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Establishment of an Axenic Strain of *Entamoeba histolytica* from Cysts in Stool, Bypassing Bacteria-Associated Cultivation

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Introduction

Although axenic trophozoites of *Entamoeba histolytica,* first produced by Diamond (1961; 1968b), have been useful for researches on amoebae and amoebiasis, several modified procedures have been devised to improve and simplify the process to obtain the axenic strains (Wittner, 1968; Raether *et al.,* 1973; Bos. 1975; Dutta and Singh, 1975; Meerovitch, 1978). Most of these procedures, however, still utilized trophozoites of *E. histolytica* grown in bacteria-associated media as the starting material. The cysts in stool could not be useful for further manipulations unless their excystation was accomplished in bacteria-associated media (Meerovitch, 1978). Although Diamond (1968a) utilized cysts of the parasite, which were formed from the trophozoites in bacteria-associated media and isolated by his microisolation technique, no attempts have been done to establish axenic strains of *E. histolytica* from the cysts in stool without employing bacteria-associated cultivation of the trophozites. Wang *et al.* (1974) produced axenic strains of the parasite bypassing bacteria-associated cultures. However, they obtained the trophozoites, as the starting material, from liver abscesses of human patients in which *E. histolytica* is usually present under aseptic conditions.

During studies on improvement of axenic cultivation of *E. histolytica,* we could isolate almost pure cysts of the parasite from a stool by a series of density gradient centrifugations. This enabled us to obtain an axenic strain of *E. histolytica* bypassing bacteria-associated cultivation of amoebae. The present communication describes details of this procedure.

Materials and Methods

As the starting material, a stool of a Laotian immigrant, who settled in Japan, was used. It contained about 100,000 cysts per 1 g, which had the same morphological characteristics as those of *E. histolytica,* and a small number of eggs of *Opisthorchis viverrini,* although the immigrant was asymptomatic. Approximately 1 g of the stool was suspended in 10 ml of physiological saline, filtered with a gauze and washed three times in 10 ml of the saline by centrifugation at 670 g for 5 min. The specimen was suspended in 5 ml of distilled water, layered on 5 ml of 35% sucrose solution (w/v) and centrifuged at 380 g for 10 min in a swinging bucket (H-103 N2, Kokusan Centrifuge Inc., Tokyo, Japan). A resulting band formed over the sucrose solution was isolated with a micropipette and diluted five-fold with distilled water.

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After washing twice and suspending the pellet in distilled water as described above, the specimen was layered on 5 ml of 25% sucrose solution (w/v) and centrifuged at 380 g for 10 min. This gradient centrifugation was repeated again in the same manner except that the second centrifugation was done at 670 g for 10 min. The band formed over the sucrose solution was isolated and diluted with distilled water as above after the second density gradient centrifugation. Subsequently, the specimen was washed once in distilled water, incubated in 1% HCl for 15 min and centrifuged at 750 g for 10 min. The pellet, which was composed of almost pure cysts of amoebae (approximately 1,000 in number) was resuspended in 15 mM phosphate-buffered saline (PBS), pH 7.4 containing 1,000 units/ml potassium penicillin G and 1 mg/ml streptomycin sulfate. After washing twice in the PBS containing the antibiotics, 700–1,000 cysts were suspended in 15 ml of BI-S-33 medium (Diamond *et al.*, 1978) in the presence of 2×10^8 epimastigotes of *Trypanosoma cruzi* (Tulahuen strain), which had been grown in LIT medium (Evans, 1978) and washed thrice in BI-S-33 medium without bovine serum. Subsequently, the whole suspension was transferred into a screw-capped culture tube of 16×125 mm (Wheaton Scientific, Millville, New Jersey, USA) in the presence of 250 units/ml penicillin and 250 μg/ml streptomycin. The culture tube was placed at 35.5 C, and the growth of amoebae was evaluated at least every 12 hours with an inverted microscope and by counting the number of trophozoites using a haemocytometer. All of these manipulations were done at 20 C, and under sterile conditions. Glasswares and solutions except for some ingredients of BI-S-33 medium were autoclaved (15 lb, 121 C, 10–15 min). The ingredients which could not be autoclaved were filter-sterilized.

Contamination of the monoxenic and axenic cultures with bacteria, fungi and mycoplasma was evaluated with fluid thioglycollate, Sabouraud's medium and Chanock's agar plate utilizing PPLO agar (Difco Laboratories, Detroit, Michigan, USA), respectively. Contamination of the axenic culture with epimastigotes of *T. cruzi* was examined with LIT medium. Repeated microscopic observations were also done to evaluate contamination of the cultures with the microorganisms.

Amoebae in the axenic culture were identified with gel diffusion precipitin (GDP) test.

Results

On 2nd day after starting the monoxenic association of cysts of amoeba with *T. cruzi,* a few actively motile trophozoites could be observed. The number of trophozoites gradually increased. On the 9th day, most of the culture fluid was aspirated, and 15 ml of fresh BI-S-33 medium, which also contained the same number of epimastigotes of *T. cruzi,* was added to the culture tube, because the motile trypanosomes decreased in number. Amoebae in the monoxenic culture were serially transferred every 4–6 days by centrifuging the whole culture fluid and suspending the pellet in the fresh medium. When the 3rd transfer was done, the parasites were concentrated by centrifugation, and divided into two almost equal parts, each of which was suspended in 15 ml of the fresh medium containing *T. cruzi* as above. Subsequently, amoebae in these two monoxenic cultures were maintained and subcultured in the same manner as above. Evaluation of sterility of the monoxenic cultures, which was usually done 3 days after the media were renewed, indicated that the cultures were not contaminated with bacteria, fungi and mycoplasma.

After 37 transfers were conducted as above during 6 months, amoebae in one of

the two monoxenic cultures were found to grow as rapidly as a previously established axenic strain of *E. histolytica* (strain H-303: NIH). On the basis of this observation, a homogeneous suspension of the amoebae (about 120,000/ml in number) was prepared from this culture by gently inverting the tube, and 0.8 ml of the suspension was inoculated into 3 culture tubes of the same type containing 15 ml of BI-S-33 medium with no trypanosomatid associates. These cultures were incubated at 35.5 C. Subsequent observations indicated that the amoebae grew in all of the three cultures. Epimastigotes of *T. cruzi* rapidly diminished in number. Moreover, these cultures were not contaminated with bacteria, fungi and mycoplasma as examined 1 day before the subculture was made, i.e., on the 5th day of the cultivation, by transferring 0.4 ml of the homogeneous suspension, prepared as above, into 15 ml of the fresh medium with no trypanosomatid associates. Immediately after the first subculture was made, the motile trypanosomes became scarcely detectable on a light microscope. Since then, the subcultures have been done every 4 days as described above for longer than 9 months, and no contamination with bacteria, fungi and mycoplasma has been demonstrated. Epimastigotes of *T. cruzi* have no more been demonstrable with LIT medium after the 3rd subculture was performed. Our preliminary studies also suggested that epimastigotes of *T. cruzi* scarcely grew in BI-S-33 medium irrespective of the concomitant presence of amoebae at 35.5 C,

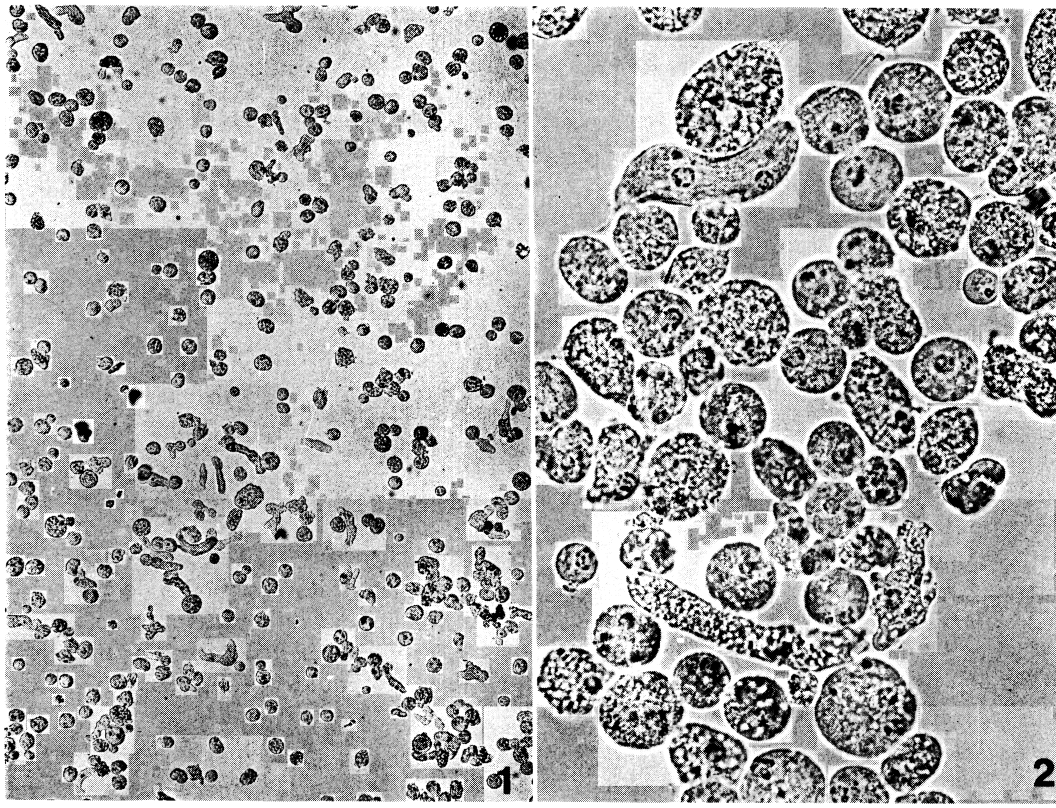

Fig. 1 The axenic strain established in the present study (strain AH-1:KEIO) (×150).
Fig. 2 An enlarged view of strain AH-1:KEIO (×750).

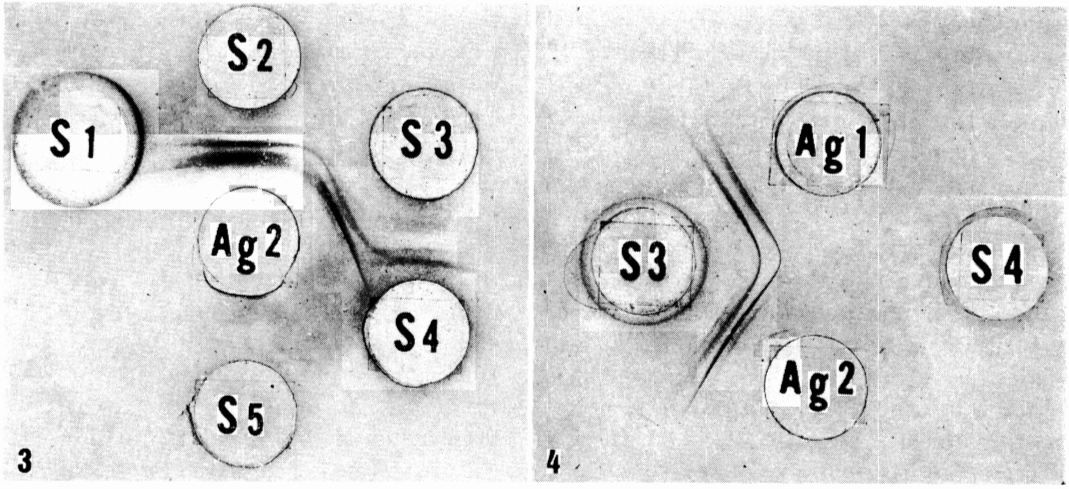

Fig. 3 GDP test for identification of strain AH-1:KEIO. S1: Negative control serum; S2: Antiserum isolated from a patient with intestinal amoebiasis before treatment. The diagnosis was performed by detection of the parasite with an endoscopic biopsy and by serological methods. Later, symptoms of the patient were completely removed by treatment with metronidazole. S3: S2 diluted twice with Dulbecco's phosphate-buffered saline (PBS); S4: S2 absorbed with the same volume of the crude extract of strain H-303:NIH of *E. histolytica* (8.0 mg protein/ml); S5: S2 absorbed with the same volume of the crude extract of strain AH-1:KEIO (8.1 mg protein/ml); Ag2: The crude extract of H-303 strain of *E. histolytica* (3.0 mg protein/ml). Trophozoites of strains H-303 and AH-1, grown in BI-S-33 medium, were washed thrice, suspended in Dulbecco's PBS and disrupted by homogenization for 2 min at 4 C. These homogenates were used for preparation of S4, S5 and Ag2 as described above.

Fig. 4 GDP test for identification of strain AH-1:KEIO. Ag1: The crude extract of AH-1 strain (3.0 mg protein/ml). Other details as in the legend to Fig. 3.

and virtually all epimastigotes died in 10 days, even if the medium was renewed every 4 days. Amoebae in the axenic cultures were demonstrated in Figs. 1 and 2.

GDP test was attempted in order to identifiy the axenic amoebae established in the present experiment. Absorption of an antiserum from a patient with intestinal amoebiasis using the extract of the established strain removed the precipitin lines between the serum and *E. histolytica* (H-303 strain) (Fig. 3). Moreover, the precipitin lines between the serum and H-303 strain seemed identical with those between the serum and the established strain (Fig. 4). On the basis of these findings, we conclude that the axenic amoebae established in this study are *E. histolytica,* and designate them "AH-1: KEIO".

Discussion

Our present investigations appear to simplify the process to establish axenic strains of *E. histolytica* from the cysts in stool by bypassing association of amoebae with bacteria in the cultures, which has been employed in most of the previous methods utilizing the cysts in stool as the starting material. It was also demonstrated that the monoxenic cultivation of *E. histoilytica* with *T. cruzi* as well as the axenic cultivation could be accomplished with only BI-S-33 medium. Although amoebae in the other monoxenic culture, which did not grow so rapidly as axenic *E. histolytica*, were also transferred into BI-S-33 medium with no trypanosomatid associates more

than 10 times during 100 subcultures, they did not exhibit growth in the medium. On the other hand, the monoxenic amoebae with a faster growth rate are still culturable in the axenic medium after more than 100 transfers in BI-S-33 medium with trypanosomes. These observations suggest that amoebae, which can grow as rapidly as axenic *E. histolytica* in the monoxenic culture, may be culturable in the axenic medium. Nothing has been known, however, about factors which controlled the growth rate of *E. histolytica* in the present monoxenic culture. Treatment of cysts of *E. histolytica* with HCl has been supposed to function in killing bacteria and promoting culturability of the parasite in the axenic medium. Although Diamond (1968a) used 0.4%, we employed 1% HCl, because treatment with this concentration of the acid for 15 min at 20 C could most effectively kill bacteria, and did not decrease culturability of the amoebae.

Our present procedure can not be applicable to the trophozoites in bacteria-associated media. Moreover, there is a possibility that this method can produce axenic trophozoites only from cysts of *E. histolytica* with some peculiar backgrounds as judged from the importance of bacteria in the culturability of this parasite. In order to examine this possibility, we are currently trying to produce axenic trophozoites of *E. histolytica* from various sources of the cysts in stool according to the procedure described in this communication.

Summary

An axenic culture of *Entamoeba histolytica* was obtained from cysts in stool bypassing bacteria-associated cultivation of trophozoites. The cysts were isolated from a stool of an asymptomatic carrier by a series of density gradient centrifugations. After incubating in 1% HCl, they were washed, transferred into BI-S-33 medium containing epimastigotes of *Trypanosoma cruzi*, and placed at 35.5 C. In a few days, actively motile trophozoites of amoeba could be observed. After 37 transfers, amoebae in one of the two monoxenic cultures, which could grow as rapidly as axenic *E. histolytica*, were inoculated into BI-S-33 medium with no trypanosomatid associates. Subsequent observations for longer than 9 months indicated that the amoebae grew under the axenic condition. The amoebae were identified as *E. histolytica* by gel diffusion precipitin test.

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細菌共棲培養を必要としない嚢子からの赤痢アメーバ無菌株の確立

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細菌共棲培養を用いることなく糞便中の嚢子から赤痢アメーバの栄養型の無菌化に成功した．嚢子は一連のショ糖密度勾配遠沈によってヒト糞便より分離し，1％塩酸にて20C，15分間処理後，*Trypanosoma cruzi* の epimastigotes を含む BI-S-33 培地に加えた．培養開始後数日目ですでに活発に運動する栄養型を確認した．栄養型の数は次第に増殖したので，培地交換によって37代継代した結果，二つの monoxenic culture のうち一方が無菌化赤痢アメーバとほぼ同様の増殖速度を示したので，この一部を *T. cruzi* を含まない BI-S-33 培地に移した．以後9ケ月以上観察を続け，アメーバが無菌状態で増殖することを確認した．尚，ゲル内沈降反応によるて無菌化されたアメーバが確かに赤痢アメーバであることも確認された．