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CHITIN SYNTHESIS INHIBITORS PREVENT CYST FORMATION BY ENTAMOEBA TROPHOZOITES

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Cysts of Entamoeba invadens obtained under axenic culture conditions have been reported to be similar to cysts of the human intestinal parasite E histolytica both in morphology and chitin presence in their walls. Mature E. invadens cyst forms, isolated from cultures following discontinuous Percoll gradient sedimentation were resistant (>80%) to detergent treatment. Addition of chitin synthesis inhibitors such as Polyoxin D and Nikkomycin (50 µg/ml) to cultures in encystation media markedly inhibited (>85%) the formation of detergent resistant cysts and prevented the incorporation of radiolabeled chitin precursor N-acetyl[^{3}H]glucosamine. These findings suggest that chitin synthesis inhibitors may serve as drugs which specifically block the life cycle of the Entamoeba parasite.

INTRODUCTION.

Axenic cultures of Entamoeba invadens trophozoites, an intestinal parasite of snakes (1) are known to form cysts upon transfer to a special encystation medium (2). These cysts are morphologically similar to cysts of Entamoeba histolytica obtained from faeces of human amoebiasis patients (3). Moreover, the cell walls of E. invadens cysts in analogy to those of the human parasite have been shown to contain microfibrils of chitin (4) the β(1-4) linked polymer of N-acetylglucosamine commonly found in fungi, crustacea and insects, but absent in the human body (5).

Polyoxin D and Nikkomycin are representatives of a growing family of structural analogues of UDP-N-acetylglucosamine which specifically block chitin synthesis in fungi (6-8) and have been reported to be non-toxic for mammals. In the present study we report that both drugs Polyoxin D and Nikkomycin inhibited the formation of mature cysts by E. invadens trophozoites. Tunicamycin which specifically blocks biosynthesis of dolichol

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or bactoprenyl diphosphate N-acetylglucosamine (9) had no effect on Entamoeba cyst formation.

MATERIALS AND METHODS

Nikkomycin, both a technical grade and a Nikkomycin X (86% purity) sample (10) were obtained from Bayer, Germany. Polyoxin D from Calbiochem, USA. Tunicamycin was from Eli Lilly. Vagisec detergent was a pharmaceutical product from Schmid Products, N.J., USA. N-acetyl[^{3}H]glucosamine (3 Ci/mM) was purchased from Radiochemical Center, Amersham, England. Percoll was obtained from Pharmacia Co., Uppsala, Sweden. Chitinase was from Sigma.

Cultivation of trophozoites and formation of cysts. Trophozoites of Entamoeba invadens strain IP1 obtained from Dr. L. Diamond, NIH, Bethesda, were grown axenically in TYI-S-33 medium according to the method of Diamond et al. (11). Optimal encystation was obtained by innoculation of trophozoites of E. invadens in Falcon plastic flasks (35 ml) or 3 ml glass tubes (5×10^5 cells/tube) containing an encystation medium which is composed of yeast extract, trypticase and dialysed serum (2). Incubations in the encystation media were carried out at 28°C for 3 or 4 days. Tubes containing different concentrations of various chitin synthesis inhibitors, (Nikkomycin, Polyoxin D or Tunicamycin) were innoculated in parallel.

Separation of subpopulations of Entamoeba cells by Percoll gradients. Each day after the initial innoculation of trophozoites into the encystation media, the cells from a tube were chilled in ice water (10 min), harvested by centrifugation (300 x g., 5 min), washed with saline (x2) and layered on top of a discontinuous Percoll gradient. The gradient consisted of layers containing 10,20,30,40,50,60,70 and 100% Percoll. We have previously shown that trophozoites are fully viable following centrifugation in a Percoll gradient and can be separated from other cells (12). Following centrifugation for 30 min at 300 x g., the cells in the various layers were removed, washed and counted in a hemacytometer under a microscope.

Incorporation of N-acetyl[^{3}H]glucosamine into Entamoeba. Amoeba were incubated in encystation medium for 4 days with and without Nikkomycin (500 µg/ml, crude preparation). In addition, each tube contained N-acetyl[^{3}H]glucosamine (3 µCi/ml, 3 Ci/mM). Cells collected from the drug containing and control tubes were each divided into two parts. One part was treated with Vagisec (1%), after which both parts were layered on Percoll gradients. Each layer was collected and mixed with a scintillation mixture, containing Triton-X-100. Radioactivity was determined in a Packard Scintillation Counter Model 3255. Results are given in counts per minute incorporated into cells of each gradient layer. [^{3}H]glucosamine was recovered from acid hydrolysates of cysts followed by paper chromatography as earlier described (13). Radiolabeled material remaining after treatment of cysts in NaOH (1M, 2h, 100°C) (14) was digested with chitinase (1 mg/ml; 37°C, 20 h).

RESULTS

Cultures of E. invadens which undergo encystation contain a mixture of the three different cell forms; trophozoites, precysts and mature cysts (Fig. 1). These forms were readily separated and quantitated by centrifugation on a discontinuous Percoll gradient. The motile trophozoites sedimented as a band between 60-70% Percoll, whereas the round and granulated precyst forms appeared in bands between 40-60%. Percoll layers of 20-30% and 30-40%

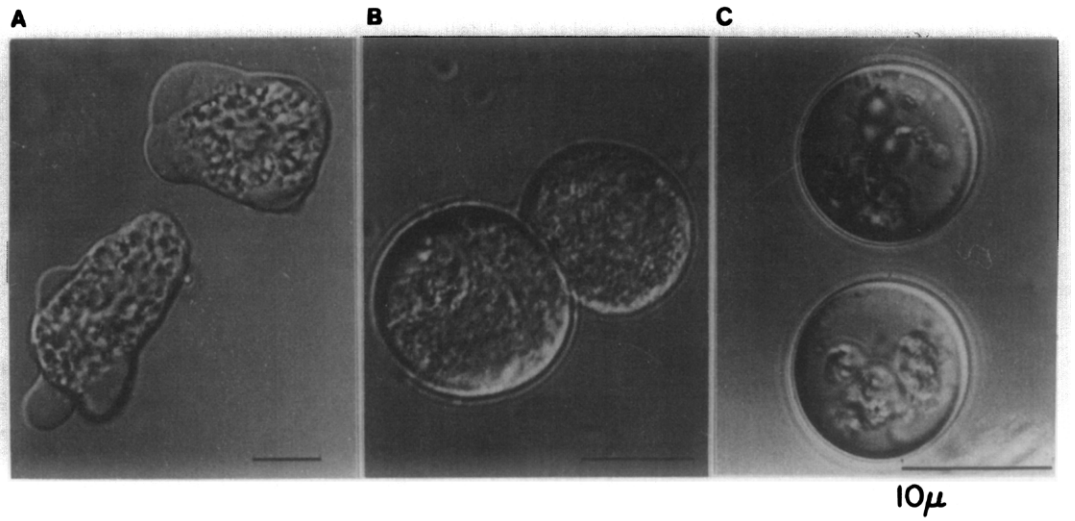

Figure 1. Entamoeba invadens cell forms. (A) Trophozoites at the time of transfer into encystation medium. (B) Rounded up precyst forms as observed in 40-50% Percoll bands (see Figure 2). (C) Mature birefringent cysts as observed in 20-30% Percoll gradient bands.

contained mostly mature cysts, whereas the layer at 10% and to some extent that at 20% contained cell debris and cyst ghost forms that could be distinguished under the microscope. Addition of detergent (Vagisec at 1% v/v final concentration) followed by vigorous mixing of the amoeba forms recovered from the various Percoll bands, disrupted and dissolved the trophozoites and precyst forms (cells that sedimented at densities higher than 40% Percoll). The detergent also solubilized the ghost and vesicle-like forms which sedimented at 10-20% Percoll. This treatment hardly affected (<20%) the mature cyst forms that sedimented at 20-40% Percoll. Maximal number of mature, detergent-resistant cysts was observed after 3-4 days of incubation in the encystation media and the yields were usually between 20-30% of the starting number of trophozoites (Fig. 2).

Addition of the chitin synthesis inhibitors Nikkomycin or Polyoxin D to cultures undergoing encystation caused a considerable reduction in the amount of cyst forms produced and an increase in the amount of cyst ghosts and cell debris. Only a slight increase in the number of motile trophozoites that remained in the cultures was observed. The biggest difference, however, was detected in the reduced amount of detergent resistant cyst forms recovered

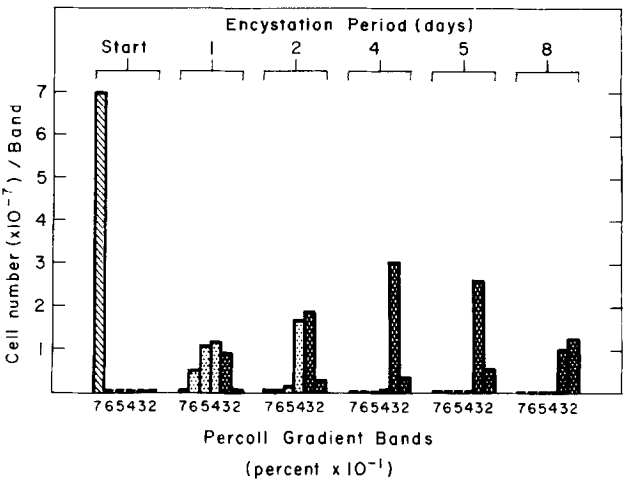

Figure 2. Encystation of Entamoeba invadens. Distribution of Entamoeba cell forms following sedimentation in a discontinuous Percoll gradient. Trophozoites ($5x10^5$/ml) were innoculated into six duplicate flasks containing encystation media. Each day the cells from a pair of flasks were harvested and sedimented on the Percoll gradient and the cells which banded in the various layers were counted. The average number of cells obtained from duplicate experiments is given. Cell debris and cyst ghosts which usually appear in 10-20% Percoll are not included. For further details see Methods.

after treatment of the cultures with Vagisec (1%). Inhibition of formation of detergent resistant cysts was considerable at concentrations of 10 µg/ml and over 85% at 50 µg/ml (Fig. 3). No effect was observed when Tunicamycin (10 µg/ml) was added to the cultures.

Entamoeba cell forms became radiolabeled following incubations in encystation media containing N-acetyl[^{3}H]-glucosamine. In the presence of Nikkomycin, total incorporation of the radiolabeled precursor into the different cell forms was considerably lower (>60% inhibition)(Table I). The amount of N-acetyl[^{3}H]glucosamine recovered in the detergent-insoluble cysts of the Nikkomycin treated cultures was approximately 75% lower than the amount of radioactivity determined in control experiments (Table I). Acid hydrolysis of the radiolabeled cells followed by paper chromatography revealed that over 90% of the radioactivity incorporated was not metabolized and was subsequently identified as [^{3}H]glucosamine (13). Moreover, the radioactivity in the detergent-resistant, mature cyst forms was mostly (>60%) contained in a NaOH-insoluble material (14) which was degraded upon digestion with chitinase (>70%).

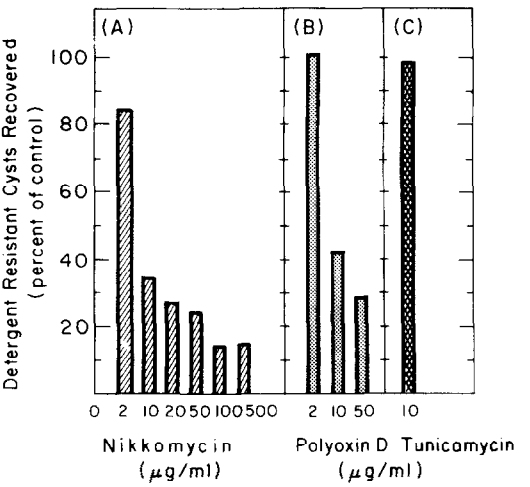

Figure 3. Detergent-resistant cyst forms recovered after 3-day cultivation in encystation media in the presence of varying amounts of Nikkomycin (A); Polyoxin D (B); or Tunicamycin (C). Duplicate tubes (3 ml) containing 1.2 x 10^6 trophozoites for each drug concentration were cultured. After collection of the cells they were vigorously treated with Vagisec (1%) and sedimented in the discontinuous Percoll gradients as described in Methods. The average yield of detergent-resistant cysts obtained in the control cultures was 2.5 x 10^5. For further details see Methods.

DISCUSSION

Cysts of the Entamoeba intestinal parasite are usually formed in the colon or rectum as a response of the motile trophozoite to hostile environmental conditions such as depletion of essential nutrients and changes

TABLE 1

Incorporation of N-acetyl[^{3}H]glucosamine by different Entamoeba forms during encystation

Cell form	Band on Percoll gradient	[^{3}H]GlcNAc incorporation (cpm) Control	+Nikkomycin
Cysts	20	488	88
	30	274	39
Precyst	40	128	11
	50	12	<10
	60	17	<10

Trophozoites of E. invadens were incubated in duplicate tubes containing 8 ml of encystation medium (5 x 10^5 cells/ml) together with [^{3}H]-GlcNAc (3 μci/ml; 3Ci/mM) for 4 days either with or without Nikkomycin (crude preparation 500 μg/ml). Cells were collected, treated with Vagisec (1%) detergent, then separated on a Percoll gradient. For further details see Methods. Duplicate samples of cells from the various layers were removed, mixed with a scintillation cocktail and counted. The calculated cpm average above background is given.

to more hypotonic conditions (3). The mechanical strength of the cyst cell wall is assumed to be due to the presence of chitin microfibrils (4,15). Formation of the cyst's rigid wall layer is critical for maintaining the parasite's life cycle as it enables it to survive outside the host for a period of several weeks, depending on the conditions. Since cysts are responsible for the transmission of amoebiasis to new hosts, drugs or compounds that specifically block the synthesis or assembly of the amoeba's cell wall chitin without affecting host cells, could be beneficial in containing the dissemination of cysts to the environment.

Our results clearly show that addition of chitin synthesis inhibitors such as Nikkomycin or Polyoxin D to trophozoites incubated in the encystation medium, had a remarkable effect on their ability to form cysts. Microscopic observation of cultures during the initial period of encystation with chitin synthesisinhibitors was quite misleading, since most of the trophozoites lost their motility, became "round" and formed granulated precysts (Fig. 1) which appeared similar to those in control cultures. The majority of these cells, however, did not mature into fully formed cysts and were incapable of withstanding the disruptive action of detergents (Fig. 3). Furthermore, many of the precysts formed initially in the presence of chitin synthesis inhibitors appeared to disintegrate, and considerable amounts of cell debris and cyst ghosts appeared in the low density Percoll gradient bands of the drug treated cultures.

The data on the inhibition of uptake of radiolabeled N-acetylglucosamine into the alkali-insoluble material of mature, detergent-resistant cysts (Table 1) indicates that the drug blocked chitin microfibril formation. Since the chitin microfibrils are important for the mechanical strength of the cyst wall, their absence may be a major cause in the inability of such cells to withstand a variety of disruptive forces.

The effect of other chitin synthesis inhibitors on cyst formation by Entamoeba is currently under study. Inhibition of E. invadens cyst formation was recently observed upon addition of Calcofluor M_2R (15), a commercial dye that is known to bind to chitin and other β-(1-3) and β-(1-4) linked

polysaccharides (16). Further studies are needed, however, in order to determine the effect of these drugs on the chitin synthase system of Entamoeba. Preliminary results obtained with a crude enzyme preparation composed of membranes of precysts indicate that Polyoxin D inhibited the incorporation of radiolabeled UDP-N-acetylglucosamine into particulate material (manuscript in preparation).

Cysts of E histolytica can be obtained only from amoebiasis patients since no in vitro axenic culture conditions have been yet developed for their production. However, in view of the numerous similarities between the cysts of Entamoeba invadens and histolytica (3,4) the results obtained so far on the inhibition of E. invadens cyst formation are most encouraging. These findings suggest that chitin synthesis inhibitors may serve as drugs which specifically block the life cycle of pathogenic Entamoeba and could prevent transmission of the disease.

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