μM. Subsequently, amoebae made resistant were able to continuously multiply in the presence of a 40 μM concentration of the drug. In contrast to mechanisms of metronidazole resistance in other protozoan parasites, resistant amoebae did not substantially down-regulate pyruvate:ferredoxin oxidoreductase or up-regulate P-glycoproteins, but exhibited increased expression of iron-containing superoxide dismutase (Fe-SOD) and peroxiredoxin and decreased expression of flavin reductase and ferredoxin 1. Episomal transfection and overexpression of the various antioxidant enzymes revealed significant reduction in susceptibility to metronidazole only in those cells overexpressing Fe-SOD. Reduction was highest in transfected cells simultaneously overexpressing Fe-SOD and peroxiredoxin. Although induced overexpression of Fe-SOD did not confer metronidazole resistance to the extent found in drug-selected cells, transfected cells quickly adapted to constant exposures of otherwise lethal metronidazole concentrations. Moreover, metronidazole selection of transfected amoebae favored retention of the Fe-SOD-containing plasmid. These results strongly suggest that peroxiredoxin and, in particular, Fe-SOD together with ferredoxin 1 are important components involved in the mechanism of metronidazole resistance in E. histolytica.

Metronidazole (1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole) is the drug of choice for the treatment of infections caused by anaerobic or microaerophilic microorganisms and has been used in clinical practice for >25 years (1). The drug enters the cell through passive diffusion, where a nitro group is subsequently reduced to reactive cytotoxic nitro radicals by reduced ferredoxin or flavodoxin. Ferredoxin and flavodoxin function as electron acceptors of pyruvate:ferredoxin oxidoreductase, hydrogenase, and some other enzymes found specifically in microaerophilic bacteria and protozoan parasites (2–4). The selective toxicity of metronidazole for anaerobic and microaerophilic organisms is due to the redox potential of their electron transport components, which are sufficiently negative to reduce the nitro group of metronidazole (3, 5). Under aerobic conditions, the nitro radicals can be oxidized by oxygen, which leads to futile cycling and detoxification of the drug. During this reaction, superoxide radical anions are formed that may also be toxic for the anaerobic organisms (6). Treatment with metronidazole is usually very effective. However, metronidazole resistance is well documented for various bacteria and protozoan species (7, 8).

Aerobic as well as anaerobic resistance to metronidazole has been described in Trichomonas vaginalis. In this organism, activation of the drug occurs in a specific organelle called the hydrogenosome, in which pyruvate:ferredoxin oxidoreductase, ferredoxin, and hydrogenase are localized. Some clinical T. vaginalis strains exhibit resistance only under aerobic conditions (9, 10). Under anaerobic conditions, these isolates are metronidazole-sensitive. They show no decrease in pyruvate:ferredoxin oxidoreductase or hydrogenase levels, which are required for reductive activation of metronidazole. These trichomonads have lowered oxygen-scavenging capabilities, leading to higher oxygen concentrations and therefore to a futile cycling of reduced metronidazole and inactivation of the drug (11, 12). Other reports have indicated decreased levels of ferredoxin in metronidazole-resistant trichomonads (13, 14) or overexpression of a gene encoding an ATPase related to the P-glycoprotein family (7).

Anaerobic resistance to metronidazole has only been induced in vitro (15, 16) and has been correlated with decreased levels or total lack of pyruvate:ferredoxin oxidoreductase and hydrogenase, leading to inactivation of the pyruvate-oxidizing pathway that is responsible for metronidazole activation (17, 18). Similar findings have been reported for metronidazole-resistant strains of Giardia accompanied by chromosomal rearrangements (19–21).

Recently, Samarawickrema et al. (22) described for the first time the in vitro induction of metronidazole resistance under microaerophilic conditions in the protozoan parasite Entamoeba histolytica, the causative agent of human amoebiasis. Like Giardia, E. histolytica has no hydrogenosomes and hydrogenase, and drug activation occurs in the cytosol (23). Interestingly, amoebae made resistant exhibited normal levels of pyruvate:ferredoxin oxidoreductase activity, but a substantial decrease in the iron-containing superoxide dismutase (Fe-SOD)† (22). Up to now, studies about the regulation of ferredoxin and P-glycoproteins have not been performed in drug-resistant E. histolytica isolates.

Besides Fe-SOD, which catalyzes the dismutation of super-
oxide radical anions to oxygen and hydrogen peroxide, two other enzymatic antioxidation, a flavin reductase (NADPH-flavin oxidoreductase) and a peroxiredoxin, have been identified in *E. histolytica*. Flavin reductase functions as a disulfide reductase, but is also able to reduce oxygen to form hydrogen peroxide (24). Peroxiredoxin was shown to remove hydrogen peroxide, which is produced during the flavin reductase reaction or during dismutation of superoxide radical ions by amoebic Fe-SOD. The peroxiredoxin-mediated hydrogen peroxide-removing reaction is dependent on the presence of thiols or flavin reductase activity (25, 26). This tight interaction of the enzymatic antioxidation might suggest involvement of the three enzymes in the mechanism of amoeba metronidazole resistance, as up-regulation of Fe-SOD should result in elevated levels of hydrogen peroxide, which are toxic and therefore need to be inactivated.

In this study, we report on the pattern of expression of the different antioxidant enzymes as well as of ferredoxin and P-glycoprotein in metronidazole-resistant *E. histolytica* trophozoites. In addition, recently developed methods for *Entamoeba* transfection were applied to elucidate whether overexpression of Fe-SOD and/or of the other antioxidant enzymes is sufficient to confer amoeba resistance to metronidazole.

**EXPERIMENTAL PROCEDURES**

**Parasite Culture**

The axenically cultured trophozoites of the *E. histolytica* isolate HM-1:IMSS were grown in TYI-S-33 medium (27) under microaerophilic (5–7% O₂) or anaerobic conditions using Anaerocult C or A, respectively (Merck).

To induce metronidazole resistance, amoebae growing in the mid-logarithmic phase under microaerophilic conditions were exposed to a low concentration of the drug (4 μM) for 24 h. Live amoebae were transferred in new medium without the drug and cultivated until mid-logarithmic phase. This procedure was repeated three times with the same concentration before higher metronidazole concentrations were used (8, 12, 16, 20, 25, 29, 33, 37, and 40 μM).

**Expression Constructs**

All plasmids used for the transfection of *E. histolytica* trophozoites are derivatives of the expression vectors pEhNEO/CAT and pEhHYG/CAT (28). The chloromphenical acetyltransferase gene was removed by restriction with the endonucleases KpnI and BamHI (pNeoCass/ pHygCass) and replaced by the *E. histolytica* genes coding Fe-SOD, peroxiredoxin, and flavin reductase, respectively (30–32). The Fe-SOD-, peroxiredoxin-, and flavin reductase-coding sequences were amplified by polymerase chain reaction of the respective DNA fragments. The transfection vectors were pBS (Stratagene)-derived plasmids containing the neomycin phosphotransferase- or hygromycin phosphotransferase-coding sequence flanked by 480 base pairs of the 5'-untranslated sequence and 600 base pairs of the 3'-untranslated sequence of an *E. histolytica* actin gene. The Fe-SOD-, peroxiredoxin-, or flavin reductase-coding sequence is flanked by 485 base pairs of the 5'-untranslated sequence of an *E. histolytica* lectin gene and 600 base pairs of the 3'-untranslated actin gene (pNeoFe-SOD, pNeoFR, pNeoPR, pHygFe-SOD, and pHygFR) (29).

**Transfection**

Transfections were performed by electroporation as described previously (28). Drug selection started 48 h after transfection, using a 10 μg/ml concentration of the neomycin analogue G418 or 5 μg/ml hygromycin. After 2 weeks, the concentration of G418 was increased to 50 μg/ml, and the concentration of hygromycin was increased to 10 μg/ml. For transfection of *E. histolytica* with two different expression vectors, the trophozoites were first transfected with the plasmid containing the *neo* gene. After 2 weeks of cultivation in the presence of 10 μg/ml G418, they were transfected with the second plasmid, which contains the *hgy* gene. These amoebae were cultivated for 2 weeks in the presence of 5 μg/ml hygromycin and 10 μg/ml G418. After 2 weeks of cultivation, a G418 concentration of 50 μg/ml and a hygromycin concentration of 10 μg/ml were used.

**Cultivation of Transfected E. histolytica Trophozoites in the Presence of Metronidazole**

Trophozoites (1 × 10⁶) transfected with pNeoCass, pNeoFe-SOD, pNeoPRD, or pNeoFR were cultivated in 50-ml culture flasks in TYI-S-33 medium supplemented with 6 μM metronidazole and 50 μg/ml G418 for 72 h. Each day, fresh culture medium, G418, and metronidazole were added to the amoebae. After 48 and 72 h of cultivation in the presence of metronidazole, viable amoebae were counted. Trophozoites transfected with pNeoCass were used as controls.

Double-transfected amoebae were cultivated in TYI-S-33 medium for 96 h without G418 and hygromycin. After 96 h, 1 × 10⁶ amoebae were cultivated in TYI-S-33 medium supplemented with 6 μM metronidazole. After 48 and 72 h of cultivation in the presence of metronidazole, viable amoebae were counted. It is important to cultivate amoebae transfected with expression vectors containing the *hgy* gene for a few days without addition of hygromycin because the combination of hygromycin and metronidazole is highly toxic for *E. histolytica*. Overexpression of Fe-SOD, peroxiredoxin, and flavin reductase after 96 h without addition of G418 and hygromycin was examined by Western blot analysis.

Each assay was done five times in duplicate. Viability of the amoebae was examined by trypan blue exclusion (0.5 mg/ml). The survival rate of the transfected amoebae relative to the controls was determined. The p value was calculated using one-way analysis of variance.

**DNA and RNA Analyses**

Total RNA was isolated using TRIzol reagent (Life Technologies, Inc.). Plasmid DNA from *Escherichia coli* was extracted by the alkaline lysis method using a plasmid mega kit (Qiagen Inc.). Northern blotting was carried out by standard protocols. Hybridizations were performed in 0.5 M Na₂HPO₄, 7% SDS, and 1 mM EDTA (pH 7.2) at 55 °C. Blots were washed in 40 mM Na₂HPO₄ and 1% SDS (pH 7.2) at 55 °C.

**Enzymatic Assays**

*E. histolytica* trophozoites were harvested in the mid-logarithmic phase of growth by chilling on ice for 10 min and centrifuged at 430 × g (4 °C, 5 min). The pellet was washed twice in ice-cold phosphate-buffered saline, freeze-thawed five times in solid CO₂/jetethanol, and sedimented by centrifugation at 150,000 × g at 4 °C for 40 min. The 150,000 × g supernatant (trophozoite extract) was used for the enzymatic assays.

**Peroxiredoxin Assay**—The consumption of H₂O₂ by *E. histolytica* peroxiredoxin was determined using a reaction mixture containing 50 μM Tris-HCl (pH 8.0), 0.2 mM diithiothreitol, 50 μM H₂O₂, and different amounts of protein. Before addition of H₂O₂, protein samples were preincubated with 0.2 mM diithiothreitol for 30 min. The reaction was stopped after 30 min by trichloroacetic acid precipitation (10% final concentration). After removal of the precipitated protein, 0.2 ml of 10 mM ferrous ammonium sulfate and 0.1 ml of 2.5 M potassium thiocyanate were added to a 1-ml aliquot of the supernatant. H₂O₂ was determined spectrophotometrically (at 480 nm) using known amounts of H₂O₂ (1–50 μM) as a standard (33). The absorption of NADPH at 340 nm (ε = 6.22 μmol) was measured. One unit of NADPH oxidase activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of NADPH/min.

**Superoxide Dismutase Assay**—The activity of superoxide dismutase was determined by the reduction of ferricytochrome c according to standard methods (35). All assays were done at least in triplicate.

**Protein Analysis**

Proteins were separated on 12% SDS-polyacrylamide gel, and immunoblotting was carried out as described (36). The rabbit polyclonal anti-Fe-SOD, anti-peroxiredoxin, and anti-flavin reductase sera were described previously (24, 25, 37). Protein concentration was determined using the bichinonic acid reagent assay (Pierce).

**RESULTS**

**Generation of in Vitro Metronidazole-resistant *E. histolytica* Trophozoites—Cultivation of wild-type *E. histolytica* trophozoites (HM-1) under microaerophilic conditions (5–7% oxygen) in the presence of various amounts of metronidazole revealed that a concentration of 12 μM was sufficient to reproducibly kill 50% of the cells within 24 h and was lethal for all cells after prolonged exposures over 72 h. In contrast, amoebae grown under
strict anaerobic conditions were less susceptible to metronidazole. At a concentration of 12 μM, only 28% of the cells died within 24 h, and only 50% did so within 72 h. As, from a clinical point of view, only those *E. histolytica* trophozoites that are resistant to metronidazole in the presence of oxygen are of medical importance, all further experiments were performed under microaerophilic conditions. To generate metronidazole-resistant amoebae, cells were challenged with increasing amounts of the drug, starting with 4 μM and ending up with 40 μM metronidazole added to the culture medium. The whole procedure took ~200 days, and afterwards, cells made resistant (HM-1/Met) were continuously maintained in the presence of 40 μM metronidazole for several months. Although the doubling time of resistant cells was slower compared with that of wild-type cells, HM-1/Met cells constantly multiplied, and microscopic inspection did not reveal any abnormalities. A further increase in the metronidazole concentration resulted in a significant decrease in the doubling time, and at a concentration of 200 μM, no further multiplication was observed. However, even at this drug concentration, cells were still viable, as evidenced by their adherence to the culture plate, regular amoeboid movement, and trypan blue exclusion.

Expression of Antioxidant Enzymes in Metronidazole-resistant *E. histolytica* Trophozoites—Expression of peroxiredoxin, flavin reductase, Fe-SOD, and pyruvate:ferredoxin oxidoreductase was compared between wild-type amoebae (HM-1) and those made resistant and continuously grown in the presence of 40 μM metronidazole (HM-1/Met). Northern blot analyses revealed a slight decrease of ~20% for pyruvate:ferredoxin oxidoreductase RNA and a more pronounced decrease of ~40% for flavin reductase RNA in resistant amoebae, whereas the levels of Fe-SOD and peroxiredoxin RNAs in these cells were found to be increased by 5.1- and 2.9-fold, respectively (Fig. 1). These results corresponded with respective protein data as evidenced by Western blotting (Fig. 1B) and enzymatic assay analyses (Table I). Compared with HM-1 cells, HM-1/Met cells exhibited 40% less NADPH oxidase activity, but 5.5 times higher Fe-SOD- and 4.0 times higher H$_2$O$_2$-removing activities.

In addition to the various antioxidant enzymes, the levels of RNA expression of the two *E. histolytica* ferredoxins (ferredoxins 1 and 2) and of the six P-glycoprotein genes (pgp1 through pgp6) were compared between metronidazole-susceptible and -resistant amoebae by Northern blot analysis (Fig. 1C). There were no differences in the expression of the various P-glycoprotein genes and the ferredoxin 2 gene, but a strong decrease of ~80% was found in the expression of ferredoxin 1-specific RNA in metronidazole-resistant amoebae.

Overexpression of Antioxidant Enzymes in *E. histolytica* Trophozoites by Stable Episomal Transfection—To further analyze the role of Fe-SOD, peroxiredoxin, and flavin reductase in the metronidazole resistance of *E. histolytica* trophozoites, mutants that stably overexpressed the various antioxidant enzymes were generated by episomal transfection. Compared with cells transfected with the respective control plasmid (pNeoCass), transfection with the expression plasmid pNeoFeSOD, pNeoPRD, or pNeoFR resulted in amoebae expressing exceedingly higher levels of Fe-SOD, peroxiredoxin, or flavin reductase RNA (Fig. 2). Likewise, extracts of these cells contained higher amounts and exhibited elevated activities of the corresponding antioxidant enzymes (Fig. 3 and Table II). Fe-SOD activity was increased by 7.8-fold, H$_2$O$_2$-removing activity by 4.0-fold, and NADPH oxidase activity by 15.2-fold. Interestingly, cells overexpressing Fe-SOD revealed induction of peroxiredoxin, whereas overexpression of peroxiredoxin did not influence Fe-SOD expression.

In addition to cells overexpressing one of the three antioxidant enzymes, mutants were generated simultaneously overexpressing peroxiredoxin and Fe-SOD or peroxiredoxin and flavin reductase. For comparison, amoebae were transfected with the two control plasmids pNeoCass and pNeoHyg. The level of expression of each of the respective enzymes in the double-transfected mutants was comparable with the level of expression in cells transfected with a single construct, with the exception that H$_2$O$_2$-removing activity was ~20–30% higher in

---

**FIG. 1.** Expression of antioxidant enzymes in metronidazole-susceptible and -resistant *E. histolytica*. A, Northern blot analyses. Total RNAs (10 μg) of the metronidazole-susceptible *E. histolytica* isolate HM-1 and of the metronidazole-resistant isolate HM-1/Met (which was cultivated for 8 weeks in the presence of 40 μM metronidazole) were submitted to electrophoresis; blotted; and sequentially hybridized with coding regions of Fe-SOD, peroxiredoxin (prd), flavin peroxidase (fr), and pyruvate:ferredoxin oxidoreductase (pfdr). For comparison, an actin probe was used as a control. B, Western blot analyses. Trophozoites of the *E. histolytica* isolates HM-1 and HM-1/Met were washed in phosphate-buffered saline, freeze-thawed five times in solid CO$_2$/ethanol, and sedimented by centrifugation at 4 °C for 40 min. The 150,000 × g supernatants were separated on a 12% SDS-polyacrylamide gel under reducing conditions. The proteins were blotted onto nitrocellulose and developed with antisera to Fe-SOD, peroxiredoxin, and flavin reductase. C, Northern blot analyses. Total RNAs (10 μg) of the metronidazole-susceptible *E. histolytica* isolate HM-1 and of the metronidazole-resistant isolate HM-1/Met (which was cultivated for 8 weeks in the presence of 40 μM metronidazole) were submitted to electrophoresis; blotted; and sequentially hybridized with coding regions of Fe-SOD, ferredoxin 1 and 2, and P-glycoprotein 1 (pgp1). For comparison, an actin probe was used as a control. PAGE, polyacrylamide gel electrophoresis.

---

**TABLE I**

Activities of antioxidant enzymes in metronidazole-susceptible (HM-1) and resistant (HM-1/Met) *E. histolytica* trophozoites

|                | Fe-SOD | NADPH oxidase | Peroxiredoxin |
|----------------|--------|---------------|---------------|
| **HM-1**      |        |               |               |
| **units/mg**  | 6.0 ± 1.1 | 113 ± 10 | 2.6 ± 0.3 |
| **HM-1/Met**  | 33 ± 6 | 68 ± 13 | 10.5 ± 3 |

---
cells transfected with pNeoPRD and pHygFe-SOD compared with those transfected with either pNeoPRD or pNeoFe-SOD (Figs. 2 and 3 and Table II).

Survival of Transfected E. histolytica—Compared with nontransfected cells, amoebae transfected with the control plasmids and selected in the presence of 50 μg/ml G418 with or without 10 μg/ml hygromycin. Total RNAs of the respective amoebae were extracted; electrophoresed on an agarose gel; blotted; and sequentially hybridized with coding regions of Fe-SOD, peroxiredoxin, flavin reductase, and actin.

**FIG. 2.** Northern blot analyses of transfected amoebae. Trophozoites of the E. histolytica isolate HM-1:IMSS were transfected with various expression plasmids allowing overexpression of Fe-SOD, peroxiredoxin (prd), and flavin reductase (fr) alone or in combination (pNeoFe-SOD, pNeoPRD, pNeoFR, pNeoPRD/pHygFR, and pNeoPRD/pHygFR). As a control, trophozoites were transfected with pNeoCas or pNeoCas/pHygCas. The transfected amoebae were selected in the presence of 50 μg/ml G418 with or without 10 μg/ml hygromycin. Total RNAs of the respective amoebae were extracted; electrophoresed on an agarose gel; blotted; and sequentially hybridized with coding regions of Fe-SOD, peroxiredoxin, flavin reductase, and actin.

**FIG. 3.** Western blot analyses of transfected amoebae. Trophozoites of the E. histolytica isolate HM-1:IMSS were transfected with the various expression plasmids (see the legend to Fig. 2). The transfected amoebae were selected in the presence of 50 μg/ml G418 with or without 10 μg/ml hygromycin. Subsequently, trophozoites were washed in phosphate-buffered saline, freeze-thawed five times in solid CO₂/ethanol, and sedimented by centrifugation at 4 °C for 40 min. The 150,000 × g supernatants were separated on a 12% SDS-polyacrylamide gel under reducing conditions. Proteins were blotted onto nitrocellulose and developed with antisera to Fe-SOD, peroxiredoxin (PRD), and flavin reductase (FR).

**FIG. 4.** Survival of transfected amoebae in the presence of metronidazole. Trophozoites of the E. histolytica isolate HM-1:IMSS were transfected with the various expression plasmids (see the legend to Fig. 2). Transfected amoebae were selected in the presence of 50 μg/ml G418 with or without 10 μg/ml hygromycin. Trophozoites (1 × 10⁶) were cultivated in the presence of 6 μM metronidazole for 48 and 72 h, and the numbers of viable cells were counted. Shown are the rates of surviving cells relative to controls. Significant survival rates (p < 0.05) are marked by asterisks.

72 h was highest for the pHygFe-SOD/pNeoPRD double-transfected amoebae (Fig. 4). Moreover, under prolonged exposures to metronidazole, only cells of the two mutants overexpressing
Fe-SOD survived and multiplied.

In general, amoebae lose their episomally transfected plasmids within a few weeks after selection with G418 or hygromycin has been omitted. To investigate whether metronidazole would favor maintenance of the Fe-SOD-containing plasmid, amoebae transfected with pNeoFe-SOD were cultivated for several weeks in the presence or absence of 6 mM metronidazole, but without further G418 selection. Total RNAs of the respective amoebae were extracted, separated by agarose gel electrophoresis; blotted; and sequentially hybridized with coding regions of Fe-SOD, ferredoxins 1 and 2 (fd1 and fd2, respectively), neo, and actin.

DISCUSSION

In an attempt to characterize the mechanism of metronidazole resistance in the protozoan parasite *E. histolytica*, we have generated, by stepwise selection over >200 days, amoebae that were able to continuously multiply in the presence of a 40 μM concentration of the drug. This concentration was ~4 times higher than the concentration necessary to reproducibly kill the respective wild-type cells within 72 h. In addition, amoebae made resistant tolerated a constant exposure to 200 μM metronidazole (but without further multiplication) for several weeks, which is >2-fold higher than the drug concentration found in serum during therapy (~80 μM after administration of 500 mg of metronidazole and a half-life of 7 h) (38). Our approach focused on the mechanism of metronidazole resistance under microaerophilic conditions since, after tissue invasion, amoebae are exposed to elevated levels of oxygen, and therefore, from a clinical point of view, only those *E. histolytica* trophozoites that are drug-resistant in the presence of oxygen are of medical importance. However, as our preliminary results indicated, amoeba grown under anaerobic conditions are less susceptible to the drug. Therefore, the mechanism of resistance under these conditions might differ. The metronidazole susceptibility of the amoeba isolates used for selection was comparable with that reported previously (39). To our knowledge, there is no documented example of drug-resistant amoebae that have been grown in the presence of 40 μM metronidazole. The only report so far available describes a metronidazole-resistant *E. histolytica* line that, after 177 days of selection under microaerophilic conditions, could be maintained in medium containing as little as 10 μM metronidazole (22). In contrast, metronidazole-resistant lines of other protozoan parasites such as *Trichomonas* and *Giardia* that could be grown in as much as several 100 μmol have repeatedly being reported (15, 40). Thus, consistent with our results, it is obviously more difficult and time-consuming to generate metronidazole-resistant *E. histolytica*, as this parasite appears to be unable to develop resistance to high concentrations of the drug. As in the previous study (22), metronidazole resistance of amoebae was found to be associated with high levels of expression of Fe-SOD and could not be attributed to down-regulation of pyruvate-ferredoxin oxidoreductase, as expression of this enzyme was only marginally decreased, or to up-regulation of P-glycoproteins, as expression of this class of proteins was unaltered in resistant amoebae. However, as was shown for the aerobic metronidazole resistance of *T. vaginalis*, resistant *E. histolytica* expressed reduced levels of ferredoxin 1-specific RNA. Interestingly, expression of a second amoebic ferredoxin (ferredoxin 2) remained unchanged in metronidazole-resistant cells, suggesting that the two ferredoxins exert different functions within the parasite. However, down-regulation of ferredoxin 1 appears to be of importance only in those *E. histolytica* trophozoites that are resistant to high concentrations of metronidazole, as this phenomenon was found only in cells resistant to a 40 μM concentration of this, but not in those resistant to 6 μM. The increase of Fe-SOD in metronidazole-resistant amoebae is most likely not the result of a general stress response as previously suggested (22) since cultivation of *E. histolytica* trophozoites under standard stress-inducing conditions such as elevated temperatures or addition of ethanol did not increase Fe-SOD activity (data not shown).

It has to be considered that transfected amoebae were more sensitive to metronidazole, most likely due to superimposing effects caused by G418 or hygromycin selection. Nevertheless, transfected trophozoites overexpressing Fe-SOD revealed a significant reduction in the susceptibility to metronidazole; they quickly adapted to constant exposures of otherwise lethal metronidazole concentrations; and metronidazole selection favored retention of the transfected plasmid. Taken together, these results strongly suggest that increased expression of Fe-SOD constitutes an important component involved in the mechanism of metronidazole resistance in *E. histolytica*.

Besides increased Fe-SOD expression, our data indicate that resistance of amoebae is also associated with increased expression of peroxiredoxin. At present, it is hard to assess whether peroxiredoxin expression in resistant amoebae is directly linked to metronidazole or whether it is a consequence of the induction of Fe-SOD, as increased peroxiredoxin expression...
was also found in cells transfected with the Fe-SOD expression plasmid. On the other hand, although transfection with pNeo-PRD did not reduce susceptibility to metronidazole, the strongest reduction in metronidazole susceptibility was obtained in cells transfected with both the Fe-SOD- and peroxyredoxin-containing plasmids, indicating that overexpression of peroxyredoxin is not sufficient, but may at least support resistance to metronidazole.

Questions remain open about the role of Fe-SOD and peroxyredoxin in mediating resistance. It may be possible that besides dismutation of superoxide radical anions, amoebo SOD is able to detoxify nitro radicals of reactive metronidazole metabolites. For the nervous system, it has been shown that excess production of reactive nitric oxide radicals leads to various neurotoxic effects, which could be reversed by overexpression of SOD (41). Alternatively, inactivation of metronidazole might simply be due to reoxidation of nitro radicals, as cultivation of E. histolytica makes reduced levels of flavin of the enzyme is oxidized by NAD(P)H:flavin oxidoreductases of various organisms such as Salmonella typhimurium and Vibrio fischeri (44, 45). (ii) Under microaerophilic conditions, amoebo flavin reductase has been shown to reduce oxygen, which leads to the formation of increased amounts of toxic hydrogen peroxide (24).

Metronidazole is a cheap, effective, and widely used drug. Although failures in the treatment of amoebiasis with metronidazole have been reported, it has been questioned whether in vivo metronidazole resistance of amoeboae does exist, as no case of clinical resistance of E. histolytica has been documented (46, 47). The difficulties in generating amoeboae in vitro that are resistant to substantial amounts of the drug might support this idea. However, as we have shown here, in principle, it is possible to select E. histolytica trophozoites that are able to tolerate a constant exposure to metronidazole levels comparable to those found in serum during therapy following recommended doses.

Acknowledgments—We thank B. Weseloh and W. Olbrich for skillful technical assistance.

REFERENCES

1. Freeman, C. D., Klutman, N. E., and Lamp, K. C. (1997) Drugs 54, 679–708
2. Müller, M. (1986) Biochem. Pharmacol. 35, 37–41
3. Biaglow, J. E., Varnes, M. E., Roizen-Towle, L., Clark, E. P., Epp, E. R., Astor, M. B., and Hall, E. J. (1986) Biochem. Pharmacol. 35, 77–90
4. Whitney, G. P., and Varghese, A. J. (1986) Biochem. Pharmacol. 35, 97–103
5. Townson, S. M., Borenah, P. F. L., Upercof, P., and Upercof, J. A. (1994) Acta Trop. 56, 173–194
6. D’ocampo, R., and Moreno, S. N. J. (1988) FASEB J. 4, 2471–2475
7. Johnson, J. P. (1993) Parasitol. Today 9, 183–186
8. Sindar, P., Britz, M. L., and Wilkinson, R. G. (1982) J. Med. Microbiol. 15, 503–509
9. Müller, M., Lesnik, G. J., and Gorrell, T. E. (1988) Sex. Transm. Dis. 15, 17–24
10. Tachezy, J., Kulda, J., and Tomkova, E. (1993) Parasitology 106, 31–37
11. Land, K. M., and Johnson, P. J. (1997) Exp. Parasitol. 87, 305–308
12. Yarlett, N., Yarlett, N. C., and Lloyd, D. (1986) Mol. Biochem. Parasitol. 19, 11–116
13. Quon, D. V. K., D’Oliveira, C. E., and Johnson, P. J. (1992) Proc. Natl. Sci. U. S. A. 89, 4402–4406
14. Yarlett, N., Yarlett, N. C., and Lloyd, D. (1986) Biochem. Pharmacol. 35, 1703–1708
15. Kulda, J., Čerkasov, J., Demes, P., and Čerkasovová, A. (1984) Exp. Parasitol. 59, 90–103
16. Kulda, J., Tachezy, J., and Čerkasovová, A. (1993) J. Eukaryot. Microbiol. 40, 262–269
17. Upercof, J. A., Healey, A., Murray, D. G., Borenah, P. F. L., and Upercof, P. (1992) Parasitology 104, 397–405
18. Čerkasovová, A., Čerkasov, J., and Kulda, J. (1984) Mol. Biochem. Parasitol. 11, 105–118
19. Ellis, J. E., Wingfield, J. M., Cole, D., Borenah, P. F. L., and Lloyd, D. (1993) Int. J. Parasitol. 23, 35–39
20. Smith, N. C., Bryant, C., and Borenah, P. F. L. (1988) Int. J. Parasitol. 18, 991–997
21. Upercof, J. A., and Upercof, P. (1993) Parasitol. Today 9, 187–190
22. Samarawickrema, N. A., Brown, D. M., Upercof, J. A., Thanamapalerd, N., and Upercof, P. (1997) J. Antimicrobiol. Chemother. 40, 833–840
23. Reeves, R. E. (1984) Adv. Parasitol. 26, 105–142
24. Bruchhaus, I., Richter, S., and Tannich E. (1998) Biochem. J. 330, 1217–1221
25. Bruchhaus, I., Richter, S., and Tannich, E. (1997) Biochem. J. 326, 785–789
26. Poale, L. B., Chae, H. Z., Flores, B. M., Reed, S. L., Rhee, S. G., and Torian, B. E. (1997) Free Radical Biol. Med. 23, 955–959
27. Diamond, L. S., Harlow, D. R., and Cunnick, C. C. (1978) Trans. R. Soc. Trop. Med. Hyg. 72, 431–432
28. Hamann, L., Nickel, R., and Tannich, E. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8975–8979
29. Hamann, L., Buß, H., and Tannich, E. (1997) Mol. Biochem. Parasitol. 84, 83–91
30. Tannich, E., Bruchhaus, I., Walter, R. D., and Horstmann, R. D. (1991) Mol. Biochem. Parasitol. 49, 61–71
31. Bruchhaus, I., Tannich, E. (1993) Trop. Med. Parasitol. 44, 116–118
32. Bruchhaus, I., and Tannich, E. (1995) Mol. Biochem. Parasitol. 70, 187–191
33. Thuman, R. G., Ley, H. G., and Schulz, R. (1972) Eur. J. Biochem. 23, 420–430
34. Lo, H.-S., and Reeves, R. E. (1980) Mol. Biochem. Parasitol. 2, 23–30
35. McCard, J. M., and Frydovich, I. (1969) J. Biol. Chem. 244, 6049–6055
36. Leippe, M., Eibel, S., Schlenker, O. L., Horstman, R. D., and Müller-Eberhard, H. J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 7659–7663
37. Bruchhaus, I., Brattig, N. W., and Tannich, E. (1992) Arch. Med. Res. 23, 27–29
38. Scully, B. E. (1988) Med. Clin. North Am. 72, 613–621
39. Gault, M. J., Reiner, D. S., and Gillin, F. D. (1985) Trans. R. Soc. Trop. Med. Hyg. 79, 60–62
40. Townson, S. M., Laqua, H., Upercof, P., Borenah, P. F. P., and Upercof, J. A. (1992) Trans. R. Soc. Trop. Med. Hyg. 86, 521–522
41. Gonzalez-Zulueta, M., Ensz, L. M., Mukhina, G., Lebovitz, R. M., Zwacks, R. M., Engberhard, J. F., Oberley, L. W., Dawson, L. V., and Dawson, T. M. (1998) J. Neurosci. 18, 2040–2055
42. Dierich, P. J., Almar, M. D., and De Jonchkerehe, J. F. (1990) Biochem. Int. 22, 593–600
43. Fahey, R. C., Newton, G. L., Arrick, B., Overdank-Bogart, T., and Aley, S. B. (1984) Science 224, 70–72
44. Inouye, S. (1994) FEBS Lett. 347, 163–168
45. Watanebe, M., Nishino, T., Taks, K., Sofuni, T., and Nohmi, T. (1998) J. Biol. Chem. 273, 23922–23928
46. Knight, R. (1980)J. Antimicrobiol. Chemother. 6, 577–593
47. Ravdin, J. I. (1995) J. Infect. Dis. 20, 1453–1464