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Research Note

Comparative Studies on Cytopathogenicity of Whole, Viable Trophozoites and Lysosome-Rich Fractions of *Entamoeba histolytica*

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Since Lushbaugh *et al.* (1979) reported the presence of a toxin-like substance, which caused detachment and rounding of tissue-cultured cells, in cell-free extracts of trophozoites of *Entamoeba histolytica,* its properties have been investigated (Mattern *et al.,* 1980; Koblier and Mirelman, 1980). We have also demonstrated that the toxin-like substance is at least partially associated with lysosome-rich fractions of this parasite (Takeuchi *et al.,* 1981a). On the other hand, the cytopathogenicity of whole, viable *E. histolytica* has also been studied (Knight, 1977; McCaul *et al.,* 1977; Ravdin *et al.,* 1979; 1980). Ravdin *et al.* (1980) suggested that destruction of chinese hamster ovary cells with whole, viable *E. histolytica* did not appear to be attributable to the toxin-like substance. They also proposed that the mechanism of cytopathogenicity of trophozoites of this parasite was composed of two steps, i.e., contact-dependent cell killing and subsequent phagocytosis.

To further examine if the toxin-like substance is actually responsible for the contact-dependent killing of tissue-cultured cells by trophozoites of *E. histolytica,* effects of cytochalasin B, colchicine and new-born calf serum on the cytopathogenicity of whole, viable trophozoites and the toxin-like substance were evaluated and compared. In the present experiments, the toxin-like substance in lysosome-rich fractions was examined, although it also seems to be present in 110,000 g supernatant fraction of disrupted amoebae (Takeuchi *et al.,* 1981a), on the basis of the possible function of lysosomes in the pathogenicity of this parasite as proposed by Eaton *et al.* (1969).

E. histolytica (strains HM-1:IMSS; HK-9; Rahman; H-303:NIH) was axenically grown in BI-S-33 medium (Diamond *et al.,* 1978) as described previously (Takeuchi *et al.,* 1977). After cultivating for 72 hours at 35.5 C, amoebae were harvested as described (Takeuchi *et al.,* 1977) and finally suspended in 50 mM Tris-HCl buffer, pH 7.4 containing 0.25 M sucrose so that the protein concentration was 20–25 mg/ml.

Cytochalasin B and colchicine were supplied by Sigma Chemical Co. (St. Louis, Missouri). New-born calf serum was obtained from Flow Laboratory (Rockville, Maryland). Other chemicals were of the highest purity commercially available. Cytochalasin B was solubilized in dimethylsulfoxide to make 1 mg/ml and further diluted in Eagle's minimum essential medium (MEM) before use.

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Lysosome-rich fractions were prepared from cell-free extracts of trophozoites of this parasite (HM-1 strain) as described previously (Takeuchi *et al.*, 1981b). The isolated fractions were washed once and finally suspended in Eagle's MEM to make 3 mg protein/ml. Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Assay of the cytopathogenicity of lysosome-rich fractions was done using NIH:3T3 cells fundamentally according to the procedure described previously (Takeuchi *et al.*, 1981a) except that Eagle's MEM was used in place of Hanks balanced salt solution as the assay medium, and that NIH:3T3 cells were grown on a cover slip (12×32 mm) placed in a Leighton tube ($35 \times 15 \times 15$ mm) containing Eagle's MEM supplemented with 10% new-born calf serum. After incubating with lysosome-rich fractions (0.2–0.4 mg protein) for 30 min at 37 C, the cells on the cover slip were rinsed with 0.1 M phosphate buffer saline, pH 7.4 (PBS), dried in air and fixed with methanol. Subsequently, these cells were stained with Giemsa and observed with a light microscope.

The cytopathogenicity of whole, viable trophozoites was also assayed using NIH:3T3 cells cultivated in the same manner as above. Amoebae, suspended in the Tris-buffered sucrose, were washed once in the PBS and suspended in the MEM to make 2×10^5 amoebae/ml. One half ml of this suspension, i.e., 10^5 amoebae, was added to confluent NIH:3T3 cells which had been rinsed several times and overlaid with 0.5 ml of the MEM containing 5 μM paramethyl sulfonyl fluoride and 5 mM β-mercaptoethanol, and the Leighton tube was incubated for 30 min at 37 C. The cells on the cover slip were processed and observed as described above.

Normal NIH:3T3 cells without any treatments were demonstrated in Fig. 1. The cells in the presence of 60 μg/ml cytochalasin B and 100 μg/ml colchicine were shown in Figs. 2 and 3 respectively as controls. Fig. 4 demonstrates the cytopathogenicity of whole, viable amoeba (HM-1 strain). Other amoebae, i.e., HK-9, Rahman and H-303 strains, appear to have little cytopathogenicity to NIH:3T3 cells under the present assay conditions (data not shown). These findings seem to be compatible with Mattern and Keister (1977) who reported that HM-1 strain was most pathogenic to the liver of new-born hamster among numerous axenic strains examined. Fig. 5 shows alterations of NIH:3T3 cells caused by B-2, which is one of two lysosome-rich fractions isolated from *E. histolytica* (Takeuchi *et al.*, 1981b). This fraction showed a higher cytopathogenicity to NIH:3T3 cells than B-3, the other lysosome-rich fraction of amoeba (data not shown).

Effects of cytochalasin B, colchicine and new-born calf serum were evaluated using HM-1 strain and B-2 as whole, viable amoeba and lysosome-rich fraction respectively because of their high cytopathogenicity.

Addition of 60 μg/ml cytochalasin B appears to almost abolish the cytopathogenicity of whole, viable trophozoites (Fig. 6) as compared with Figs. 2 and 4. Moreover, this compound inhibited motility (Kobayashi *et al.*, 1981) and phagocytic activity of this parasite at 60 μg/ml (unpublished data). Although lower concentrations of this compound decreased motility of *E. histolytica*, its cytopathogenicity was not completely inhibited. These findings seem to be in accord with Michel and Schupp (1976) who reported that 40 μg/ml cytochalasin B was needed to abolish motility and phagocytic activity of this parasite, and that amoebae only changed motility at a concentration between 10 and 30 μg/ml of this compound. However, Ravdin *et al.* (1980) reported that destruction of chinese hamster ovary cells with wohle, viable *E. histolytica* was significantly inhibited by 5 μg/ml cytochalasin B during observation for 2–6 hours. These differences may be attributable to target cells and/or the number of amoebae employed for evaluation of the cytopathogenicity. In contrast to the cytopathogenicity of whole, viable

amoebae, that of lysosome-rich fraction did not appear to be affected by this compound (Fig. 7) as compared with Figs. 2 and 5.

Addition of colchicine did not appear to affect the cytopathogenicity of lysosome-rich fraction (Fig. 8) as well as of whole, viable trophozoites (Fig. 9) at 100 μg/ml. Lower concentrations of this compound were also ineffective. Although one may consider that colchicine enhances destruction of NIH:3T3 cells by whole, viable amoebae, when Fig. 9 is compared with Fig. 4, we suggest that this rseults from direct effects of this compound on NIH:3T3 cells. As demonstrated in Fig. 3, NIH:3T3 cells changed their morphology, and some part of the cells appeared to detach from the cover slip in the presence of 100 μg/ml colchicine. Motility as well as phagocytic activity of *E. histolytica* did not appear to be inhibited by 100 μg/ml colchicine (unpublished data).

Addition of 10% new-born calf serum seemed to abolish the cytopathogenicity of lysosome-rich fraction (Fig. 10), whereas that of whole, viable trophozoites did not appear to be affected by this serum (Fig. 11).

Although not quantitative, these findings suggest that the contact-dependent cell killing by *E. histolytica* is probably not due to the toxin-like substance localized in lysosome-rich fractions. Our observations on the effects of cytochalasin B lead us to envision that microfilament-like structures found by electron microscopical studies (Rosenbaum and Wittner, 1970; Michel and Schupp, 1976) are primarily responsible for the cytopathogenicity of trophozoites of *E. histolytica*. In this sense, motility and phagocytic activity of amoeba, which seem sensitive to cytochalasin B, may be deeply related with its cytopathogenicity.

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短　報

赤痢アメーバの栄養型とライソゾーム分画の組織培養細胞に対する病原性の比較

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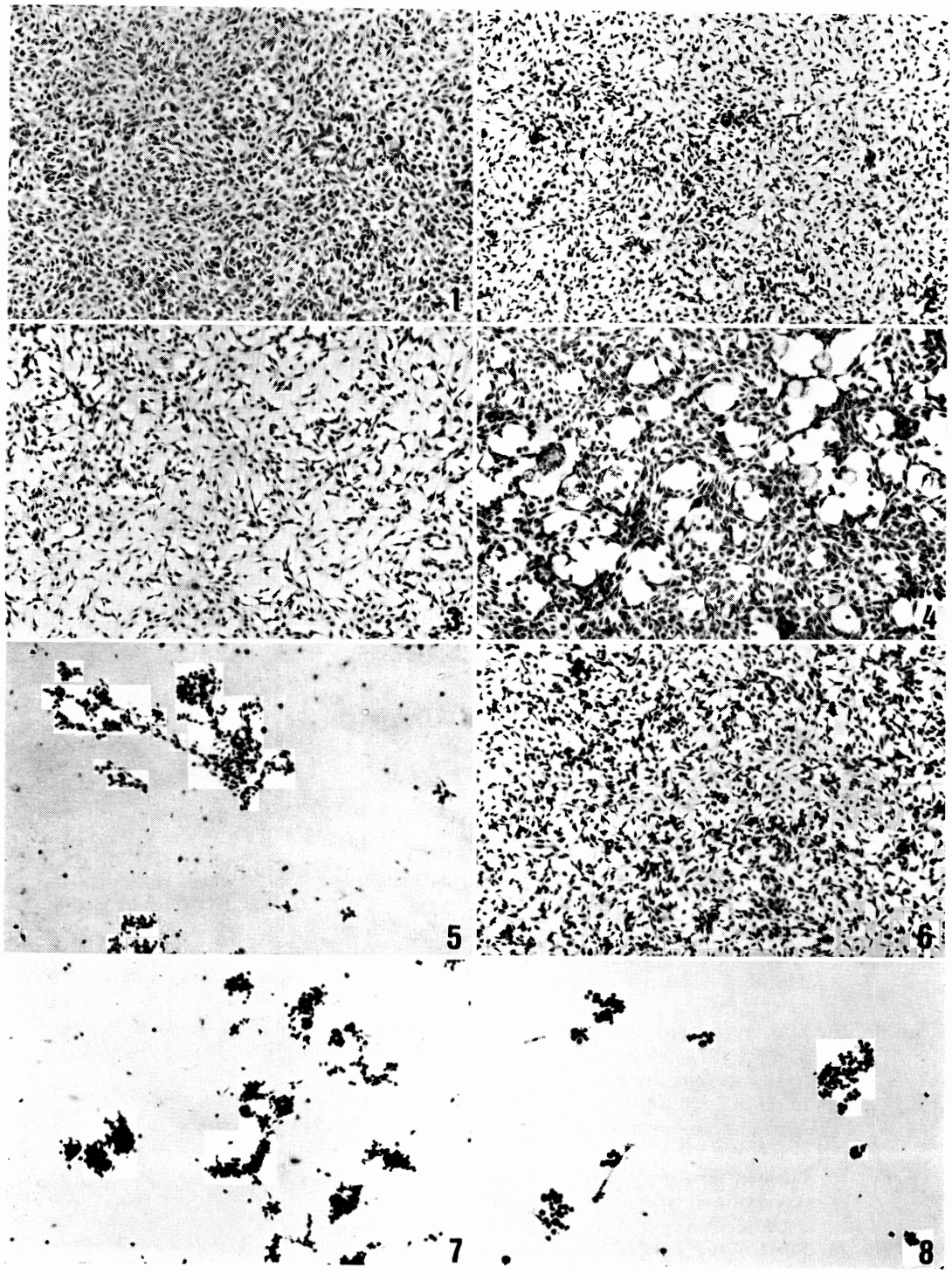
赤痢アメーバ栄養型の組織培養細胞に対する病原性にライソゾーム分画に局在しているトキシン様物質が関与しているか否かを知るため，NIH：3T3細胞を標的細胞として実験を行なつた．その結果サイトカラシンBは栄養型の，また新生仔牛血清はライソゾーム分画の培養細胞に対する病原性を減弱させるように思われた．一方，コルヒチンは両者に対して効果を示さなかつた．これらのデータよりライソゾーム分画のトキシン様物質は赤痢アメーバ栄養型の培養細胞に対する病原性に関与していないものと推測された．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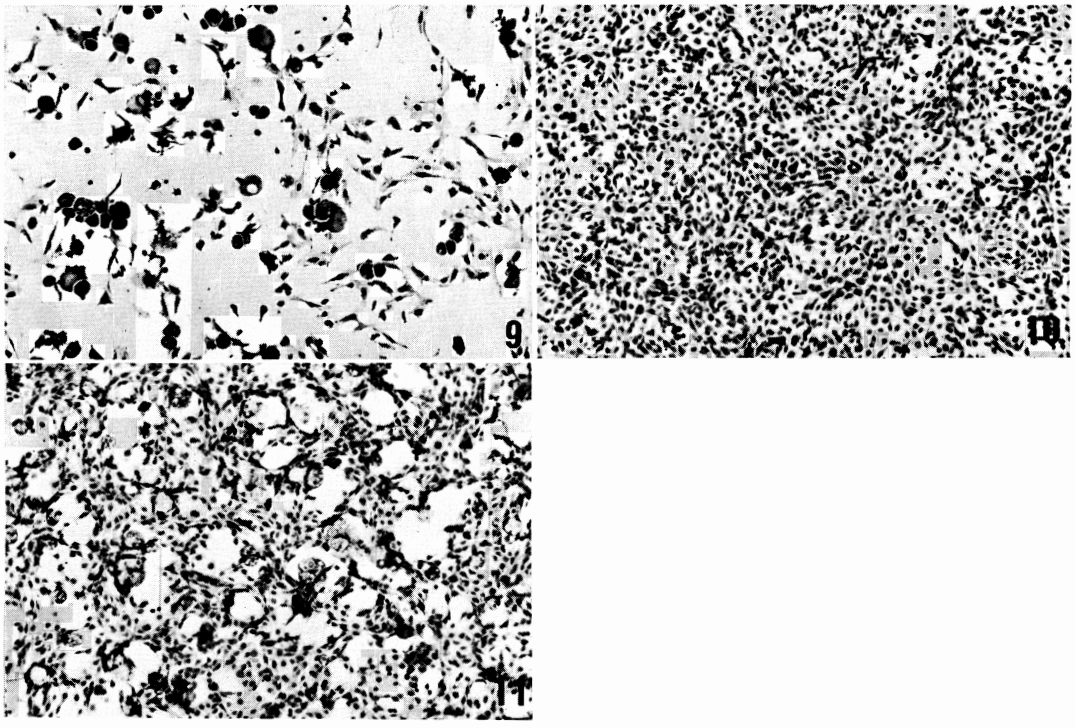

Legends of Figures

Fig. 1 Normal NIH:3T3 cells. (×40).
Fig. 2 NIH:3T3 cells in the presence of 60 μg/ml cytochalasin B. (×40).
Fig. 3 NIH:3T3 cells in the presence of 100 μg/ml colchicine. (×40).
Fig. 4 Cytopathogenicity of whole, viable amoebae (HM-1 strain). (×40).
Fig. 5 Cytopathogenicity of lysosome-rich fraction (B-2). (×40).
Fig. 6 Effect of cytochalasin B (60 μg/ml) on the cytopathogenicity of whole, viable trophozoites. Amoebae were incubated with cytochalasin B for 60 min at 37 C before adding to NIH:3T3 cells. (×40).
Fig. 7 Effect of cytochalasin B (60 μg/ml) on the cytopathogenicity of lysosome-rich fraction. The fraction was processed in the same manner as above before adding to NIH:3T3 cells. (×40).
Fig. 8 Effect of colchicine (100 μg/ml) on the cytopathogenicity of lysosome-rich fraction. The fraction was incubated with colchicine for 60 min at 37 C before adding to NIH:3T3 cells. (×40).
Fig. 9 Effect of colchicine on the cytopathogenicity of whole, viable trophozoites. Amoebae were also processed in the same manner as in Fig. 8 before adding to NIH:3T3 cells. (×40).
Fig. 10 Effect of new-born calf serum on the cytopathogenicity of lysosome-rich fraction. The serum was present in the assay medium before lysosome-rich fraction was added. (×40).
Fig. 11 Effect of new-born calf serum on the cytopathogenicity of whole, viable trophozoites. The serum was also present in the assay medium before amoebae were added. (×40).