

Antisense Inhibition of Expression of Cysteine Proteinases Affects *Entamoeba histolytica*-Induced Formation of Liver Abscess in Hamsters

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Trophozoites of virulent *Entamoeba histolytica* transfected with the antisense gene encoding cysteine proteinase 5 (CP5) have only 10% of the CP activity but retain their cytopathic activity on mammalian monolayers. In the present study we found that the transfected trophozoites with low levels of CP activity were incapable of inducing the formation of liver lesions in hamsters.

Several cysteine proteinases (CP) with molecular masses in the range of 16 to 96 kDa have been detected in trophozoite extracts of pathogenic *Entamoeba histolytica* strains (9, 12, 15). CP are considered an important virulence factor in the pathogenesis of amebiasis and have been suggested to play a key role in tissue invasion, disruption of host tissues, and modulation of the cell-mediated immune response (4, 12–14). Bruchhaus and colleagues have identified up to six different CP genes which encode typical papain family proteinases with a high degree of conservation in all active site residues (3). One of them, CP5, was found to be associated with the trophozoite membranes and has been suggested to have a potential role in host tissue destruction. Interestingly, the gene encoding CP5 (*cp5*) is missing in the closely related but nonpathogenic species *Entamoeba dispar* (7). Recently, we have generated a transfectant of *E. histolytica* HM-1:IMSS in which the transcribed *cp5* antisense RNA (*E. histolytica* HM-1:IMSS pSA8 transfectant) strongly reduces the expression of CP (2). In spite of their low levels of CP activity, the pSA8 transfectants are, however, not impaired in their ability to destroy tissue culture monolayers (cytopathic activity). On the other hand, pSA8 transfectants have significantly lower erythrophagocytosis activity than does the parental strain (2).

In this study we examined the ability of the *E. histolytica* HM-1:IMSS pSA8 transfectant to induce the formation of liver abscesses in hamsters. The level of CP in detergent lysates of pSA8 trophozoites grown in the presence of 60 µg of G418 per ml was determined, prior to intrahepatic inoculation into the hamsters, by monitoring the digestion of the fluorogenic substrate Z-Arg-Arg-pNA (8). The total CP activity in lysates of the pSA8 transfectant was approximately 10% of the level of CP activity determined in the control lysates of the pEhAct-Neo (1) transfectant. The generation times of the two transfected trophozoite cultures (pEhAct-Neo and pSA8) grown in TYI-S-33 medium (5) in the presence of 60 µg of G418 per ml and the nontransfected parental HM1:IMSS strain were 8 and 7 h, respectively. This difference is most likely due to some inhibitory effects of the neomycin derivative G418. Syrian golden hamsters (6 weeks old) were intrahepatically inoculated (18) with 10⁶ trophozoites from the following cultures: (i) *E.*

histolytica HM-1:IMSS pSA8 grown in the presence of 60 µg of G418 per ml, (ii) *E. histolytica* HM-1:IMSS pEhAct-Neo grown in the presence of 60 µg of G418 per ml, and (iii) *E. histolytica* HM-1:IMSS nontransfected trophozoites. Hamsters (eight in each group) were sacrificed 1 week after intrahepatic inoculation, and the formation of lesions was evaluated (Table 1). All eight hamsters injected with *E. histolytica* HM-1:IMSS trophozoites as well as those inoculated with the HM-1:IMSS pEhAct-Neo transfectant presented extensive necrotic lesions. The sizes of the lesions obtained with HM-1:IMSS trophozoites were larger (>2 cm) than those obtained with HM1:IMSS pEhAct-Neo (1 to 2 cm). The smaller size may be due to the above-mentioned difference in growth rates. Only one of the eight hamsters injected with the HM-1:IMSS pSA8 transfectant presented a small isolated lesion, whereas none of the others had any detectable hepatic lesions. A section of amoeba-infected liver was inoculated under sterile conditions in TYI-S-33 medium. For lesions obtained with the *E. histolytica* HM-1:IMSS trophozoites and the HM-1:IMSS pEhAct-Neo transfectant, a full trophozoite culture was obtained 24 h after inoculation into the TYI-S-33 medium in the absence of G418. For the small and isolated lesion obtained with the *E. histolytica* HM-1:IMSS pSA8 transfectant, it took 2 weeks to get a satisfactory culture of trophozoites in the absence of G418. Plasmid DNA was rescued from pSA8- and pEhAct-Neo-transfected trophozoites and introduced into *Escherichia coli* cells to yield ampicillin-resistant transfectants, showing that in both cases the plasmid was still present in trophozoites recovered from liver abscess (Fig. 1). As a control, we were also able to rescue pSA8 and pEhAct-Neo plasmid from transfected trophozoites initially grown in TYI-S-33 medium in the presence of 60 µg of G418 per ml and then cultivated for 2 weeks in TYI-S-33 medium in the absence of G418 (Fig. 1). Interestingly, lysates of pSA8-transfected amoebae that were recovered from the single abscess had 35% of the level of CP activity

TABLE 1. Hamster liver abscess formation by transfected trophozoites

Trophozoite source	No. of animals with abscess	Mean size of abscess (cm)
HM1:IMSS pEhAct-Neo	8/8	1–2
HM1:IMSS pSA8	1/8	<0.2
HM1:IMSS	8/8	>2

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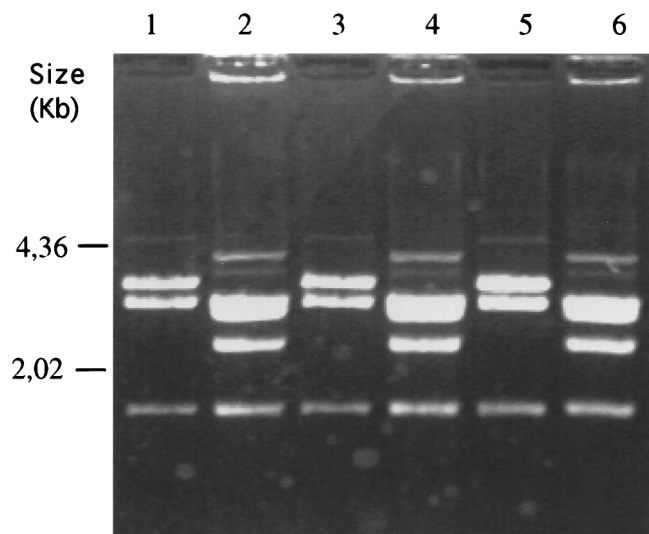


FIG. 1. Restriction analysis of plasmid rescued in *E. coli* XL1-blue from pEhAct-Neo- and pSA8-transfected trophozoites isolated from liver abscess. Plasmid preparations were digested by restriction enzymes (*Xho*I-*Sal*I) and size fractionated on agarose gels. Lanes: 1, plasmid pEhAct-Neo; 2, plasmid pSA8; 3, plasmid rescued from pEhAct-Neo transfectant isolated from liver abscess; 4, plasmid rescued from pSA8 transfectant isolated from liver abscess; 5, plasmid rescued from pEhAct-Neo transfectant cultivated in TYI-S-33 medium for 2 weeks in the absence of G418; and 6, plasmid rescued from pSA8 transfectant cultivated in TYI-S-33 medium for 2 weeks in the absence of G418.

found in pEhAct-Neo-transfected trophozoites. This level of CP activity is slightly higher than the level of CP activity (10%) found in pSA8-transfected trophozoites before their passage through the livers of hamsters as demonstrated in this study as well as in our previous study (2). This result could be due to selection in the liver of a subpopulation of pSA8-transfected trophozoites that have slightly higher CP activity. The selection of amoeba clones with higher levels of CP activity by hamster liver passages was previously reported by Navarro-Garcia et al. (10).

Our results suggest an important role for amoeba CP in liver abscess formation in hamsters. This conclusion is in agreement with the results observed upon inhibition of CP by the specific inhibitor E-64 and reduction of liver abscess formation in SCID mice (17). Our present results clearly indicate that the *in vitro* cytopathic assay is not sufficiently indicative of amoebic virulence. In our previous study with tissue-cultured monolayers, we demonstrated that in contrast to intact trophozoites, lysates of the pSA8-transfected trophozoites had very poor cytolytic activity. These findings indicate that while intact trophozoite CP are not a main factor for destruction of monolayers, CP most likely play an important role in the mammalian liver where amoebae must battle the immunological defenses of the host (16). Based on the differences that we find between the *in vitro* and *in vivo* determinations of transfectant virulence, the presence and release of CP by intact and lysed amoeba are important *in vivo* components involved in tissue damage in the host. Other elements involved in amoebic virulence are resistance to complement and phagocytosis activity. It has been shown that after long-term cultivation *in vitro* pathogenic isolates were susceptible to lysis by the alternative pathway of complement (6). No significant difference was found in the susceptibility of pSA8 transfectants and that of the pEhAct-Neo control or untransfected strain HM-1:IMSS to

complement. In all cases lysis was approximately $\pm 70\%$. Another aspect that may also be of importance is the impaired rate of erythrophagocytosis of the pSA8 transfectants (2), which could also contribute to its inability to induce liver abscesses. However, in view of the conflicting reports about the correlation between the phagocytic activity of the trophozoites and their ability to induce liver abscesses (11, 19), more studies are needed for conclusions about the involvement of phagocytic activity in the formation of liver abscesses.

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