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Effect of cholesterol on multiplication, lipid metabolism and lysosomal enzymes of *Hartmanella culbertsoni**

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Abstract. Addition of sonicated dispersions of cholesterol to peptone-salt-vitamin medium resulted in the metabolism of the sterol by *Hartmanella culbertsoni.* Trophozoite multiplication was stimulated at 1–5 mg/litre, but retarded at 10-20 mg/litre. When cholesterol was added to the medium, incorporation of [1,2—^{14}C] –acetate into neutral lipid, phospholipid, non-saponifiable and cholesterol fractions of the amoebae was significantly reduced. Cholesterol ester was detected in the medium but phospholipids were not released. Addition of cholesterol stimulated the activity of lysosomal acid phosphatase, acid deoxyribonuclease and cathepsin B but did not affect 5′-nucleotidase, adenosine triphosphatase, alkaline phosphatase, glucose-6-phosphatase, succinate dehydrogenase and cytochrome C oxidase.

Keywords. Cholesterol; trophozoite-multiplication; phospholipid; lysosomal enzymes.

Introduction

Cholesterol is present in *Entamoeba histolytica* and *E. invadens* (Sawyer *et al.*, 1967) and is an absolute requirement for axenic cultivation of the later organism (Van Vliet *et al.*, 1975). It is known to restore the virulence of attenuated and avirulent strains of *E. histolytica* (Singh, 1975; Das and Ghoshal, 1978; Meerovitch and Ghadirian, 1978). *H. culbertsoni*, a free living pathogenic amoeba does not require cholesterol for its growth in peptone-vitamin-salt medium (Kaushal and Shukla, 1975), but is exposed to an environment of cholesterol when it invades host brain and causes meningoencephalitis. Rai and Rao (1977, 1978) reported decrease in the cholesterol levels of brain in experimental meningoencephalitis in mice by *H. culbertsoni.* We have also observed that cholesterol of mouse brain infected with this organism is catabolised (Lal and Garg, 1979). Eaton *et al.* (1969) and Corosi (1976) have reported that lysosomes are ejected from the pseudopodia of virulent *E. histolytica.* It appears that there may be some correlation between the infection of *E. histolytica* and other amoebae in an environment of cholesterol and their virulence as well as the activity of lysosomal enzymes.

In this paper, we report the effect of exogenous cholesterol on trophozoite multiplication, lipid metabolism and the activity of lysosomal enzymes of *H. culbertsoni* cultured in peptone-vitamin-salt medium.

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Materials and methods

Growth and harvesting of trophozoites

H. culbertsoni, (Culbertson strain A–l) (Singh and Das, 1970) was maintained axenically in medium containing peptone (2% w/v), NaCl (0·5% w/v), thiamine (10 mg/litre) and cyanocobalamine (5 μg/litre) (Kaushal and Shukla, 1975), in Roux bottles containing 100 ml of the medium at 37 ± 2° C. Subcultures were made asceptically into fresh medium after every seven days. For large scale cultivation amoebae were grown in 1 litre Erlenmeyer flasks containing 300 ml of the medium with constant shaking at 37 ± 2° C (Emenvee shaker) for 4 days, 2×10^7 trophozoites were used as inoculum. Aliquots of culture were withdrawn at 24, 48, 72, 96 and 120 h and trophozoites counted in a haemocytometer. Cells were harvested after 96 h growth by centrifugation at 2000 g for 20 min and washed with normal saline.

Preparation of sonicated dispersion of cholesterol

Chromatographically pure cholesterol (Centron Research Laboratories, Bombay) was dissolved in a minimum volume of methanol and added to phosphate buffer (20 mM, pH 7·2) and sonicated at 20 Kc/sec (energy output 1·5 amp) for 20 min. The dispersion was centrifuged at 2000 g for 20 min, cholesterol was assayed in the supernatant solution and aliquots added to the media to give a final concentration of 1,5,10 and 20 mg/litre.

Addition of radioactive precursors

[1,2-^{14}C]-Acetate (sp. act. 33·7 mCi/mmol) obtained from Bhabha Atomic Research Centre, Bombay and [1,2-^{3}H]-cholesterol from New England Nuclear Company, Boston, Mass, USA. (sp. act. 40 Ci/mmol) were added before sterilisation, $3{\cdot}5 \times 10^6$ cpm of [^{14}C] acetate or $1{\cdot}5 \times 10^7$ cpm of [$_3$H]-cholesterol/100 ml of medium. After 96 h growth, the trophozoites were harvested and washed with fresh medium till no radioactivity was detected in the washings. They were then suspended in normal saline and aliquots digested with hymine hydroxide (Packard Instruments Company, Zurich, Switzerland) in scintillation vials at 60° C overnight. After digestion, a few drops of hydrogen peroxide were added followed by 15 ml of scintillation fluid (4g PPO and 100 mg POPOP/litre in toluene diluted with 2-methoxy ethanol 1 : 1 v/v). Radioactivity in total lipid, neutral lipid, phospholipid and nonsaponifiable fractions was directly scanned in a liquid scintillation spectrometer (Packard Model 3330; counting efficiency for ^{14}C was 90% and for ^{3}H was 56%).

Extraction and fractionation of lipids from the amoebae

Lipids of the trophozoites were extracted with chloroform: methanol (2 : 1 v/v) according to Folch *et al.* (1951). Fractionation of total lipids was achieved by chromatography on silicic acid column, neutral lipids were first eluted with chloroform and subsequently phospholipids were eluted with methanol. Total lipids were saponified and nonsaponifiable fractions, were extracted with hexane.

Extraction of lipids from culture filtrates

The culture medium obtained after harvesting the trophozoites was lyophylised and the lipids extracted. Thin layer chromatography of non-saponifiable fractions was carried out on activated silica gel G in chloroform: acetone (9:1 v/v), using *ortho*phosphoric acid : water (1 :1 v/v) for detection of sterols (Liebemann–Burchardt positive compounds).

Analysis of lipids

Free fatty acids were estimated according to Mosinger (1965), sterols according to Zlatkis *et al.* (1953) and lipid phosphorous according to Wagner *et al.* (1962). Radioactivity in the various fractions of the non-saponifiable fraction resolved on thin layer chromatography was measured by adding the scrapped silica gel of un-sprayed zones corresponding to Liebermann-Burchardt positive spots, directly into scintillation vials.

Enzyme assay

Trophozites were homogenised in 0·25 M sucrose buffered with tris-HCl pH 7·2, 20 mM. The homogenate was centrifuged at 105,000 g for 1 h and the pellet resuspended and 5′ nucleotidase (E.C. 3·1·3·5, Leon and Hilmoe, 1955), ATPase (E.C. 3·6·1·3, Post and Sen, 1967), glucose-6-phosphatase (E.C. 3·1·3·9, Swanson, 1950), succinate dehydrogenase (E.C 1·3·99·1 Slater and Bonner, 1952) and cytochrome C oxidase (E.C. 1·9·3·1, Whartson and Tzagolof, 1967) were assayed in this suspension. Another aliquot of the homogenate was centrifuged at 800 g for 12 min to remove nuclear fraction and the supernatant was centrifuged again at 12,000 g for 10 min to remove the mitochondrial fraction. This supernatant was frozen and thawed 6 times to rupture lysosomes and lysosomal enzymes assayed: Cathepsin B (E.C. 3·4·22·1) according to Mycek (1970), acid phosphatase (E.C. 3·1·3·2) according to Wooton (1964), acid deoxyribonuclease (E.C. 3·1·4·5) according to De Duve *et al.* (1955). Protein was assayed according to Lowry *et al.* (1951) using bovine serum albumin as standard.

Results

Lipid composition

H. culbertsoni grown in peptone-mineral-salt-vitamin medium contains 4·26 mg total lipid, 1·96 mg phospholipid, 0·042 mg free fatty acid and 0·46 mg cholesterol equivalent per 10^8 cells. When 1 mg cholesterol/litre of medium was added, the corresponding values were 4·00, 1·42, 0·038 and 0·57 while at 10 mg cholesterol/litre, they were 3·20, 1·12, 0·024 and 0·60 respectively. The non-saponifiable fraction on thin layer chromatography was resolved into five Liebermann-Burchardt positive spots with R_j values, 0·17, 0·38, 0·5, 0·56 and 0·9. The mobility of spot III was identical to an authentic sample of cholesterol on silica gel G as well as on silver nitrate impregnated silica gel G and gave an infrared spectrum superimposable with that of cholesterol. The other fractions are presumably precursors or metabolites of cholesterol or other sterols.

Trophozoite multiplication

Trophozoite multiplication, in the absence of cholesterol continued upto 96. h, beyond which period the cell counts registered a decrease. Addition of 1 mg cholesterol/litre stimulated trophozoite multiplication upto 96 h, while at. 5 mg/ litre cholesterol the stimulation of trophozoite multiplication was upto 48 h. At higher concentrations (10 mg and 20mg/litre) there was no stimulation in growth upto 48 h beyond which period the trophozoites counts were significantly reduced. Presumably, lysis or rupture of the amoebae took place (table 1).

Table 1. Effect of cholesterol on the multiplication of *H. culbertsoni.*

Incubation (h)	Cholesterol added (mg/litre)				
	0	1	5	10	20
	(Trophozoite counts × 10^4/ml)				
0	6·6	6·6	6·6	6·6	6·6
24	25±6	50±8	35±6	20±4	18±4
48	100±10	145±8	130±6	95±5	70±8
72	220±8	280±10	155±8	129±6	138±6
96	280±10	340±14	150±6	100±4	95±4
120	240±8	300±10	120±8	90±6	80±4

All values are Mean ± S.E. of four sets.

Lipid turnover

When traces of [1,2-^{3}H]-cholesterol undiluted with nonradioactive cholesterol were added to the medium, 14% of the radioactivity was recovered in the nonsaponifiable fraction of the amoebae, which was randomly distributed in various fractions (I, 24·6%; II, 6·20%; III, 14·0%; IV, 16·3% and V, 25·8%). Incorporation of [l,2–^{14}C] -acetate in trophozoites, total lipids and phospholipids was significantly, reduced only at 10 mg cholesterol/litre, but incorporation of radioactivity in the non-saponifiable fractions was inhibited at 1 and 10 mg cholesterol although there was no significant reduction in radioactivity of the neutral lipids (table 2). Distribution of radioactivity in various Liebermann-Burchardt positive nonsaponifiable fractions showed that acetate incorporation was specifically inhibited in fraction II and III (cholesterol), while in fractions I, IV and V, acetate incorporation was either not affected or slightly stimulated (table 3).

Addition of cholesterol to the medium did not result in the detection of phosphorlipids in the culture filtrates obtained after harvesting the cells; however, two Liebermann-Burchardt positive spots, one corresponding to cholesterol and the other moving faster than cholesterol were detected on thin layer chromatography The latter, on alkali hydrolysis, gave Liebermann-Burchardt positive spot corresponding to cholesterol and was characterised as cholesterol ester. This ester was also detected in culture-filtrates where cholesterol was not added in the medium.

Table 2. Effect of cholesterol on [1,2-^{14}C] acetate incorporation.

System	Cholesterol (mg/L)		
	None	1·0	10·0
	Incorporation (cpm × 10^4)/1 × 10^8 cells		
Trophozoites (6)	42·0±5·6	35·7±3·0^{NS}	24·5±3·1^a
Total lipids (6)	21·8±2·1	22·0±3·5^{NS}	15·7±2·0^a
Neutral lipids (6)	3·6±0·3	4·2±0·8^{NS}	2·6±0·6^{NS}
Phospholipids (6)	18·0±1·1	17·7±1·6^{NS}	13·6±1·0^b
Nonsaponifiables (3)	0·93±0·05	0·73±0·02^b	3·36±0·02^c

All values are Mean ± S.E. Figures in parenthesis indicate number of experiments.
NS = not significant; *P* values : $a > 0{\cdot}05$, $b > 0{\cdot}02$, $c > 0{\cdot}001$ as compared to controls.

Table 3. Distribution of radioactivity in the five spots obtained on thin layer chromatography of the nonsaponifiable fraction.

	Cholesterol added (mg/litre)		
	none	1·0	10·0
	Distribution of radioactivity (%)		
I	54·0	53·4	69·5
II	21·5	4·1	2·8
III (cholesterol)	7·5	5·5	5·6
IV	3·2	4·1	5·5
V	4·3	5·5	5·6

Effect of cholesterol on lysosomal enzymes

H. culbertsoni contains 13·3 mg protein/1 × 10^8 trophozoites and this was not affected by addition of cholesterol but the activity of lysosomal acid Phosphatase, cathepsin B and acid deoxyribonuclease were increased in presence of exogenous cholesterol (table 4), while membrane-bound 5′ nucleotidase, adenosine triphosphatase, glucose-6-phosphatase, succinate dehydrogenase and cytochrome C oxidase remained unaffected (data not given).

Discussion

The results presented in this paper show that exogenous cholesterol is incorporated and metabolised by *H. culbertsoni.* Incorporation of exogenous cholesterol in the amoebae inhibits lipid biosynthesis (table 2), but there is cholesterol-enrichment

Table 4. Effect of cholesterol on lysosomal enzymes.

Cholesterol added mg/litre	Acid phosphatase[1]	Cathepsin B[2]	Acid deoxy ribonuclease[3]
None	0·52±0·06	0·08±0·01	1·4±0·12
1·0	1·60±0·12[a]	0·11±0·01[a]	14·1±1·2[a]
10·0	1·98±0·10[a]	0·16±0·03[a]	12·1±1·0[a]

[1] and [2] μmol of phenol and tyrosine released/min/mg protein

[3] $\frac{\triangle \text{ OD at 260 nm}}{0{\cdot}001}$ /min/mg protein

All values are Mean ± S.E. of five experiments. Enzyme units/min/mg protein.
P values : *a* > 0·001, *b* > 0·05 as compared to sets without exogenous cholesterol.

of the amoebae. At low concentrations, cholesterol stimulates trophozoite multiplication. This is in agreement with the earlier reports that cholesterol added to TPS-2 axenic medium (Singh *et al.,* 1973) stimulates multiplication of *E. histolytica* (Das and Garg, 1977). Cholesterol-enrichment of the amoebae may lead to expansion of the cell membrane (Jackson and Gotto, 1976), and this may eventually increase cellular proliferation.

It has been observed earlier by Bruckdorfer *et al.* (1969) and Zaidi and Garg (1979) that incubation of erythrocytes or mitochondrial fractions of rat kidney with sonicated dispersion of cholesterol leads to release of membrane-bound phospholipids into the medium. However, in the present study, incubation of the amoebae with exogenous cholesterol for as long as 96 h does not lead to release of membrane phospholipids into the medium, but cholesterol of the medium is esterified. This could be an adaptation for disposing free fatty acids and/or cholesterol. Lysosomal enzymes are also activated when the amoebae is grown in presence of exogenous cholesterol. These results suggest that a similar pattern of events take place when *H. culbertsoni* is exposed to cholesterol of host brain during meningoencephalitis.

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