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Specific Labeling of Cysteine Proteinases in Pathogenic and Nonpathogenic *Entamoeba histolytica*

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Growth of *Entamoeba histolytica* trophozoites was inhibited by 50% at low concentrations (2.0 μg/ml) of the diazopeptidyl inhibitor benzyloxycarbonyl-leucyl-L-tyrosyldiazomethane (Z-L-Leu-L-Tyr-CHN_2). Iodination of the tyrosine residue lowered the growth inhibitory efficacy of the diazopeptidyl inhibitor (50% inhibition, approximately 10 μg/ml). However, even at this concentration, practically all of the cysteine proteinase activity of the cells was irreversibly inactivated as shown by fluorescence microscopy with the dipeptide substrate L-Arg-L-Arg-4-methoxy-β-napthylamide or colorimetrically with azocasein as the substrate. Growth of trophozoites of *E. histolytica* from various strains, including both pathogenic and nonpathogenic zymodemes, was similarly inhibited. The concentration of inhibitor required to inactivate the proteinase activity of nonpathogenic cells was lower. Lysates from trophozoites grown in the presence of sublethal concentrations of ^{125}I-labeled protease inhibitor (10 μg/ml) showed as many as eight radioactive bands by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (molecular sizes, 73, 68, 56, 40, 39, 35, 29, and 27 kilodaltons). Two of these bands (molecular sizes, 29 and 27 kilodaltons) could be seen in gels of the cytoplasmic fraction, whereas the high-molecular-size bands were mostly associated with the membrane fraction. The radioactive bands in pathogenic and nonpathogenic strains were very similar with only minor differences. The results obtained show that *E. histolytica* cells, irrespective of their pathogenicity, possess a number of cysteine proteinases of similar molecular sizes which are vital for cell growth.

It is generally accepted that the histolytic activity of trophozoites of *Entamoeba histolytica* is related to the action of proteolytic enzymes (9, 11, 13–15, 21, 22, 25–27). This activity, however, has not yet been sufficiently well characterized. A correlation between invasive activity and the amount of cysteine proteinase present in the strains of *E. histolytica* exhibiting various degrees of virulence has been observed (8). Several previous reports have described the partial or complete purification of several cathepsin-like cysteine proteinases of *E. histolytica*, as well as their substrate specificities (11, 13, 14, 21, 22, 26).

Specific inhibitors which covalently bind to the active site of lytic enzymes have been shown in numerous cases to be excellent tools for the identification of the active sites, as well as for studying the metabolic role and cellular localization, of the proteinases (2, 17, 23). In this study, we used a specific diazopeptidyl inhibitor, Z-L-Leu-L-Tyr-CHN_2 (6), to detect active forms of thiol proteinases in trophozoites of *E. histolytica* or in cell lysates. This inhibitor is a member of a group of reagents, peptidyl diazomethyl ketones, shown to be specific for cysteine proteinases (6, 10, 12), with the peptide sequence providing the affinity required for targeting the active site. This inhibitor reacts covalently with the cysteine residue at the active site and does not react with inactive or denatured enzymes (2, 17). Iodination of the tyrosine residue of the inhibitor (Z-L-Leu-L-[^{125}I]-Tyr-CHN_2) also provided a very useful tool for in vivo labeling (17). Radiolabeled cysteine proteinase bands with high molecular sizes were found in the membrane fraction, whereas the low-molecular-size bands, especially the well-known and abundant 27- to 29-kilodalton thiol protease, as described previously (13), were located in the cytoplasmic fraction, presumably concentrated in cytoplasmic vacuoles. A parallel study comparing pathogenic strains of different virulence (9, 24) and a strain with nonpathogenic zymodeme allowed us to assert that a minimum of cysteine proteinase activity is present in all strains and that this is apparently vital for amebic growth. The relationship between the level of proteinase activity of *E. histolytica* strains and their virulence and pathogenicity (9) requires careful reevaluation.

MATERIALS AND METHODS

***E. histolytica* cultures.** *E. histolytica* SAW 1734R clAR, isolated from an asymptomatic carrier and possessing a nonpathogenic zymodeme, originally isolated by and obtained from P. G. Sargeaunt (London School of Hygiene and Tropical Medicine) was grown in TYI-S-33 culture medium (7) together with its original bacterial flora in the absence of antibiotics. Under these growth conditions, the amebae retained their nonpathogenic zymodeme (18) and were avirulent. All of the axenic strains of *E. histolytica* were grown in TYI-S-33 medium (7). The axenic strains studied were HM-1:IMSS c16 and Rahman cl1, both isolated from patients suffering from symptoms of amebiasis and possessing a pathogenic zymodeme. Strain Rahman, in spite of being pathogenic, is avirulent. Strain HM-1:IMSS is very virulent (4). Another axenic and virulent strain used for some experiments was SAW 1734R clAR, which originally was a

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nonpathogenic zymodeme that had converted to a pathogenic zymodeme during the process of axenization (18).

Trophozoites were cultured in 35-ml Falcon plastic flasks for 48 h in the presence or absence of different concentrations of diazopeptidyl inhibitors (see below). Growth and viability of the amebic trophozoites were monitored by removing samples and counting the viable cells which did not incorporate eosin (20). Trophozoites were harvested by chilling the flasks in ice-water for 5 min, followed by sedimentation (600 × *g*) and repeated washing (three times) with phosphate-buffered saline (pH 7.2; 380 osmol kg^{-1}). The pellet was suspended in water. The trophozoite suspension was freeze-thawed in a solid CO_2-acetone bath three times, and the cell lysate was sedimented in a Beckman centrifuge at 100,000 × *g* for 30 min at 4°C to separate the membrane and cytoplasmic fractions. Controls to detect the possible presence of proteins and radiolabeled components that can originate from the bacterial flora that accompanied the xenic cultures were as follows. A loopful containing bacterial flora was taken from the supernatant suspension (600 × *g*) of harvested trophozoites, inoculated in the same medium, and grown for another 48 h. Care was taken to check that no trophozoites were present in the culture. The bacterial cells were harvested by sedimentation (10,000 × *g*) for 15 min, washed three times with phosphate-buffered saline by sedimentation, suspended in water, and freeze-thawed, as described above.

Proteinase activity. (i) Colorimetric assay. Proteinase activity was determined with azocasein (Sigma Chemical Co., St. Louis, Mo.) as the chromophoric substrate. Typically, a freeze-thawed lysate (see above) prepared from 5×10^4 trophozoites (10 μl) was incubated in a 0.5% azocasein mixture (300 μl) containing 2 mM dithiothreitol and 1 mM EDTA at 37°C for 1 h. Proteolysis was stopped by the addition of 750 μl of 10% trichloroacetic acid. The sample was cooled at 4°C and was centrifuged at 3,000 × *g* for 10 min. The A_{336} of the supernatant solution was monitored by a spectrophotometer.

(ii) Fluorimetric assay. The dipeptide derivative L-arginyl-L-arginyl-4-methoxy-β-naphthylamide (Sigma Chemical Co.) was used as a proteinase substrate to determine protease activity in intact cells by a fluorescence microscope. Trophozoites (strain HM-1:IMSS; 10^6) were harvested and washed twice with phosphate-buffered saline and twice with a cacodylate buffer (100 mM; pH 6.8) containing 5% sucrose (CacoS). The trophozoites were suspended in 200 μl of the CacoS buffer, and simultaneously, 100 μl of the substrate mixture (5 mg/ml in CacoS buffer) and 200 μl of a coupling reagent (5-nitro-2-salicylaldehyde; 2 mM in CacoS buffer) were added. The mixture was incubated for 15 to 30 min at room temperature, centrifuged (600 × *g*), and washed (four times) with cacodylate buffer without sucrose. The trophozoites were observed with an Olympus BH-2 fluorescence microscope (excitation, 360 to 430 nm; emission, 550 to 600 nm).

The diazopeptidyl inhibitors used were benzyloxycarbonyl-leucyl-L-tyrosyldiazomethane (Z-L-Leu-L-Tyr-CHN_2) and benzyloxycarbonyl-leucyl-L-(iodo)-tyrosyldiazomethane (Z-L-Leu-L-[I]-Tyr-CHN_2) derivatives (6).

Growth inhibitory studies of the various *E. histolytica* strains with the two peptide inhibitors were done as previously described (20). Viable trophozoites which did not include eosin were counted under the microscope. The sublethal concentration was determined for each compound. The diazopeptidyl inhibitors at concentrations used appeared not be toxic to mammalian cells, as there was no observable damage to tissue-cultured baby hamster kidney cells. The effect of proteinase inhibitors on the rate of nucleic acid synthesis of the trophozoites was investigated by monitoring the incorporation of [^{3}H]thymidine. Trophozoites of strain HM-1:IMSS or of the nonpathogenic strain SAW 1734R clAR were grown in small vials (4 ml, in the presence or absence of the proteinase inhibitor Z-L-Leu-L-(I)-Tyr-CHN_2 [10 μg/ml]) together with [^{3}H]thymidine (50 μCi; specific activity, 80 Ci/mM; Dupont, NEN Research Products, Boston, Mass.) as described previously (5). Samples (100 μl) of cells in culture were removed every 6 to 12 h, and the level of radioactive incorporation into 10% trichloroacetic acid-precipitable material was followed for 48 h by counting the samples in a scintillation counter.

^{125}I labeling of protease inhibitor. Z-L-Leu-L-Tyr-CHN_2 was iodinated by the Iodogen method (Pierce Chemical Co., Rockford, Ill.) (16, 17). Solutions of sodium phosphate buffer (10 μl; 50 mM; pH 7.5), Na^{125}I (10 μl; 1 mCi), and Z-L-Leu-L-Tyr-CHN_2 (25 μl; 1 mM in 25% ethanol) were added to an Iodogen-coated glass tube and incubated at 0°C for 10 min. Sodium phosphate buffer (455 μl of 50 mM; pH 7.5) was added, and the reaction was stopped by removing the mixture from the Iodogen-coated tube. The concentration of active inhibitor in the solution was determined by titration with a preparation of papain that had previously been titrated and standardized with the unlabeled inhibitor Z-L-Leu-L-(I)-Tyr-CHN_2 and with commercially available azocasein (Sigma Chemical Co.) as a substrate. The specific activity of the ^{125}I-labeled inhibitor was determined by titration of papain activity. The radioactivity was determined in the trichloroacetic acid-precipitable material after the addition of sufficient labeled inhibitor to reduce the activity of a known papain-containing solution by 80%. The specific activity obtained for the inhibitor was 4 mCi/μmol.

In vivo labeling of cysteine proteinase. Experiments in which the cysteine proteinases were inactivated and radiolabeled during in vivo growth were as follows. Radiolabeled Z-L-Leu-L-(^{125}I)-Tyr-CHN_2 (10 μCi), diluted with unlabeled Z-L-Leu-L-(I)-Tyr-CHN_2 to give a final concentration of 10 μg/ml, was added to culture flasks of freshly inoculated trophozoites of *E. histolytica* from pathogenic or nonpathogenic strains (10^5 cells/ml), and incubation at 37°C continued for 24 or 48 h. Control cultures of the bacterial cells that were isolated from the nonpathogenic strain SAW 1734R clAR were incubated with radiolabeled inhibitor as described above.

Gel electrophoresis. Slab gels (sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) were prepared basically as described previously (3). Samples containing approximately 200 μg of protein were run, and protein bands were detected with Coomassie brilliant blue. A standard protein mixture (Sigma Chemical Co.) served as the molecular size marker. Autoradiographs were prepared after exposure of the dried gels to X-ray film (Agfa-Gevaert, Curix PR-2) for 3 days with two intensifying screens at −70°C.

RESULTS

Growth of cultures of *E. histolytica* trophozoites (strain HM-1:IMSS, Rahman, or SAW 1734R clAR) in the presence of the diazopeptidyl inhibitors Z-L-Leu-L-Tyr-CHN_2 (at 2 μg/ml) and Z-L-Leu-L-(I)-Tyr-CHN_2 (at 10 μg/ml) was inhibited by approximately 50%. The iodinated derivative was an approximately fivefold less-efficient inhibitor; however,

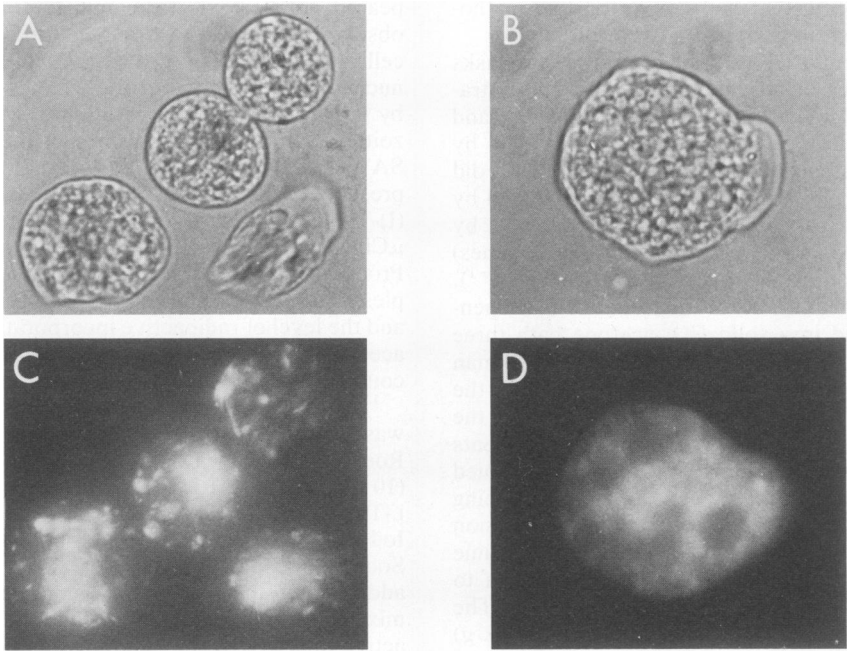

FIG. 1. Light and fluorescence microscopy of trophozoites from *E. histolytica* HM-1:IMSS grown for 24 h in the presence or absence of the cysteine proteinase inhibitor Z-L-Leu-L-(I)-Tyr-CHN_2 (10 μg/ml). The fluorescent substrate for the cysteine proteinases was L-Arg-L-Arg-4-Methoxy-β-napththylamide. Growth of trophozoites in the absence (A) or presence (B) of inhibitor; fluorescence microscopy of cells in the absence (C) or presence (D) of inhibitor.

even at concentrations of 10 μg/ml, practically all of the cysteine proteinase activity of the trophozoites was inactivated (>90%). This was shown either by fluorescence microscopy (Fig. 1) with the dipeptide substrate L-Arg-L-Arg-4-methoxy-β-naphthylamide or by determining the remaining activity with azocasein (Fig. 2). Very significant proteinase activity was observed in the control trophozoites, whereas cells grown in the presence of sublethal concentrations (10 μg/ml) of the iodinated inhibitor, even after prolonged incubation with the substrate, showed practically no fluorescence, even though the cell morphology appeared quite normal. A significant difference in the levels of inhibition of cysteine proteinase activity was found at 2 μg/ml between the pathogenic strain (HM-1:IMSS) and the less-pathogenic strains (SAW 1734R clAR and Rahman) (Fig. 2). Only a slight inhibition (<10%) in the proteinase activity was detected in strain HM-1:IMSS, whereas in the less-pathogenic strains, the inhibition was approximately 50%. At 10 μg/ml, the proteinase activity of all strains was inhibited to approximately the same extent. At this concentration of iodinated inhibitor, the rate of nucleic acid synthesis, as determined by following the [^{3}H]thymidine incorporation into trophozoite cultures for 48 h (5) was almost unaffected (<10%; data not shown). At higher concentrations of the inhibitor Z-L-Leu-L-(I)-Tyr-CHN_2 (50 μg/ml), cell morphology, especially of the axenically grown cells (strains HM-1:IMSS and Rahman), was dramatically changed, and these cells appeared very small with little motility. Most of the cells died within 24 h, as determined by eosin incorporation.

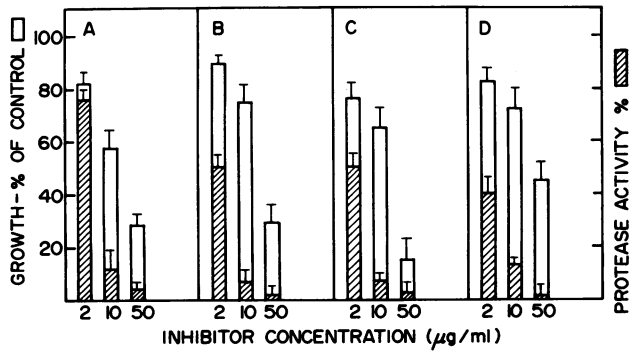

FIG. 2. Effects of different concentrations of the cysteine proteinase inhibitor Z-L-Leu-L-(I)-Tyr-CHN_2 on the growth and proteinase activity of the harvested trophozoites. Trophozoites of strains HM-1:IMSS (A), Rahman (B), SAW 1734R clAR (pathogenic zymodeme, axenic culture) (C), and SAW 1734R clAR (nonpathogenic zymodeme, xenic culture) (D) were incubated for 48 h in the presence of three different concentrations of inhibitor. After 48 h, cells were harvested and counted in triplicate. Bars indicate standard deviation of the mean. The total protease activity present in the lysates, in comparison with that of controls grown in the absence of inhibitor, was determined in duplicates after digestion of azocasein, as described in Materials and Methods.

SDS-PAGE of membrane and cytoplasmic fractions of trophozoites from the pathogenic strains HM-1:IMSS (virulent) and Rahman (avirulent) and the nonpathogenic strain SAW 1734R clAR (avirulent) (4, 9, 18) grown in the presence (24 h) of the proteinase-radiolabeled inhibitor Z-L-Leu-L-(^{125}I)-Tyr-CHN_2 (10 μg/ml) were compared (Fig. 3). Autoradiograms of gels clearly show three areas of radiolabeled bands, three of them at molecular sizes of 73, 68, and 56 kDa, others at molecular sizes of 40, 39, and 35 kDa, and the smaller molecular species at molecular sizes of 29 and 27 kDa. The soluble cytoplasmic proteins remaining after sedimentation by high-speed centrifugation contained only the lower-molecular-size bands (molecular sizes of 27 and 29

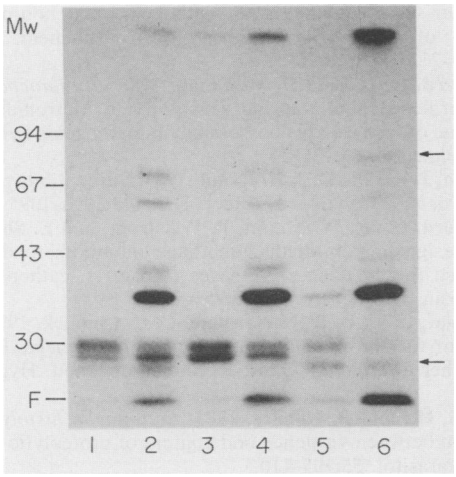

FIG. 3. Autoradiograms by SDS-PAGE of three *E. histolytica* strains grown in the presence of the radiolabeled inhibitor Z-L-Leu-L-[^{125}I]-Tyr-CHN$_2$ for 24 h. Lanes: 1 and 2, strain Rahman (pathogenic zymodeme, avirulent in several assays) (4) cytoplasmic fraction and membrane fraction, respectively; 3 and 4 strain HM-1:IMSS (pathogenic zymodeme, virulent) cytoplasmic fraction and membrane fraction, respectively; 5 and 6 SAW 1734R clAR (xenic culture, nonpathogenic zymodeme) (4) cytoplasmic fraction and membrane fraction, respectively. The SDS-PAGE was run in 10% acrylamide. Controls which contained only the bacterial flora exposed to the radiolabeled inhibitor did not show any bands. For Coomasie brilliant blue staining, see Fig. 4a. Arrows indicate extra and missing bands in the nonpathogenic SAW 1734R clAR strain (4). Molecular sizes are indicated on the left. F, Front.

kDa), whereas the sedimented membrane fractions contained the higher- and middle-molecular-size radiolabeled bands. The similarity between the radiolabeled bands seen in the strains with pathogenic and nonpathogenic zymodemes was striking. Slight differences were observed in a high-molecular-size band present in nonpathogenic SAW 1734R clAR and not in the others, and a small-molecular-size band was missing in the same strain. Controls containing lysates of the isolated bacterial associates obtained from the xenic cultures of the nonpathogenic strain did not show any radioactive bands (data not shown).

Coomassie brilliant blue staining of SDS-PAGE revealed a few band differences between the pathogenic and nonpathogenic strains, as well as a few protein pattern differences between lysates of trophozoites grown in the presence or absence of the inhibitor, mainly in the 60- to 80-kDa region (Fig. 4).

DISCUSSION

A number of diazopeptidyl inhibitors which specifically and irreversibly bind to the active site of cysteine proteinases at very low concentrations have been synthesized and described (6, 10, 12). Growth of *E. histolytica* strains of both pathogenic and nonpathogenic zymodemes in the presence of low concentrations of the peptide inhibitor Z-L-Leu-L-Tyr-CHN$_2$ (5 μg/ml) was lethal to the cells. Iodination of the tyrosine residue reduced the inhibitory efficacy of the peptide, probably because of the introduction of a bulky molecule making it more difficult for the peptide to penetrate into the active site of the enzyme. Sublethal concentrations (10 μg/ml) of the iodinated substrate Z-L-Leu-L-(I)-Tyr-CHN$_2$ could be attained in which the remaining cysteine proteinase activity of the trophozoites was very low (<10%). Under these conditions, no significant morphological alterations could be observed, other than a slowdown in trophozoite motility and vacuole migration with very little effect on the rate of nucleic acid synthesis. SDS-PAGE of trophozoite lysates grown in the presence of the inhibitor revealed a number of additional protein bands at the 60- to 80-kDa range, suggesting that these are proteins which were not modified because of the absence of cysteine proteinase activity (23). The function of these proteins is not yet known. Metabolic labeling of trophozoites from various *E. histolytica* strains of different pathogenicity with the Z-L-Leu-L-([^{125}I]Tyr-CHN$_2$ cysteine proteinase inhibitor revealed a number of radiolabeled protein bands with molecular sizes from 27 to 73 kDa. The higher-molecular-size forms were

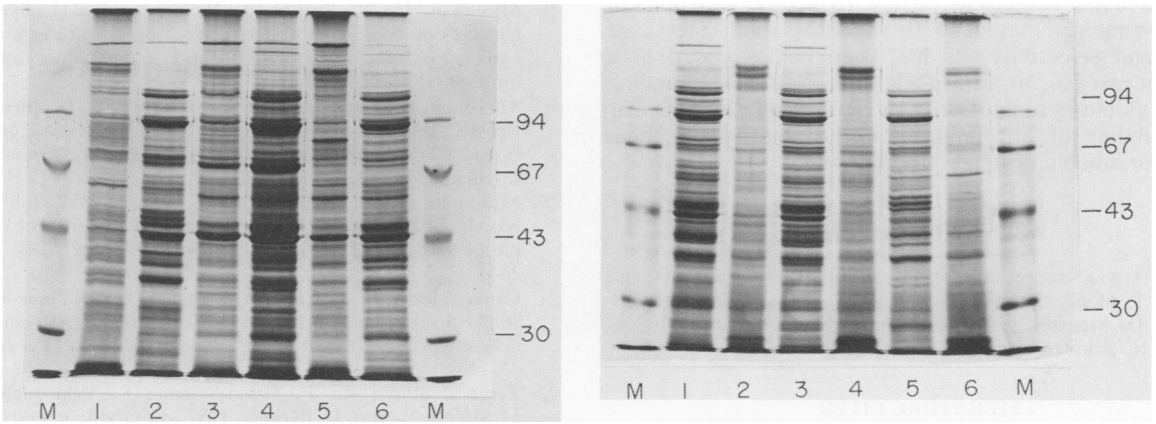

FIG. 4. (Left) Coomassie brilliant blue staining by SDS-PAGE of three *E. histolytica* strains grown in the presence of the proteinase inhibitor Z-L-Leu-L-[^{125}I]-Tyr-CHN$_2$ at 10 μg/ml for 24 h. M, Molecular size markers. Strain SAW 1734R clAR (xenic culture, nonpathogenic zymodeme) membrane (lane 1) and cytoplasmic fraction (lane 2); Strain HM-1:IMSS membrane (lane 3) and cytoplasmic fraction (lane 4); strain Rahman membrane (lane 5) and cytoplasmic fraction (lane 6). (Right) Coomassie brilliant blue staining by SDS-PAGE of the same three *E. histolytica* strains grown in the absence of proteinase inhibitor. M, Molecular size markers. Strain Rahman cytoplasmic fraction (lane 1) and membrane fraction (lane 2); strain HM-1:IMSS cytoplasmic fraction (lane 3) and membrane fraction (lane 4); strain SAW 1734R clAR (xenic) cytoplasmic fraction (lane 5) and membrane fraction (lane 6).

present almost exclusively in the sedimentable membrane fraction, whereas the soluble cytoplasmic fraction contained the lower-molecular-size species of which the 29- to 27-kDa band was one of the most abundant. Several molecular forms of an active cysteine proteinase of *E. histolytica* have been reported (11, 13, 21, 22, 26). A 27- to 29-kDa cysteine proteinase was recently purified from a pathogenic strain of *E. histolytica* (200:NIH), and the sequence of the N-terminal region was identified (13). Zymograms have been used to demonstrate the presence of protease activity in a number of protein bands with molecular sizes of 66, 56, 40, and 27 kDa (21, 22). The evidence, which indicated that the 66-kDa molecule was a thiol protease, was, however, not conclusive. Surprisingly, the higher-molecular-size bands were reported to be located in the cytoplasmic fraction, whereas the lower molecular-size ones were in the membrane (21). It is possible that the isolation of the trophozoite membrane and cytoplasmic fractions, which was done by the concanavalin A procedure (1), may have caused aggregation or cosedimentation artifacts which could not be resolved in the zymograms.

The novel radiolabeled diazodipeptide inhibitor has helped us to detect several components with cysteine proteinase activities in the membrane and cytosol fractions of *E. histolytica* cells. Irreversible inhibition of these activities in vivo leads to cell death. Trophozoites could survive, however, if levels of proteinase activity were at least 10% of those present in noninhibited cells. Contrary to what was previously assumed (8), strains of *E. histolytica* with pathogenic and nonpathogenic zymodemes (9, 18, 24) seem to have similar cysteine proteinase components although they apparently differ in their absolute amounts in the cell. Strains with a pathogenic zymodeme, which are known to be more virulent, had more proteinase activity than did the less-virulent strain or the strain with nonpathogenic zymodeme, as evidenced by the higher concentration of diazopeptidyl inhibitor required in order to obtain 50% inhibition of activity. The slight difference in the radioactively labeled cysteine proteinase band patterns of the nonpathogenic zymodeme deserves a more detailed study.

The diazomethane dipeptide inhibitors may not be useful as novel antiamebic drugs because of possible interactions with mammalian cathepsins (2, 17). However, variations in peptide sequence may lead to inhibitors with increased activity and selectivity, as has been demonstrated in a number of cases (6, 10, 12). In addition, the covalent binding of these inhibitors to inert drug carriers, in a fashion similar to the binding of nitroimidazoles (19), may make them specific for amebic cysteine proteinases.

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