The 29-Kilodalton Thiol-Dependent Peroxidase of *Entamoeba histolytica* Is a Factor Involved in Pathogenesis and Survival of the Parasite during Oxidative Stress*†

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The 29-kDa surface antigen (thiol-dependent peroxidase; Eh29) of *Entamoeba histolytica* exhibits peroxidative and protective antioxidant activities. During tissue invasion, the trophozoites are exposed to oxidative stress and need to deal with highly reactive oxygen species (ROS). In this investigation, attempts have been made to understand the role of the 29-kDa peroxidase gene in parasite survival and pathogenesis. Inhibition of *eh29* gene expression by antisense RNA technology has shown approximately 55% inhibition in *eh29* expression, maximum ROS accumulation, and significantly lower viability in 29-kDa downregulated trophozoites during oxidative stress. The cytopathic and cytotoxic activities were also found to decrease effectively in the 29-kDa downregulated trophozoites. Size of liver abscesses was substantially lower in hamsters inoculated with 29-kDa downregulated trophozoites compared to the normal HM1:IMSS. These findings clearly suggest that the 29-kDa protein of *E. histolytica* has a role in both survival of trophozoites in the presence of ROS and pathogenesis of amoebiasis.

*Entamoeba histolytica*, the enteric protozoa, is a well-established causative agent of amoebic dysentery and liver abscesses in humans (37). *E. histolytica* is well known for its high potential for invading and destroying human tissue, leading to diseases such as hemorrhagic colitis and extraintestinal abscesses (30). The parasite usually lives and multiplies within the human gut, which constitutes an environment of reduced oxygen pressure. During tissue invasion, *E. histolytica* is exposed to elevated amounts of exogenous reactive oxygen species (ROS), such as superoxide radical anions (*O₂⁻*), hydrogen peroxide (*H₂O₂*) (14, 26). These highly toxic molecules cause severe damage to biological macromolecules, leading to metabolic dysfunctions. In addition, *E. histolytica* has to inactivate the ROS produced by endogenous enzymes for its survival. Several defense mechanisms exist in which a wide array of enzymatic and nonenzymatic antioxidants, including superoxide dismutase (SOD), glutathione peroxidase, catalase, and glutathione, play an active role in cell survival during oxidative stress. *E. histolytica* produces an iron-containing SOD that is induced by superoxide anions to produce *H₂O₂* (9). Hydroperoxides produced during oxidative stress could be detoxified by a bifunctional NADPH:flavin oxidoreductase containing NADPH-dependent disulfide reductase and a *H₂O₂*-forming NADPH oxidase activity (10, 12). Catalase and glutathione reductase systems are absent in *E. histolytica*, but it encodes a 29-kDa cysteine-rich antigen (thiol-dependent peroxiredoxin) located on the surface of the trophozoites (8). The 29-kDa thiol-dependent peroxiredoxin of *E. histolytica* (Eh29) is homologous to AhpC (alkyl hydroperoxide C-22 protein) of *Salmonella enterica* serovar Typhimurium and *Saccharomyces cerevisiae* thiol-specific antioxidant protein (17). In our previous study it was clearly demonstrated that the enzymes SOD and Eh29 increased by 1.7- and 2.1-fold, respectively, in 1-hour high-oxygen-exposed trophozoites compared to controls. This suggests the synchronous involvement of SOD and Eh29 in detoxification of ROS (1) and survival of the parasite under stressed conditions. Thus, it could be said that Eh29 plays an important role in survival of the parasite in highly oxidative environments.

Therefore, the aim of this study was to decrease the expression of Eh29 in *E. histolytica* using an antisense RNA technique and to show that the trophozoites become susceptible to oxidative stress. For antisense RNA inhibition, a series of plasmid vectors both inducible and constitutive has been successfully used to study the in vivo effects of certain genes in *Entamoeba* spp. (2, 3, 6, 19, 21, 24, 32). In this study a cloned fragment of the *E. histolytica eh29* gene was inserted in the antisense orientation in a tetracycline-inducible expression vector (18, 27, 29). Tetracycline-induced transfected trophozoites of *E. histolytica* showed reduced levels of *eh29* mRNA and protein expression. The Eh29-deficient parasites were more sensitive than the wild type to an oxygenated environment as well as *H₂O₂* in axenic culture.

**MATERIALS AND METHODS**

Cultivation and maintenance of *E. histolytica*. Trophozoites of *E. histolytica* strain HM1:IMSS were cultured axenically in TYI-S-33 medium (16), supplemented with 10% heat-inactivated bovine serum and 3% complete vitamin mix at 37°C. Transfected trophozoites were grown in the presence of 5 µg/ml hygro-
mycin. The dose of hygromycin was increased gradually until the growth rates of the transfected trophozoites were similar to that of the control cells.

**Construction of plasmid.** The tetracycline-controlled gene expression vector pEHHYG-tetR-O-CAT (18, 29) was used as the basic construct for our experiment (kind gift from Egbert Tannich, Bernhard Nocht Institute for Tropical Medicine, Germany) for regulation of the eh29 gene in *Entamoeba histolytica*. A 422-bp fragment from the 5’ end of the eh29 gene was amplified using primers AF (5’-GGGCTGCTGCAACGATCTACCTCTGCTGCAATGTCGA-3’) and AR (5’-GGGCTG

**Transfection and selection of drug-resistant *E. histolytica* phenotype.** Transfection of plasmids in *E. histolytica* was performed by electroporation as described by Purdy et al. (28), with slight modifications under electroporation conditions (3). Briefly, *E. histolytica* trophozoites in logarithmic phase were kept in ice for 5 min, centrifuged at 400 × *g* for 5 min, and washed once in incomplete cytoxin buffer (120 mM KCl, 0.15 mM CaCl₂, 10 mM KH₂PO₄/K₂HPO₄, pH 7.5; 1% glucose). *E. histolytica* trophozoites were resuspended in 0.8 ml of incomplete cytoxin buffer, supplemented with 2 mM ATP and 5 mM glutathione, at a concentration of 1.5 × 10⁷ per ml. Immediately, 2.5 µl of DEAE-dextran (1 mg/ml) was added to the cell suspension and it was transferred to a 0.4-cm electroporation cuvette (Bio-Rad) and incubated with 100 µg purified circular plasmid. Transfections were carried out in a Bio-Rad Gene Pulser II at 1.2-kV voltage, 3,000-V/cm field strength, and 25-cm cuvette capacitance with a time constant of 0.4 ms. After transfection, the cuvette was immediately placed on ice for 15 min and trophozoites were transferred to 15-ml sterile screw-cap culture tubes containing 11 ml TYI-S-33 medium. The transfected trophozoites were initially incubated with 5 µg/ml hygromycin, and the dose was gradually increased to 10 µg/ml to obtain stable transfectedants. The medium was changed every 24 h with fresh addition of hygromycin.

**Selection of tetracycline dose.** The control and transfected trophozoites were incubated for 24 h, 48 h, and 72 h with a tetracycline dose of 0.5 µg/ml, 1 µg/ml, and 5 µg/ml (18, 29). Trophozoites were washed once with prewarmed complete TYI-S-33 medium and chilled on ice for 5 min to dislodge the cells from tubular surfaces. The viability of the collected trophozoites was examined by trypan blue exclusion (0.5 mg/ml).

**Isolation of total RNA.** Total RNA was isolated from nontransfected wild-type HM1 trophozoites, transfected control trophozoites (pEHHYG-tetR-O-CAT vector only), transfected uninduced trophozoites (pEHHYG-tetR-O-Reveh29) incubated with 0.5 µg/ml hygromycin, and 5 µg/ml hygromycin for 20 h. Transiently transfected trophozoites (pEHHYG-tetR-O-Reveh29) and from 3-h oxidative stress-induced trophozoites were subjected to 3 h of oxidative stress as described earlier. Normal and stressed derived cells collected after incubation were washed twice in phosphate-buffered saline (pH 7.4) and labeled using the Annexin V-FLUOS staining kit (Roche, Germany) following the manufacturer’s protocol. The cells were analyzed using a FACSCalibur (BD Biosciences) for early stage (Annexin V positive, PI negative) and late stage (Annexin V positive, PI positive) apoptotic cells.

**Detection of reactive oxygen species.** Intracellular oxidant levels were determined by the use of 2’,7’-dichlorodihydrofluorescein diacetate (H₂DCF-DA), which is oxidized inside the cell to the fluorescent dichlorofluorescein (DCF). Control and stressed-derived *E. histolytica* trophozoites were incubated with 0.4 mM (final concentration) DCFH-DA for 15 min in the dark. The cells were washed once in phosphate-buffered saline (pH 7.4) and immediately examined under a confocal microscope (LSM510; Zeiss) (1). Measurement of the fluorescently labeled cells was performed with a Leica TCS SP2 confocal microscope equipped with an argon laser at 488 nm and an argon/krypton laser at 568 nm (1, 4). The mean fluorescence intensity was analyzed using a Beckman Coulter (Cytoflex FC 500) flow cytometer at 530 ± 30 nm. A total of 10,000 cells were analyzed per sample.

**Viability assay.** Trophozoites in the logarithmic growth phase were washed once with prewarmed complete TYI-S-33 medium and chilled on ice for 5 min to dislodge the cells from tube surfaces. Briefly, 2 × 10⁷ trophozoites in 20 ml complete TYI-S-33 medium were incubated at 37°C for different time periods (5 to 6 h). After each hour, culture medium was collected and the trophozoites were collected. The viability of trophozoites was examined by trypan blue exclusion (0.5 mg/ml).

**Measurement of cell viability by MTT assay.** Viability of stress-induced cells was determined using an in vitro toxicology assay kit based on 3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma). MTT is an organic compound which is converted to the blue formazan salt derivative by the mitochondrial dehydrogenase enzymes. The rate of conversion of MTT to formazan is proportional to viable cell number. The absorbance of formazan salt is measured spectrophotometrically at 570 nm (25). Nontransfected, transfected control, uninduced transfected, tetracycline-induced transfected, and 5 µM catalase-treated tetracycline-induced trophozoites were subjected to oxidative stress for 3 h. The stress-induced cells were incubated with MTT and assayed following the kit protocol.

**No monolayers.** Monolayers of baby hamster kidney (BHK-1) cultured cells were grown in 24-well plates in Dulbecco’s modified Eagle’s (DME) medium supplemented with 5% fetal calf serum for 72 h until confluence. A total of 2 × 10⁵ trophozoites (2 × 10⁵/ml suspended in DME medium) were added to the wells containing a BHK-1 cell monolayer (2 × 10⁵/well) with serum-free DME medium. Incubation for 1 h was continued at 37°C in a CO₂ incubator. The reaction was stopped by incubating the plate at 4°C for 10 min, washed with cold saline twice, fixed with 4% formaldehyde for 10 min, and finally washed with cold saline. The cells were then stained with methylene blue (0.1% in borate buffer, 0.1 M, pH 8.7) for 10 min. The excess stain was washed with 0.01 M borate buffer, and the incorporated stain was extracted using HCl (1 ml, 0.1 M) at 37°C for 30 min. The intensity of color was measured at 660 nm (5, 7).

**Cytotoxic activity of trophozoites on cell monolayers.** Monolayers of baby hamster kidney (BHK-1) cultured cells were grown in 24-well plates in Dulbecco’s modified Eagle’s (DME) medium supplemented with 5% fetal calf serum for 60 to 72 h until confluence. A total of 2 × 10⁵ trophozoites (2 × 10⁵/ml suspended in DME medium) were added to the wells containing a BHK-1 cell monolayer (2 × 10⁵/well) with serum-free DME medium. Incubation for 1 h was continued at 37°C in a CO₂ incubator. The reaction was stopped by incubating the plate at 4°C for 10 min, washed with cold saline twice, fixed with 4% formaldehyde for 10 min, and finally washed with cold saline. The cells were then stained with methylene blue (0.1% in borate buffer, 0.1 M, pH 8.7) for 10 min. The excess stain was washed with 0.01 M borate buffer, and the incorporated stain was extracted using HCl (1 ml, 0.1 M) at 37°C for 30 min. The intensity of color was measured at 660 nm (5, 7).

**Cytotoxic activity.** The cytotoxic activity of viable trophozoites was determined by vital dye exclusion (7, 22). Freshly harvested BHK cells were washed and resuspended in TYI-S-33 medium without serum. The BHK cells were incubated with trophozoites in the ratio 6:1 (9 × 10⁵ BHK cells, 1.5 × 10⁶ trophozoites) at 37°C. Viability was assayed by examining the trypan blue-treated cells in a hemocytometer. The cytotoxic activity was calculated as the percentage of
stained cells in the sample minus the percentage of stained cells in the sample with BHK cells alone. The experiment was done in triplicate.

**Enzyme assay.** The enzymatic activity of alcohol dehydrogenase in the trophozoite lysates from the different types of trophozoites was determined as described previously (23). One unit of activity is defined as 1 μmol of substrate reduced per min per mg of protein.

**Ingestion of GFP-labeled *Escherichia coli* cells.** *E. coli* cells (1 × 10⁹) containing a plasmid which expresses green fluorescent protein (GFP) were associated in culture medium with different types of trophozoites (1 × 10⁶). The trophozoites with bacteria were harvested after 1 h and washed by centrifugation, and a portion (25%) was fixed with 3.7% formaldehyde. The remaining trophozoites were resuspended in fresh culture medium and were harvested after 24 h, washed, and fixed with formaldehyde. The trophozoites containing fluorescent bacteria were examined under a confocal microscope (7), and the mean fluorescence intensity was measured in a spectrofluorimeter (Perkin-Elmer) at 470-nm excitation and 510-nm emission.

**Induction of amoebic liver abscesses.** Female Syrian golden hamsters (6 weeks old) were inoculated intrahepatically with 5 × 10⁵ nontransfected, transfected...
control, uninduced transfected, and tetracycline-induced transfected trophozoites. Hamsters (four in each group) were sacrificed 1 week after intrahepatic inoculation, and formation of lesions was evaluated. Hamsters used in this study were approved by the Institutional Review Board, and animal experiments were conducted under appropriate regulatory guidelines.

Statistical analysis. Data are means ± standard errors of the means (SEM) of multiple experiments. Statistical differences were analyzed by one-way analysis of variance, with statistical significance being set at 0.05 ($P < 0.05$).

RESULTS

Dose selection of tetracycline. The viabilities of the transfected and control trophozoites were assayed with increasing doses of tetracycline in a time-dependent manner. A 24-h incubation with a 0.5-μg/ml or 1-μg/ml dose of tetracycline revealed around 97% viability. The viability was significantly decreased with a dose of 5 μg/ml tetracycline when incubated for same time period (Fig. 1A). With an increase in the period of incubation with different doses of tetracycline, the viability of the trophozoites decreased gradually. The viability was significant only with 24-h incubation on exposure to tetracycline doses of 0.5 μg/ml and 1 μg/ml, but the expression of the inserted gene was maximum with a dose of 1 μg/ml when incubated for a period of 24 h (50% downregulation in eh29 expression) compared to induction with 0.5 μg/ml (11% down-regulation).

FIG. 2. Expression of the eh29 gene product in different trophozoites. A and B: Semiquantitative RT-PCR analysis. The PCR products from trophozoites without (A) and with (B) 3-h oxidative stress were separated on a (1%) agarose gel and stained with ethidium bromide. Eh29 and actin products are marked. Ethidium bromide-stained PCR products were photographed and then images were analyzed densitometrically. PCR products were quantitated and expressed as percentages with respect to actin band density. Data represent means ± SEM of three independent experiments ($P < 0.05$). C and D: Immunoblot analysis of the $E. histolytica$ 29-kDa protein. The immunoblot was prepared by resolving amoeba lysates from nontransfected and different transfected trophozoites without (C) and with (D) 3-h oxidative stress. The blot was developed with NICED 11 antibody specific for Eh29 and with fibronectin to confirm equal loading of proteins. Data represent means ± SEM of several trophozoites from three independent experiments ($P < 0.05$). Bars: 1, nontransfected trophozoites; 2, pEhHYG-tetR-O-CAT-transfected control trophozoites; 3, uninduced pEhHYG-tetR-O-Reveh29-transfected trophozoites; 4, tetracycline-induced pEhHYG-tetR-O-Reveh29-transfected trophozoites.
Antisense eh29 significantly reduces the 29-kDa gene product. The mRNA levels of the eh29 gene in nontransfected, transfected control (pEhHYG-tetR-O-CAT-containing trophozoites), uninduced transfected trophozoites, and tetracycline-induced transfected trophozoites under normal and stress-inducing conditions were quantified using semiquantitative RT-PCR. The expression level of eh29 in tetracycline-induced trophozoites was found to be reduced by 55% from control trophozoites. The mRNA levels of eh29 in nontransfected and transfected control trophozoites were quite similar. No prominent change was noticed in the expression level of actin in the four different types of trophozoites (Fig. 2A).

FIG. 3. Generation of reactive oxygen species production and viability analysis of stressed trophozoites. E. histolytica trophozoites were subjected to 3 h of oxidative stress, and ROS generation was analyzed by confocal microscopy and flow cytometry using DCFH-DA (see Materials and Methods for details). Viability was examined by trypan blue exclusion. A: Relative expression of ROS in E. histolytica trophozoites as viewed under a laser confocal microscope. B: Histogram representing relative expression of ROS as obtained with a flow cytometer. C: Mean fluorescence intensity indicating expression of ROS of the trophozoites. D: Percentage of dead cells. Data represent means ± SEM of three independent experiments (P < 0.05). Bars: 1, nontransfected trophozoites; 2, pEhHYG-tetR-O-CAT-transfected control trophozoites; 3, uninduced pEhHYG-tetR-O-Reveh29-transfected trophozoites; 4, tetracycline-induced pEhHYG-tetR-O-Reveh29-transfected trophozoites.

NICED 11 monoclonal antibody specific for the 29-kDa protein of E. histolytica was used in an immunoblot analysis. Identical amounts of protein from nontransfected, control, uninduced transfected, and tetracycline-induced transfected trophozoites revealed a 50% decrease in the 29-kDa protein in tetracycline-induced transfected trophozoites (Fig. 2C). Nontransfected, control, and uninduced trophozoites produced nearly similar amounts of the 29-kDa protein. The same effect was also maintained in the oxidatively stressed trophozoites (Fig. 2D).

Downregulation of Eh29 affects survival of the trophozoites under O2-stressed conditions. Previously, our group showed that the maximum ROS level accumulated at 3-h, high-oxygen-stressed trophozoites compared to normal trophozoites, and the level increased in the order of 1 h < 2 h < 3 h (1). In the present investigation, it was found that the mean fluorescence
Effect of antisense eh29 on physiological activity and death of the trophozoites during oxidative stress. A: Viability assay of stressed trophozoites using MTT (for details, see Materials and Methods). B: Loss of membrane integrity during oxidative stress. Release of the cytosolic enzyme pyruvate:ferredoxin oxidoreductase served as an indicator of loss of membrane integrity and thus cell viability. Bars: 1, nontransfected trophozoites; 2, pEhHYG-tetR-O-CAT-transfected control trophozoites; 3, uninduced pEhHYG-tetR-O-Reveh29-transfected trophozoites; 4, tetracycline-induced pEhHYG-tetR-O-Reveh29-transfected trophozoites; 5, 5 μM catalase-treated tetracycline-induced transfected trophozoites. Data represent means ± SEM of three independent experiments (P < 0.05). C: The cell death assay was done using an Annexin V-FLUOS kit. The left column represents propidium iodide-positive trophozoites, the middle column represents Annexin-positive trophozoites, and the right column represents a combined image. Panels: 1, nontransfected trophozoites; 2, pEhHYG-tetR-O-CAT-transfected control trophozoites; 3, uninduced pEhHYG-tetR-O-Reveh29-transfected trophozoites; 4, tetracycline-induced pEhHYG-tetR-O-Reveh29-transfected trophozoites; 5, 5 μM catalase-treated tetracycline-induced transfected trophozoites; 6, nontransfected trophozoites exposed to oxidative stress for 6 h.
intensity was maximum in tetracycline-induced pEhHYG-tetR-O-Reveh29-containing trophozoites. The comparative analysis in ROS levels demonstrated 1.672-, 1.8-, and 7-fold increases in transfected trophozoites without antisense, transfected trophozoites with antisense, and tetracycline-induced pEhHYG-tetR-O-Reveh29-transfected trophozoites with antisense, respectively (Fig. 3A and B).

Survivability of these stressed trophozoites was studied by vital dye exclusion, and it showed that tetracycline-induced pEhHYG-tetR-O-Reveh29-transfected trophozoites were 55% viable, compared to 97.5% in nontransfected trophozoites. The uninduced transfected trophozoites and the transfected control trophozoites showed 93% and 95% viability, respectively, after 3 h of oxidative stress (Fig. 3C).

FIG. 5. Pathogenic effects of different trophozoite groups. A: Cytopathic activity of trophozoites. Trophozoites (2 × 10⁵) were incubated with BHK cells. After 1 h of interaction, the destruction of the BHK tissue culture monolayer was determined as described in Materials and Methods. B: Cytotoxic activity of E. histolytica trophozoites (1.5 × 10⁶) incubated in suspension with BHK cells (9 × 10⁶). Viability was determined by trypan blue exclusion as described in Materials and Methods. ●, BHK cells alone; ■, nontransfected parent HM1 strain; ○, tetracycline-induced pEhHYG-tetR-O-Reveh29-transfected trophozoites. C: Confocal fluorescence microscopy of trophozoites that ingested GFP-labeled E. coli cells. Trophozoites were associated with the bacteria for 1 h, after which they were harvested and washed. A portion of the total amount of trophozoites was then fixed in formaldehyde, and the image obtained by phase contrast with the fluorescent image shows the bacterial location in the vacuoles. D: Fluorescence intensity emitted by GFP-labeled bacteria phagocytosed by the different trophozoites, as measured in a spectrofluorimeter at 470-nm excitation and 510-nm emission. Panels (C) and bars (D): 1, nontransfected trophozoites; 2, pEhHYG-tetR-O-CAT-transfected control trophozoites; 3, uninduced pEhHYG-tetR-O-Reveh29-transfected trophozoites; 4, tetracycline-induced pEhHYG-tetR-O-Reveh29-transfected trophozoites. Data represent means ± SEM of three independent experiments (P < 0.05).
Downregulation of Eh29 affects physiological activities in trophozoites. An MTT assay of the trophozoites showed that under stressed conditions the tetracycline-induced transfected trophozoites were 62.6% less metabolically active than the nontransfected trophozoites. Catalase-treated cells showed 72% viability. The transfected control trophozoites showed 99% viability, and uninduced transfected trophozoites showed 97.5% viability compared to nontransfected trophozoites (Fig. 4A).

Membrane integrity as determined by the release of pyruvate:ferredoxin oxidoreductase from the cells was found to be significantly less in the tetracycline-induced transfected trophozoites than in the nontransfected, transfected control, and uninduced transfected trophozoites. The pyruvate:ferredoxin oxidoreductase activity was almost five times higher in the induced trophozoites than the control. In catalase-treated trophozoites the pyruvate:ferredoxin oxidoreductase release was quite similar to the nontransfected trophozoites (Fig. 4B).

The cell death assay using the Annexin V-FLUOS staining kit revealed that the mode of cell death due to oxidative stress in the tetracycline-induced transfected trophozoites is necrosis, as nearly 75% of the cells were propidium iodide positive. No significant increases in dead cells were encountered in the nontransfected trophozoites. Two percent of transfected control trophozoites, 3.5% of uninduced transfected trophozoites, and 2.5% of the catalase-treated tetracycline-induced transfected trophozoites gave a propidium iodide-positive signal (Fig. 4C). In the 6-h oxidatively stressed normal cells, Annexin V-positive signal was found.

**Antisense Eh29-downregulated trophozoites exhibit decreased cytopathic and cytotoxic effects.** A significant decrease in cytopathic activity was observed in the tetracycline-induced transfected trophozoites (>55%) compared to normal HM1:IMSS trophozoites when allowed to interact with a monolayer of BHK cells for 60 min (Fig. 5A). Transfected control trophozoites exhibited cytopathic activity very similar to nontransfected trophozoites of the parent *E. histolytica* strain. The uninduced transfected trophozoites showed cytopathic activity quite similar to nontransfected trophozoites.

The cytotoxic effect of the trophozoites on BHK cells was measured by the damage to the membranes of suspended BHK cells, as detected by trypan blue exclusion. The nontransfected trophozoites showed 92% cytotoxic activity at 90 min compared to 41.6% cytotoxic activity in tetracycline-induced transfected trophozoites (>55% decrease in cytotoxicity) (Fig. 5B). The control transfected trophozoites showed very similar results (85%) as nontransfected trophozoites, and uninduced transfected trophozoites showed 74.6% cytotoxic activity.

**Effect of antisense Eh29 on phagocytic ability of trophozoites.** A marked decrease in the ingestion of fluorescent-labeled bacteria (Fig. 5C) was noticed in the induced transfected trophozoites (69.7%) (Fig. 5D) compared to the nontransfected, control transfected, and uninduced transfected trophozoites.

**Effect of antisense inhibition of Eh29 on liver abscess formation in hamsters.** To correlate the decrease in cytopathic effect with the general decrease in virulence, liver abscess formation in hamsters was observed after inoculating the animals intrahepatically with $5 \times 10^7$ nontransfected, transfected control, uninduced transfected, and tetracycline-induced transfected trophozoites. Hamsters inoculated with parent HM1:IMSS trophozoites, the control transfected trophozoites, or uninduced transfected trophozoites showed extensive necrotic lesions (>20 mm), while of the four hamsters inoculated with tetracycline-induced transfected trophozoites only two showed minor lesions (<10 mm) (Table 1).

**DISCUSSION**

Earlier, our lab demonstrated that in oxidatively stressed trophozoites, ROS levels increased in a time-dependent manner ($3 \ h > 2 \ h > 1 \ h$). A 2.1-fold overexpression of the *eh29* gene was noticed in 1-h-stressed trophozoites compared to the control trophozoites. Increased levels of the 29-kDa protein were also noticed in oxidatively stressed trophozoites compared to normal trophozoites in an immunoblot assay against NICED 11 monoclonal antibody (1). These observations clearly suggested a possible role of Eh29 in the survival of the parasite and maintaining the pathogenic effect during invasion in the highly oxygenated environment of the host. Catalase- and glutathione-dependent peroxidase activities are absent in *E. histolytica*, but *E. histolytica* contains a peroxiredoxin on the outer surface of the cell which reduces and detoxifies peroxides and peroxynitrites under oxidatively stressed conditions (11). This protein is reported to localize to the parasite host cell contact and can effectively counteract the free radicals generated by the host cell and facilitates invasion (13). Therefore, it could be predicted or possibly true that Eh29, a membrane-associated peroxiredoxin in nature, takes part in the removal of $H_2O_2$, formed under stressed conditions and helps in the survival of the parasite. To establish the involvement of Eh29 in survival and removal of ROS from the parasite, an antisense regulation technique to inhibit the expression of the *eh29* gene was used.

In the tetracycline-induced *eh29* antisense transfected trophozoites, the expression of the 29-kDa protein was downregulated by almost 50% (Fig. 2). This downregulation affected the survival rate of the parasite under oxidative stress conditions. In 3-h oxidatively stressed cells, the survival rate of the *eh29* downregulated trophozoites was only 65% compared to 97.5% in normal trophozoites. ROS accumulation in the 29-kDa antisense transfected trophozoites showed a sevenfold increase under stress conditions compared to the control trophozoites, suggesting an involvement of the 29-kDa protein in removal of ROS, particularly $H_2O_2$. In the Eh29 downregulated trophozoites, cellular physiological activities were impaired and membrane integrity was lost under stressed conditions, but the cells were viable in the presence of catalase (Fig. 4). The above evidence confirmed that when the 29-kDa protein was downregulated in the trophozoites, the $H_2O_2$ level was high, as the

**TABLE 1. Induction of liver abscess formation in hamsters**

| Type of trophozoites | Abscess size (mm) |
|----------------------|------------------|
| Nontransfected HM1:IMSS | 20–25 |
| pEHHYG-tetR-O-CAT-transfected control | 20–22 |
| Uninduced pEHHYG-tetR-O-Reveh29 transfected | 17–22 |
| Tetracycline-induced pEHHYG-tetR-O-Reveh29 transfected | 6–9 |

*Six-week-old female Syrian Golden hamsters were inoculated with $5 \times 10^7$ trophozoites after laparotomy and sacrificed after 7 days. Four animals were inoculated with each type of trophozoites.*
H$_2$O$_2$ formed could not be neutralized by the inhibited peroxidase. It is known that increased H$_2$O$_2$ levels alter membrane properties and disrupt membrane-bound proteins significantly, thereby killing the trophozoites. Further, almost all the eh29 downregulated trophozoites were propidium iodide positive, while no such observation was found in the normal trophozoites subjected to oxidative stress. Normal cells were Annexin V positive only after 6 h of oxidative stress, thus suggesting a possible necrotic death in the downregulated trophozoites. Therefore, the 29-kDa membrane-bound protein removes the toxic ROS during oxidative stress and is an essential factor for parasites survival.

Earlier reports have also shown the involvement of Eh29 in pathogenesis (15, 31). We have also made similar observations (unpublished data). To confirm the role of the 29-kDa protein in pathogenesis, cytopathic and cytotoxic effects on BHK1 cells were studied in Eh29 antisense and control trophozoites. The BHK monolayer destruction was only 20% by the 29-kDa-downregulated trophozoites compared to 75% by the normal HMI. The cytotoxic activity was also significantly lower with the 29-kDa-inhibited trophozoites. The phagocytic capability of 29-kDa-inhibited trophozoites decreased to 69.7%, and liver abscess size in hamsters was significantly lower, confirming the result obtained by Soong et al. (35). All these findings clearly revealed that Eh29 has a definite role in pathogenesis. However, growth culture rates, protein electrophoretic patterns, and basal levels of alcohol dehydrogenase were almost similar ever, growth culture rates, protein electrophoretic patterns, revealed that Eh29 has a definite role in pathogenesis. How-toxic ROS during oxidative stress and is an essential factor for parasites survival.

Eh29 is a thiol-specific antioxidant and is a GalNAc lectin-associated protein (20); therefore, it may be concluded that during the host-parasite interface the lectin recruits Eh29 (thiol-specific antioxidant protein), which serves as a mechanism by which the parasite protects itself during tissue adherence and invasion from oxidative attack by the activated host phagocytic and epithelial cells, facilitating invasion.

Thus, in the future the 29-kDa protein can be used as a target for rational drug design for treatment of amoebic liver abscess.

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