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# Changes in Isoenzyme Patterns of a Cloned Culture of Nonpathogenic *Entamoeba histolytica* during Axenization

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**The axenization of an *Entamoeba histolytica* isolate with a nonpathogenic isoenzyme electrophoretic pattern (zymodeme) was recently achieved for the first time (15). Forty days after the cells were transferred to the medium used for axenic cultivation, the amebae developed virulence properties, and the zymodeme converted to a pathogenic pattern. To exclude the possibility that the original isolate consisted of two zymodeme populations and that conditions of growth selected for a particular population, the experiment was repeated with a cloned culture of a nonpathogenic (zymodeme III) strain, *E. histolytica* SAW 1734R clAR, isolated by and obtained from P. G. Sargeaunt. Axenization was accomplished, as before, by transferring trophozoites to TYI-S-33 medium containing a mixture of antibiotics to suppress the growth of the associated bacterial flora and a nutritional supplement consisting of γ-irradiated bacteria. A change in the hexokinase and phosphoglucomutase isoenzyme pattern was observed 21 days after the amebae had been transferred to the axenic medium but before complete axenization of the amebae had occurred. The change in zymodeme was accompanied by an increase in virulence, as evidenced by the ability of fewer amebae to induce hepatic abscesses in hamsters. A reverse conversion to a nonpathogenic zymodeme was also accomplished by reassociating and subculturing the newly converted pathogenic trophozoites of strain SAW 1734R clAR with the bacterial flora that accompanied this ameba in the original xenic culture. The electromobilities of the hexokinase isoenzymes changed back to their original pattern 7 days after the amebae were returned to xenic growth conditions. Our in vitro results demonstrate that culture conditions and bacterial flora can cause changes in the zymodeme and virulence of a cloned ameba isolate and raise the concern that this could happen also in vivo. Thus, the finding of a particular zymodeme in a culture of *E. histolytica* isolated from a carrier should not be used to predict a clinical condition or serve as a basis for the recommendation of therapy.**

Studies of isoenzymes of *Entamoeba histolytica* trophozoites isolated from thousands of asymptomatic and symptomatic human cases around the world have resulted in the characterization of at least 20 distinct zymodemes (21–24, 26, P. G. Sargeaunt, Letter, J. R. Soc. Med. 75:920, 1982). Based on the clinical picture of the source of the isolate, these zymodemes are placed in one of two categories: pathogenic or nonpathogenic (9). The nonpathogenic zymodemes, which are the majority of cases, are in isolates from asymptomatic carriers with negative serology, whereas the pathogenic zymodemes are derived from persons with positive serology and presence or history of some clinical symptoms. The finding of a nonpathogenic zymodeme in conjunction with a pathogenic one in any single host has never been reported (21, 25). Moreover, no alteration of isoenzyme patterns, i.e., shift from nonpathogenic to pathogenic or vice versa, was ever demonstrated in any of the longitudinal culture studies (21, 25) that were conducted in the presence of viable bacteria in Robinson medium (19). Based on these studies, it has been suggested that zymodemes can be used as biochemical markers to distinguish between the two different *E. histolytica* subspecies, and that persons harboring amoebae with nonpathogenic zymodemes may not require treatment (9, 24).

In contrast to pathogenic isolates, all attempts to cultivate nonpathogenic *E. histolytica* under axenic conditions failed. This feat has recently been accomplished (15); during the process of axenizing such an isolate, *E. histolytica* CDC:0784:4, a change in the pattern of the isoenzymes from nonpathogenic to pathogenic was observed. This occurred approximately 35 days after the amebae were transferred to a medium for axenic cultivation, but before complete axenization had actually occurred. The change in zymodeme was accompanied by an enhancement in virulence as evidenced by the ability of the amebae to induce the formation of hepatic abscesses in hamsters and to destroy monolayers of tissue culture cells (3, 15).

Two explanations were offered for the observed changes in zymodeme and virulence; the change could be explained by an alteration within the amebae. Alternatively, if the original amebal isolate consisted of a mixed population of zymodemes, the conditions of growth could select for one or the other of the population. To exclude the latter possibility, the experiment was repeated with a cloned culture of a nonpathogenic strain isolated by P. G. Sargeaunt from an asymptomatic carrier in Israel. Our present findings clearly show that isoenzyme patterns are not a stable and inherent property of the ameba (16) and that alterations in culture conditions and bacterial flora can cause changes in isoenzyme electromobilities and virulence properties. Moreover, our results force us to question the advisability of using isoenzyme patterns to predict a clinical condition or as the basis for the recommendation of therapy (9).

## MATERIALS AND METHODS

*E. histolytica* SAW 1734R clAR was isolated in March 1985 by P. G. Sargeaunt from an asymptomatic carrier in Israel and cultured in Robinson (19) growth medium. It was

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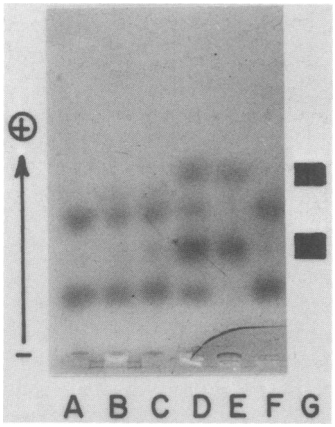


FIG. 1. Electrophoretic pattern of hexokinase isoenzymes from *E. histolytica* SAW 1734R clAR. Trophozoite samples were harvested from each growth condition, and lysates were prepared and applied to agarose minigels (14, 15). (A) Nonpathogenic migration pattern of *E. histolytica* SAW 1734R clAR cultured together with their accompanying bacterial flora. (B) Nonpathogenic pattern of SAW 1734R clAR 17 days after transfer to a medium for axenic growth (TYI-S-33) supplemented with γ-irradiated bacteria (15). (C) Nonpathogenic pattern of cultures 18 days after transfer to TYI-S-33 medium. Notice the beginning of a pathogenic pattern too. (D) Trophozoite sample taken 20 days after transfer to TYI-S-33 medium. Notice that this culture contains already a mixture of nonpathogenic and pathogenic pattern of isoenzyme. (E) Pathogenic pattern of trophozoite sample of *E. histolytica* SAW 1734R clAR 21 days after transfer to TYI-S-33 medium. (F) Nonpathogenic pattern of hexokinase from *E. histolytica* CDC:0784:4 (group I) used as a nonpathogenic marker. (G) Pathogenic pattern from *E. histolytica* HM-1:IMSS used as a pathogenic marker. Samples containing lysates of bacterial cells without amebae did not show any hexokinase bands in this region.

identified as belonging to the nonpathogenic zymodeme group III. Cloned cultures of amebae were isolated by P. G. Sargeaunt of the London School of Hygiene and Tropical Medicine in June 1985 by diluting a 48-h culture growth sufficiently so that one drop delivered onto a piece of microscope slide by a capillary pipette and examined under a microscope using a X10 objective contained a single ameba (21). Only after agreement by two independent workers that not more than one ameba was present in the drop was the glass slide dropped into new culture medium. The presence of the nonpathogenic zymodeme III in the clone was confirmed by electrophoresis. This cloned strain was given to us by P. G. Sargeaunt in January 1986.

Three pathogenic strains served as controls for pathogenic and virulence properties: the two axenically grown strains *E. histolytica* HM-1:IMSS clone 6 (10) and 200:NIH, as well as the xenic strain *E. histolytica* SAW 408, which was isolated from a symptomatic patient (18) and was never axenized. This last strain was also obtained from P. G. Sargeaunt.

**Cultivation of *E. histolytica*.** Strains HM-1:IMSS and 200:NIH were grown axenically in TYI-S-33 medium (8). Strain SAW 1734R clAR was transferred from Robinson medium to glass tubes containing a modified Jones (13) monophasic medium (11 ml) composed of phosphate buffer (66 mM, pH 7.0), NaCl (0.8%), yeast extract (BBL Microbiology Systems, Cockeysville, Md., 0.66%), rice starch (BDH, Poole, England; 2 mg/ml), ox serum (33%), and erythromycin (50 μg/ml). Trophozoites ($10^6$) grown together with their accompanying bacterial flora were harvested from a number of tubes by sedimentation (600 × *g*) and transferred to a tube with TYI-S-33 medium containing 15% bovine serum with an antibiotic mixture composed of erythromycin (Sigma Chemical Co., St. Louis, Mo.), amikacin (Bristol Myers, Syracuse, N.Y.) and cefotaxime (Hoechst, Frankfurt, Federal Republic of Germany) at final concentrations of 50 μg/ml as well as an antimycotic fungizone solution II (50 μl; BioLabs, Jerusalem, Israel). The culture medium was supplemented with a suspension of freshly sterilized (γ irradiated from a cobalt source with 500 kilorads) bacterial flora ($2 \times 10^8$ bacteria per ml) which was associated with strain SAW 1734R clAR and was grown separately in the modified Jones medium.

Every 48 h the culture medium was carefully decanted, the trophozoites which adhered to the glass were retained, and the tube was replenished with fresh medium and additives as described above. After the third refeeding, the cells multiplied well and were transferred to plastic flasks containing 35 ml of the above medium. After the 10th subculture the addition of γ-irradiated bacteria at each subculture was no longer required for growth. Antibiotic mixtures were still added, however, since in their absence background bacterial growth could be occasionally detected.

The return to xenic growth of amebae which had grown in TYI-S-33 medium in the absence of bacteria for over 30 days was as follows. Bacterial flora which had grown together with *E. histolytica* SAW 1734R clAR in Diamond TYSGM-9 medium (6) was separated from the trophozoites by chilling the tubes and repeated differential centrifugation (800 × *g*, 10 min). Most of the bacterial cells did not sediment under these conditions. A 2-ml sample of the supernatant bacterial suspension was inoculated into fresh TYSGM-9 medium (8 ml) in glass tubes. Three control tubes in which only bacterial flora was inoculated were incubated in parallel. No trophozoites were ever detected in such tubes. Examination of the bacterial species present (done at the Sheba Medical Center, Ramat Gan) revealed only two organisms, *Escherichia coli* and *Pseudomonas aeruginosa*. Trophozoites ($10^6$) of strain SAW 1734R clAR which had grown in TYI-S-33 medium for axenic growth were harvested by centrifugation and added to the TYSGM-9 medium containing the bacteria and erythromycin (50 μg/ml). Every 48 h the tubes were chilled, the trophozoites were sedimented by centrifugation (600 × *g*), and the supernatant was decanted, leaving behind approximately 1 ml of the old medium. The sedimented amebae were suspended, fresh TYSGM-9 medium (10 ml) was introduced, and the tubes were reincubated. After the second feeding, the amebae multiplied well, and subcultures were repeated every 48 h.

Another source of bacterial flora used for returning axenically grown trophozoites to xenic growth conditions was the flora that accompanied a pathogenic strain, *E. histolytica* SAW 408 (17, 18). This strain and its flora were maintained and grown also in TYSGM-9 medium. The bacterial flora accompanying this strain was examined by D. G. Owen (17) and found to contain the following organisms: *E. coli*, *Streptococcus faecalis*, *Flavobacter* sp., and *Clostridium* sp.

**Virulence determinations.** Hepatic abscesses were induced in 2-month-old male Syrian golden hamsters, after laparatomy, by direct inoculation of trophozoites into the right lobe of the liver. Six days later the animals were sacrificed, and their livers were inspected for abscesses. Trophozoites were isolated aseptically from the abscess and grown axenically in TYI-S-33 medium. Trophozoite virulence was also determined by the rate of destruction of tissue

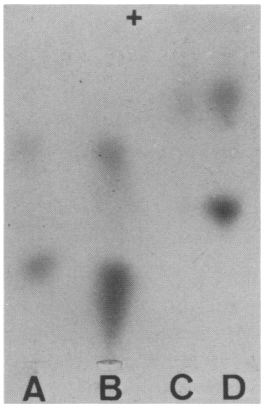


FIG. 2. Electrophoretic pattern of phosphoglucomutase isoenzymes from *E. histolytica* strain SAW 1734R clAR. Trophozoite samples were harvested from each growth condition, and lysates were prepared and applied to agarose minigels (14, 15). (A) *E. histolytica* SAW1734R clAR grown in a medium for axenic cultivation for 25 days; (B) *E. histolytica* 200:NIH grown axenically and used as a pathogenic marker; (C) lysate containing only the bacterial flora that grew in the xenic cultures of *E. histolytica* SAW 1734R clAR; (D) *E. histolytica* SAW 1734R clAR original culture grown in TYSGM-9 medium together with its associated bacterial flora.

cultured baby hamster kidney cell monolayers in quantitative assays as previously described in detail (3).

For isoenzyme electrophoresis, water lysates of trophozoites grown in and harvested from the various cultures were prepared by methods described previously (22, 23). Lysis was accomplished by freezing and thawing, and the soluble material was stored in liquid nitrogen (−196°C). The lysates were subjected to agarose gel electrophoresis as previously described for DNA minigels (14), after which the enzymes were revealed by formazan development. The isoenzymes identified were phosphoglucomutase (PGM; EC 2.7.5.1) and hexokinase (HK; EC 2.7.1.1).

The mobility of hexokinase was monitored routinely for all cultures, since this enzyme was reported previously to be the best marker for pathogenicity (9).

## RESULTS

A cloned culture of *E. histolytica* SAW 1734R clAR originally isolated from an asymptomatic carrier and possessing zymodeme group III was cultured together with its associated bacterial flora in modified Jones medium as well as in TYSGM-9. Examination of the hexokinase isoenzyme pattern of the trophozoites grown in both of these xenic media showed that they were nonpathogenic (Fig. 1, lane A). Trophozoites were transferred to and maintained in TYI-S-33 medium together with antibiotics and γ-irradiated bacterial cells. Under these new conditions the amebae gradually increased their division rate, and 20 days later the supplement of irradiated bacteria was no longer required. Examination of the hexokinase isoenzyme pattern of samples of trophozoites harvested 17, 18, 20, and 21 days after their transfer to TYI-S-33 showed that up to day 20 they had slow-migrating bands, indicating that they were nonpathogenic (Fig. 1, lanes B and C). On day 20 a mixture of slow- and fast-migrating bands appeared in the culture (Fig. 1, lane D), and on day 21 only the fast-migrating bands could be seen, indicating that the culture now had a pathogenic zymodeme (Fig. 1, lane E). At this stage the cultures were not yet axenic; the presence of viable bacteria from the associated bacterial flora could still be detected through the use of sterility test media (7). Controls containing only lysates of the bacterial flora did not show any isoenzyme bands in the same region. Further examination of the migration of the hexokinase isoenzymes from trophozoite cultures which were grown for over 30 days in the medium for axenic growth showed no further changes, and the cultures possessed only pathogenic isoenzyme patterns. Examination of the electromobility of the phosphoglucomutase isoenzyme showed that on day 21 a change in pattern had also occurred (Fig. 2). Lysates containing only the bacterial flora showed bands that could be easily distinguished from those belonging to the trophozoite.

The shift in the isoenzyme patterns was accompanied by a distinct change in the morphology of the amebae. Trophozoites grown in xenic culture are characterized by having a relatively smooth endoplasm with only a few large vesicles. Most trophozoites also ingested rice particles (Fig. 3A). In contrast, trophozoites cultivated in axenic medium are characterized by the presence of multiple small vesicles (Fig. 3B). In the process of axenizing strain SAW 1734R clAR, trophozoites with an endoplasm characteristic of axenically grown organisms made their first appearance in the cultures coincident with the ability of the amebae to multiply in the absence of the irradiated bacteria (day 21).

Trophozoites of strain SAW 1734R clAR grown with their associated bacterial flora in TYSGM-9 medium produced only small liver abscesses (<2 mm) in two out of five hamsters when injected at a concentration of $10^6$ organisms per liver. On the other hand, trophozoites that were harvested on day 25, after their transfer to TYI-S-33, and

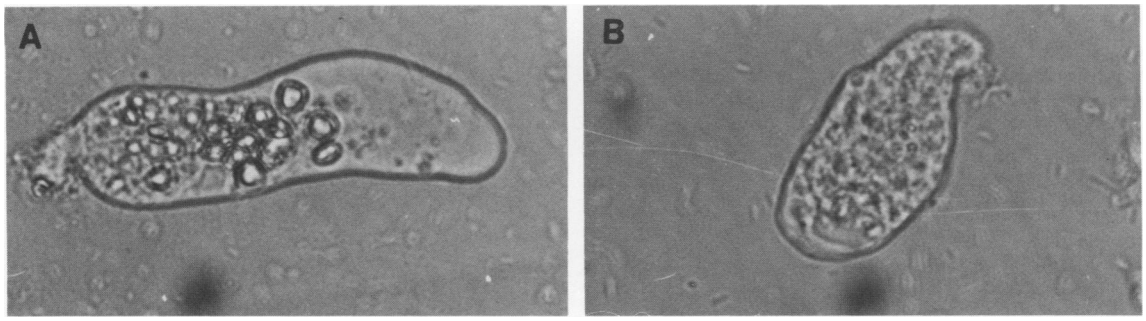


FIG. 3. (A) Trophozoite of *E. histolytica* SAW 1734R clAR (×400) grown in TYSGM-9 medium together with its accompanying bacterial flora and rice particles. (B) Trophozoites of *E. histolytica* SAW 1734R clAR (×400) cultured in a medium for axenic growth (TYI-S-33) for 21 days. See the text.

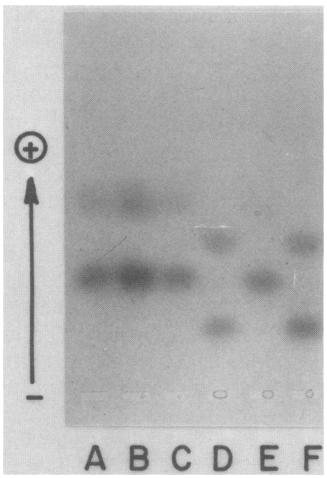


FIG. 4. Electrophoretic pattern of hexokinase isoenzymes from *E. histolytica* SAW 1734R clAR. Trophozoite samples were harvested from each growth condition, and lysates were prepared and applied to agarose minigels (15). (A) Pathogenic pattern of trophozoite sample of *E. histolytica* HM-1:IMSS used as a pathogenic marker. (B) Pathogenic pattern of *E. histolytica* SAW 1734R clAR 30 days after transfer to a medium for axenic growth (TYI-S-33) (see Fig. 1). (C) Pathogenic pattern of trophozoites of *E. histolytica* SAW 1734R clAR (as in B) after culturing for 2 days in TYSGM-9 medium, together with the bacterial flora that accompanied this strain in its original culture. (D) Nonpathogenic pattern of trophozoites of *E. histolytica* 1734R clAR (as in B) after culturing for 7 days in TYSGM-9 medium together with the bacterial flora that accompanied it in its original culture. (E) Pathogenic pattern of trophozoites (as in B) after culturing for 7 days in TYSGM-9 medium together with the bacterial flora that accompanied the pathogenic strain *E. histolytica* SAW 408. (F) Marker of nonpathogenic pattern of original culture of *E. histolytica* 1734R clAR grown in TYSGM-9 medium together with its accompanying bacterial flora.

already possessed a pathogenic zymodeme produced hepatic abscesses (>5 mm) in all five animals at $2 \times 10^5$ organisms per animal. Trophozoites were successfully subcultured from these abscesses, and examination of their isoenzymes indicated that they were pathogenic. Furthermore, trophozoites of strain SAW 1734R clAR grown with their associated bacterial flora caused hardly any damage (about 10% destruction) of baby hamster kidney cell monolayers at $10^6$ ameba per well (3), whereas trophozoites harvested on day 40 after their transfer to TYI-S-33 medium caused 50% destruction of monolayers at $2 \times 10^5$ amebae per well, similar to that observed for the axenically grown strain HM-1:IMSS (3) (data not shown).

Trophozoites of strain SAW 1734R clAR that had grown in the medium for axenic cultivation, TYI-S-33, for 30 days and had a pathogenic zymodeme were harvested and introduced into two tubes containing TYSGM-9 medium together with two types of bacterial flora, one obtained from cultures of *E. histolytica* SAW 1734R clAR growing in TYSGM-9 and the other from *E. histolytica* SAW 408 (17, 18) grown in the same medium. The culture medium was replaced every 48 h, after the third time trophozoites appeared to multiply and change their morphology to that usually seen in xenic cultures. Examination of their hexokinase electromigration 7 days after their reassociation with bacteria in TYSGM-9 medium showed that trophozoites grown with the bacterial flora that accompanied strain SAW 1734R clAR had reverted to a nonpathogenic zymodeme, whereas those grown with the bacterial flora that was associated with the pathogenic strain SAW 408 remained pathogenic (Fig. 4). Further cultivation for 14 days under these conditions did not cause any further change in the isoenzyme mobility.

## DISCUSSION

Numerous methods have been developed over the years for the in vitro culture of *E. histolytica* trophozoites (7). During the early work continuous propagation was impossible in the absence of living bacteria, but only certain species of bacteria could support multiplication (4, 12). The development by Diamond (5) of an axenic medium in which amebae could grow and divide in the absence of any other living organism or cell has provided the basis by which studies of various aspects of the ameba-bacteria-host interrelation can be undertaken. Even so, not all amebae could be grown under axenic conditions of growth, and before our recent success (15) there were no reports on the axenization of *E. histolytica* having a nonpathogenic zymodeme. In attempting to axenize such an isolate, we used sterile $\gamma$-irradiated bacterial flora as a supplement for growth in TYI-S-33 medium. After the 15th transfer, the amebae became capable of multiplying in the absence of added bacteria. At the same time, a gradual change in the virulent properties of the trophozoites present in the culture was observed. Interestingly, this was also accompanied by a shift in the isoenzyme pattern from nonpathogenic to pathogenic (15).

We considered two explanations for the observed changes in zymodeme and virulence. The first one was that the isolate we had consisted of two zymodeme populations and that conditions of growth selected for one or the other of the populations. Although this possibility was remote, especially since neither Sargeaunt et al. (21–24, 26) nor others (11) who studied the isoenzymes of numerous amebal isolates had ever reported the finding of a nonpathogenic zymodeme in conjunction with a pathogenic one in any single patient, we could not completely disregard it. To exclude such a possibility the experiment was repeated with a cloned culture of a nonpathogenic zymodeme isolated by P. G. Sargeaunt; the results reported herein prove that a second explanation was correct, i.e., that zymodemes are not a stable biochemical marker of the ameba. In a previous report (16) we had shown that short-term interaction of axenically grown amebae with bacteria (for 1 h), which markedly enhanced their virulence (3), did not cause any change in their pathogenic zymodeme. Our present results demonstrate that shifts in zymodeme can occur not only from the nonpathogenic to the pathogenic zymodeme but also from the pathogenic to the nonpathogenic zymodeme. This latter shift seems to be dependent, however, on the nature of the bacterial flora with which the axenically grown pathogenic amebae become reassociated in culture. In the case studied here, where the bacteria (*E. coli* and *P. aeruginosa*) were those which were associated with an isolate possessing a nonpathogenic zymodeme, a conversion of the culture was observed, but when the bacterial flora was that which accompanied the pathogenic isolate SAW 408 (*E. coli*, *S. faecalis*, *Flavobacter* sp., *Clostridium* sp.), the zymodeme of the introduced ameba remained pathogenic. The electrophoretic bands belonging to the bacterial isoenzymes did not interfere or affect in any case the readings of the patterns given by the amebae. The nature of

the bacterial contribution (from the pathogenic isolate), which did not permit the conversion to the nonpathogenic zymodeme, is under investigation.

At the moment we do not have a good explanation for the mechanism of isoenzyme alterations observed in our experiments or for the many zymodeme groups that have been discovered in different isolates of *E. histolytica*. Sargeaunt et al. (21, 25) never noticed alteration of isoenzyme patterns from nonpathogenic to pathogenic or vice versa in any of their longitudinal culture studies. All of their studies, however, were performed with ameba cultures initiated and maintained in the presence of viable bacteria in Robinson medium (19). In our experiments, significant changes in the culture growth conditions were made; this may have caused the induction of metabolic systems, which led to changes in the isoenzyme electromobilities and the appearance of virulent properties.

*E. histolytica* is not the first organism in which changes in zymodemes have been observed. Somewhat similar changes have been shown to happen in a number of protozoa (*Paramecium* and *Tetrahymena* species), where variations in isoenzyme electrophoretic mobilities and expression of certain enzymes are subject to conditions of growth, media components, and the presence or absence of bacteria in the medium (1, 2, 20).

One important point that should be stressed at this stage, however, is that alterations in isoenzyme patterns and increased virulence of *E. histolytica* trophozoites that result due to changes in laboratory growth conditions could also occur in the microenvironment of the human intestine, where variations in bacterial flora and other conditions are known to happen (27).

In principle, we support the notion that amoebae in the pathogenic state are capable of causing disease in humans. However, contrary to the established hypothesis and previous beliefs (9, 16), classification of pathogenic and nonpathogenic isolates by their isoenzyme electrophoretic patterns could be misleading. Our results clearly demonstrate that isoenzymes of cloned cultures can change from one state to the other, and consequently we must conclude that zymodeme analysis of human isolates may be of little diagnostic value and should not be used to predict a clinical condition or serve as a basis for recommendation of therapy (9, 24). Shifts from nonpathogenic to pathogenic *E. histolytica* could be extremely important for the development of symptomatic amebiasis. It is possible that asymptomatic carriers may harbor amoebae in a nonpathogenic state for even long periods, but these could rapidly change and become pathogenic if suitable conditions, such as a change in bacterial flora, develop in the intestine. The ability of amebae to shift back from pathogenic to nonpathogenic is also very interesting, because it may help us understand the multitude of asymptomatic carriers. Learning more about these conversions, what regulates them, and whether they are of a phenotypic or genotypic nature may allow us in the future to better control this parasite and the disease it causes.

## ACKNOWLEDGMENTS

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## LITERATURE CITED

1. **Allen, S. L.** 1968. Genetic and epigenetic control of several isozymic systems in *Tetrahymena*. Ann. N.Y. Acad. Sci. **151**:190–207.
2. **Allen, S. L., and T. A. Nerad.** 1978. Effect of acetate on esterase C activity during the growth cycle of *Paramecium*. J. Protozool. **25**:273–279.
3. **Bracha, R., and D. Mirelman.** 1984. *Entamoeba histolytica* trophozoites: effects of bacteria, microaerobic conditions and metronidazole. J. Exp. Med. **160**:353–369.
4. **Cleveland, L. R., and E. P. Sanders.** 1930. The production of bacteria-free amoebic abscesses in the livers of cats and observations on the amoebae in various media with and without bacteria. Science **72**:149–152.
5. **Diamond, L. S.** 1968. Techniques of axenic cultivation of *Entamoeba histolytica* and *Entamoeba histolytica*-like amoebae. J. Parasitol. **54**:1047–1056.
6. **Diamond, L. S.** 1982. A new liquid medium for xenic cultivation of *Entamoeba histolytica* and other lumen dwelling protozoa. J. Parasitol. **68**:958–959.
7. **Diamond, L. S.** 1983. Lumen dwelling protozoa: *Entamoeba*, trichomonads and giardia, p. 65–110. *In* J. B. Jensen (ed.), *In vitro* cultivation of protozoan parasites. CRC Press, Boca Raton, Fla.
8. **Diamond, L. S., D. R. Harlow, and C. C. Cunnick.** 1978. A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. Trans. R. Soc. Trop. Med. Hyg. **72**:431–432.
9. **Editorial.** 1985. Is that amoeba harmful or not? Lancet **i**:732–734.
10. **Gillin, F. D., and L. S. Diamond.** 1978. Clonal growth of *Entamoeba* in agar: some applications of this technique to the study of their cell biology. Arch. Invest. Med. **9**:237–246.
11. **Jackson, T. F. H. G., and V. Gathiram.** 1985. Frequency distribution of *Entamoeba histolytica* zymodemes in a rural South African population. Lancet **i**:719–722.
12. **Jacobs, L.** 1950. The substitution of bacteria in cultures of *Entamoeba histolytica*. J. Parasitol. **32**:128–130.
13. **Jones, W. R.** 1946. The experimental infection of rats with *Entamoeba histolytica* with a method for evaluating the antiamoebic properties of new compounds. Ann. Trop. Med. Parasitol. **40**:130–140.
14. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Gel electrophoresis, p. 150–185. *In* Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Mirelman, D., R. Bracha, A. Chayen, A. Aust-Kettis, and L. S. Diamond.** 1986. *Entamoeba histolytica*: effect of growth conditions and bacterial associates on isoenzyme patterns and virulence. Exp. Parasitol. **62**:142–148.
16. **Mirelman, D., R. Bracha, and P. G. Sargeaunt.** 1984. *Entamoeba histolytica*: virulence enhancement of isoenzyme-stable parasites. Exp. Parasitol. **57**:172–177.
17. **Owen, D. G.** 1985. A mouse model for *Entamoeba histolytica* infection. Lab. Anim. **19**:297–304.
18. **Reed, S. L., P. G. Sargeaunt, and A. I. Braude.** 1983. Resistance to lysis by human serum of pathogenic *Entamoeba histolytica*. Trans. R. Soc. Trop. Med. Hyg. **77**:248–253.
19. **Robinson, G. L.** 1968. The laboratory diagnosis of human parasitic amoeba. Trans. R. Soc. Trop. Med. Hyg. **62**:285–293.
20. **Rowe, E., I. Gibson, and A. Cavill.** 1970. Effects of growth conditions on the esterases of *Paramecium aurelia*. Biochem. Gen. **5**:151–159.
21. **Sargeaunt, P. G.** 1985. Zymodemes expressing possible genetic exchange in *Entamoeba histolytica*. Trans. R. Soc. Trop. Med. Hyg. **79**:86–89.
22. **Sargeaunt, P. G., T. F. H. G. Jackson, and A. E. Simjee.** 1982. Biochemical homogeneity of *Entamoeba histolytica* isolates, especially those from liver abscess. Lancet **i**:1386–1390.
23. **Sargeaunt, P. G., and J. E. Williams.** 1978. Electrophoretic isoenzyme patterns of *Entamoeba histolytica* and *Entamoeba coli*. Trans. R. Soc. Trop. Med. Hyg. **72**:164–166.
24. **Sargeaunt, P. G., and J. E. Williams.** 1982. A study of intestinal protozoa including nonpathogenic *Entamoeba histolytica* from

patients in a group of mental hospitals. Am. J. Public Health **72**:178–179.

25. **Sargeaunt, P. G., J. E. Williams, R. Bhojnani, J. Kumate, and E. Jimenez.** 1982. A review of isoenzyme characterization of *Entamoeba histolytica* with particular reference to pathogenic and non-pathogenic stocks isolated in Mexico. Arch. Invest. Med. **13**(Suppl.):89–94.
26. **Sargeaunt, P. G., J. E. Williams, and R. A. Neal.** 1980. A comparative study of *Entamoeba histolytica* (NIH-200, HK9, etc.), "*Entamoeba histolytica*-like" and other morphologically identical amoebae using an isoenzyme electrophoresis. Trans. R. Soc. Trop. Med. **74**:469–474.
27. **Savage, D. C.** 1977. Microbial ecology of the gastrointestinal tract. Annu. Rev. Microbiol. **31**:107–133.