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Research brief

Entamoeba histolytica: Differences in phagosome acidification and degradation between attenuated and virulent strains

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Abstract

Phagocytosis is the important virulent determinant of the enteric protozoan parasite *Entamoeba histolytica*. We compared the kinetics of phagosome maturation of attenuated and highly-virulent strains of *E. histolytica* using video microscopy. Phagosomes of attenuated strains were acidified rapidly within 2 min after phagosome formation (at the rate of 0.96 pH/min), persisted at pH 4.46 ± 0.13, and degraded ingested GFP-*Leishmania* very efficiently (90–94% GFP fluorescence was lost in 30 min), while phagosomes of highly-virulent strains were acidified slowly (0.69 pH/min), persisted at 5.11 ± 0.23, and degraded GFP less efficiently (60–71% decrease). These results suggest that efficiency of phagosome maturation is most probably inversely correlated with apparent virulence.

Index Descriptors and Abbreviations: *Entamoeba histolytica*; Attenuation; Virulence; Phagocytosis; Vacuolar type ATPase

The intestinal protozoan parasite *Entamoeba histolytica* is a major cause of morbidity and mortality in developing countries (Stanley, 2003). Trophozoites of *E. histolytica* phagocytose bacteria in the large intestine (Bracha et al., 1982), as well as red blood cells (Tsutsumi et al., 1992) and apoptotic immune cells (Huston et al., 2003) during tissue invasion. Their killing and phagocytosis by *E. histolytica* plays an important role in virulence (Huston et al., 2003). The pathogenesis of *E. histolytica* also depends on the concerted action of numerous virulence factors (Mirelman et al., 2000). The relative virulence of different strains varies upon culture conditions (Diamond, 1983). It was shown that apparent virulence was attenuated by a prolonged axenic cultivation (Bos and Van de Griend, 1977; Mattern et al., 1982; Phillips, 1973), while it is regained by passage through the hamster liver (Das and Ghoshal, 1976; Diamond et al., 1974; Lushbaugh et al., 1978) or co-cultivation with bacteria (Padilla-Vaca et al., 1999). However, the mechanism for such variations in virulence is still unknown. It was previously shown that maintenance of acidic intracellular endocytic vesicles in *E. histolytica* trophozoites was necessary for contact-mediated killing of host epithelial cells (Ravdin et al., 1986). In 'professional' phagocytes (e.g., macrophages and neutrophils) the phagosome acidification plays an important role for the dissociation of internalized ligand-receptor complexes and recycling of receptors to the plasma membrane (Futai et al., 2000), recruitment of hydrolases (Griffiths, 2004), microbicidal action (Hackam et al., 2003), and the degradation of phagosomal content (Grinstein et al., 1992). In all eukaryotic cells, acidification of intracellular compartments including phagosomes, endosomes, and lysosomes is mediated by the vacuolar proton pump, vacuolar ATPase (V-ATPase, Grabe and Oster, 2001). Recently, it has been shown that V-ATPase (Vph1p)

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is involved in the virulence mechanism of the AIDS-related opportunistic fungal pathogen, *Cryptococcus neoformans* (Erickson et al., 2001). Very recently, we have shown remarkable differences in the kinetics of phagosome acidification and degradation between pathogenic *E. histolytica* and closely-related but non-pathogenic *E. dispar* (Mitra et al., 2005). In the present study we established attenuated and highly-virulent strains of *E. histolytica* and compared the kinetics of phagosome maturation between them with video microscopy.

For attenuated strains, trophozoites of *E. histolytica* HM-1: IMSS cl6 (HM1, Diamond et al., 1972; Landa et al., 1970) and a clinical isolate, HATAJI (Okada et al., 2005) were grown axenically in BI-S-33 medium for over two years. For highly-virulent strains, HM1, HATAJI, SAW755 (Sargeaunt et al., 1978), and KU33 (Haghighi et al., 2003) strains were used. To regain the virulence of HM1 strains, and maintain the virulence of HATAJI, SAW755 and KU33, 10^5–10^6 trophozoites were inoculated into the left lobe of the liver of Syrian golden hamsters (*Mesocricetus auratus*) as previously described (Ghosh et al., 2004). Seven days later, animals were anesthetized with ether and sacrificed by cardiac bleeding. The livers were then cut into slices, transferred to BI-S-33 and cultivated in BI-S-33 medium with *Crithidia fasciculata* at 35 °C for 7–10 days. Subsequently, the amoebae were harvested and passaged to the hamster liver ten more times for HM1 and four times for HATAJI, SAW755, and KU33. Although attenuation of SAW755 and KU33 strains was also attempted by continuous axenic cultivation, these strains did not significantly lose either in vitro or in vivo virulence as measured described below.

Trophozoites of attenuated *E. histolytica* HM1 and HATAJI strains (A-HM1 and A-HATAJI) were cultured axenically at 35 °C in 13 × 100 mm screw-capped Pyrex glass tubes in BI-S-33 medium as previously described (Diamond et al., 1972). Trophozoites of virulent HM1, HATAJI, KU-33, and SAW755 strains (L-HM1, L-HATAJI, L-KU33 and L-SAW755) were cultured monoxenically with live *C. fasciculata* in BI-S-33 medium for 2 weeks after isolation from liver abscesses, and then maintained in the absence of *C. fasciculata* for 4 days prior to the experiments. Promastigotes of green fluorescent protein (GFP)-expressing *L. amazonensis* (Chan et al., 2003), a gift from K. P. Chang and S. Kawazu, were cultured in 199 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum, 25 mM Hepes, and 5 μg/ml tunicamycin. Chinese hamster ovary (CHO) cells were grown in F-12 medium (Invitrogen-Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Sigma–Aldrich, Irvine, UK) on a 10 cm-diameter tissue culture dish (IWAKI, Tokyo, Japan) under 5% CO_2 at 37 °C.

We evaluated in vitro pathogenicity of the individual strains by measuring the destruction of CHO monolayer, phagocytosis, and cysteine proteases (CP). CHO cells were grown to confluence in 24-well flat-bottomed tissue culture plates, washed with PBS, and labeled with 40 μM CellTracker blue (7-amino-4-chloromethylcoumarin, Molecular Probes, Eugene, OR) in the growth medium at 37 °C for 2 h. After unincorporated dye was removed by washing CHO cells twice with PBS, CHO cells were further grown in 500 μl of the growth medium for 2 h. After the medium was replaced with pre-warmed OPTI-MEM medium (Invitrogen-Gibco) supplemented with 137 mM L-cysteine and 19 mM ascorbic acid, pH 6.65, approximately 1.5×10^5 of *E. histolytica* trophozoites were added to each well and the plates were incubated at 35 °C for 1.5 h. After incubation the plate was carefully washed three times with cold PBS supplemented with 111 mM glucose and 100 mM galactose. Remaining CHO cells were incubated in 200 μl of trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA) at 35 °C for 10 min and then collected in an Eppendorf tube. The CHO cells were further lysed by sonication for 15 s and fluorescence intensity of CellTracker blue was measured using a fluorometer (F-2500, Hitachi, Japan) with excitation and emission at 353 and 465 nm, respectively. The number of adherent CHO cells was proportional to the intensity of CellTracker blue staining and expressed as percentage of the remaining fluorescence of untreated CHO cells. We evaluated in vivo virulence of the *E. histolytica* strains using a hamster liver abscess model. Five hamsters were challenged by the direct injection of 1×10^6 *E. histolytica* trophozoites in the liver as described above. After seven days, animals were sacrificed and the weight of amoebic abscesses and normal portion of the liver was measured. The activity of CP was measured as described (Nakada-Tsukui et al., 2005; Nowak et al., 2004). Phagocytosis and kinetics of phagosome acidification and degradation were monitored as described previously (Mitra et al., 2005).

Apparent virulence of the six established amoeba lines used in this study was examined in vitro and in vivo. First, the ability to destroy the monolayer of cultured CHO cells was examined (Ravdin et al., 1986). The destruction of CHO cells was linear for two hours under the conditions described above (data not shown). At 90 min, the liver-passaged strains (L-HM1, L-HATAJI, L-KU33, and L-SAW755) destroyed 85–90% of CHO cells, whereas attenuated strains (A-HM1, A-HATAJI) destroyed only 17–34% (Fig. 1A). The capacity to ingest inert FITC-yeast particles (Mitra et al., 2005) or human red blood cells (RBC) was also examined in trophozoites of attenuated and liver-passaged strains. Liver-passaged strains were more efficient in the engulfment of both yeasts (Fig. 1B) and erythrocytes (data not shown) than attenuated strains. Phagocytosis efficiency also varied amongst the liver-passaged strains: L-HATAJI showed the highest ability to phagocytose both yeasts (Fig. 1B) and erythrocytes (data not shown), while L-HM1 showed only slight increase in yeast phagocytosis compared with attenuated strains. Since CPs have been proposed to play a major role in amoebic invasion and tissue destruction due to their activities towards proteins in the extracellular matrix (Keene et al., 1986; Singh et al., 2004; Stanley et al., 1995), CP activities in the total lysate and the culture