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Research note

Inhibition by Halogenated Bisphenols of the Growth *in Vitro* of *Entamoeba coli* with a Low Susceptibility to Metronidazole

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Previous investigations in our laboratory disclosed that halogenated bisphenols like bithionol, dichlorophene and hexachlorophene potently inhibited the growth *in vitro* of axenic and xenic strains of *Entamoeba histolytica* (Takeuchi *et al.*, 1984; Kawasaki and Takeuchi, 1984), *Trichomonas vaginalis* (Takeuchi *et al.*, 1985) and *Giardia lamblia* (Takeuchi *et al.*, 1985). Through these studies, we proposed that such bisphenolic derivatives are worth further evaluation, because metronidazole-resistant strains of these protozoa have been increasingly important. Until now, the resistant strains of *T. vaginalis* have been found (Müller *et al.*, 1980), although resistant *E. histolytica* and *G. lamblia* have not been reported.

We previously isolated a strain of *Entamoeba* sp. (strain Olivia) from a patient who had a repeated administration of metronidazole because of the cysts in stool (Kobayashi *et al.*, 1982). Initial preliminary characterization led us to conceive that this was *E. histolytica*; however, later detailed investigations utilizing electron microscopy and immunological techniques indicated that this strain was *Entamoeba coli*. More recently, we isolated strain Olivia II of amoeba from the same patient, which was also characterized to be *E. coli* in the same procedures. Although *E. coli* is not pathogenic, we attempted, on the basis of this history, to examine the susceptibility to metronidazole and the bisphenolic derivatives of this parasite.

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Halogenated bisphenols, i.e., bithionol [2, 2′-thiobis (4,6-dichlorophenol)], dichlorophene [2, 2′-methylenebis (4-chlorophenol)] and hexachlorophene [2, 2′-methylenebis (3, 4, 6-trichlorophenol)], were purchased from Tokyo Kasei Inc. (Tokyo, Japan). Metronidazole was generously supplied by Shionogi Pharmaceutical Co. (Tokyo, Japan). All chemicals were of the highest purity commercially available.

A xenic strain of *E. histolytica* (strain HJ-2: KEIO) and Olivia II strain of *E. coli* were examined in this experiment. Both strains were grown at 35.5°C for 72 hr in screw-capped culture tubes (16×125 mm) containing 15 ml of Balamuth's medium enriched by supplementing 10 % heat-inactivated horse serum. The amoebae were harvested, washed once and finally suspended in the medium to yield 2.5×10^5 trophozoites/ml. One-tenth ml of each suspension was inoculated into 12 screw-capped culture tubes (10×100 mm) containing 5 ml of the medium, and the cultures were centrifuged. Two-tenths ml of the supernatant fluid was removed, and three different concentrations of metronidazole dissolved in dimethyl sulfoxide in amount of 0.1 ml were added in triplicate. The cultivation was further conducted at the same temperature as above. Regarding the bisphenols, 0.175 ml of the supernatant fluid was removed after centrifugation. Subsequently,

Table 1 Effects of metronidazole on xenic *Entamoeba histolytica* (strain HJ-2 : KEIO) and *Entamoeba coli* (strain Olivia II)

Strain	Concentrations of metronidazole (mM)	No. of viable parasites ($\times 10^4$)/culture after starting cultivation (mean ± SD)				
		at 0 (hr)	24	48	72	96
HJ-2	0	2.5	6.5±0.6	34.1±2.8	49.6±6.0	27.3±1.1
	0.012	2.5	2.2±0.2	1.5±0.1	0	0
	0.03	2.5	0.1±0.01	0.1±0.01	0	0
Oliva II	0	2.5	4.3±0.4	8.2±0.6	21.0±2.3	32.2±3.5
	0.12	2.5	4.1±0.7	8.2±0.9	20.5±1.9	32.4±4.4
	0.3	2.5	4.1±0.7	7.4±1.8	20.0±2.5	31.2±5.5

The number of viable parasites was counted thrice on different specimens from the same cultures, and the average number of the three cultures was calculated. The experiments were repeated thrice. The data on Olivia II strain of *E. coli* obtained in the presence of higher concentrations of metronidazole, e. g., 0.6 mM, were not included in this table.

0.075 ml of the solutions of the derivatives prepared as described previously (Takeuchi *et al.*, 1984) was added, and the cultures were processed in the same manner as above. The number of viable parasites in cach culture was counted as described (Takeuchi *et al.*, 1984; 1985) at appropriate time intervals. The twelve cultures were divided into four groups, one of which was set as the control supplemented with the same amounts of the solvents free from the compounds. Another control experiment was attempted by adding the compounds prepared as above and determining the resulting changes in the pH of the medium. Moreover, since rapid death of the concomitant bacteria would result in sterilization of amoebae, it was also examined if metronidazole and the bisphenolic derivatives affected the growth of bacteria by adding the same amounts of the compounds to the medium free from amoebae.

Table 1 summarizes the effects of metronidazole on xenic *E. histolytica* and *E. coli*. The growth of HJ-2 strain was significantly inhibited by 0.012 mM of the compound. However, Olivia II strain exhibited the growth at the same velocity as the control in the presence of 0.3 mM metronidazole. More than 0.6 mM of the compound was needed for killing Olivia II strain under the present experimental conditions (data not shown).

Table 2 indicates the effects of bithionol, dichlorophene and hexachlorophene on the growth of HJ-2 and Olivia II strains. Addition of 0.14 mM bithionol killed virtually all trophozoites of both strains of amoebae within 24 hr. Dichlorophene and hexachlorophene also sterilized the amoebae at comparable concentrations within the same period.

Addition of metronidazole and the bisphenolic derivatives as above did not significantly affect the pH of the medium. Moreover, addition of 0.045 mM metronidazole and 0.28 mM of either of the halogenated bisphenols did not affect the number of concomitant bacteria in the enriched Balamuth's medium.

Our previous experiments on the basis of Weinbach and Garbus (1966) showed that the inhibitory effects of the bisphenols on the growth of axenic *E. histolytica* were enhanced by removing bovine serum from the medium (Takeuchi *et al.*, 1984); accordingly, similar experiments were attempted utilizing Balamuth's medium free from horse serum. The growth of Olivia II strain became significantly slower in this medium, and addition of either of the bisphenolic derivatives at 0.14 mM killed virtually all trophozoites of Olivia II strain in shorter than 8 hr (data not shown).

These findings suggest that xenic *E. histol-*

Table 2 Effects of bithionol, dichlorophene and hexachlorophene on the growth of *Entamoeba histolytica* (strain HJ-2 : KEIO) and *Entamoeba coli* (strain Olivia II)

Strain	Compounds added (mM)	No. of viable parasites ($\times 10^4$)/culture after starting cultivation (mean ± SD)			
		at 0 (hr)	24	48	72
HJ-2	None	2.5	5.8±0.5	31.0±6.4	45.3±2.3
	Bithionol 0.14	2.5	0	0	0
	Bithionol 0.28	2.5	0	0	0
	Dichlorophene 0.14	2.5	0.5±0.09	0.07±0.01	0
	Dichlorophene 0.28	2.5	0	0	0
	Hexachlorophene 0.14	2.5	0	0	0
	Hexachlorophene 0.28	2.5	0	0	0
Olivia II	None	2.5	4.6±0.4	8.2±0.6	21.8±1.9
	Bithionol 0.14	2.5	0	0	0
	Bithionol 0.28	2.5	0	0	0
	Dichlorophene 0.14	2.5	0.9±0.2	0	0
	Dichlorophene 0.28	2.5	0	0	0
	Hexachlorophene 0.14	2.5	0.4±0.2	0.2±0.05	0
	Hexachlorophene 0.28	2.5	0	0	0

Details as described in the text and in the legend to Table 1.

ytica was highly susceptible to metronidazole. Our unpublished data also indicated that axenic strains of *E. histolytica*, *T. vaginalis* and *G. lamblia* were sterilized at comparable concentrations of metronidazole (Takeuchi *et al.*, unpublished observation). However, significantly higher concentrations of metronidazole were needed for killing Olivia II strain. In contrast, xenic *E. histolytica* and *E. coli* were sterilized at the same concentrations of the bisphenolic derivatives as those needed for killing axenic and another xenic strains of *E. histolytica* (Takeuchi *et al.*, 1984; Kawasaki and Takeuchi, 1984). It seems plausible that Olivia II strain of *E. coli* is less susceptible to metronidazole than *E. histolytica*, because both parasites were assayed under the same conditions, and the amounts of metronidazole needed for killing such sensitive parasites as *E. histolytica*, *T. vaginalis* and *G. lamblia in vitro* were always 0.012–0.045 mM as noted above.

At present, we are not aware if most strains of *E. coli* essentially have the lower susceptibility to metronidazole than *E. histolytica*. Our previous experiences on Indo-Chinese refugees suggested that both *E. histolytica* and *E. coli* were removed by administrating the same dose of metronidazole (Tateno *et al.*, unpublished observation), whereas Krishna Prasad (1972) indicated that the amount of metronidazole for sterilizing *E. invadens* and *E. moshkovski* was larger than that for killing *E. histolytica*. The present findings in combination with our previous data on the potent anti-*E. histolytica* action of the bisphenolic derivatives (Takeuchi *et al.*, 1984; Kawasaki and Takeuchi, 1984), however, lead us to conceive that these bisphenols are worth further detailed evaluation against metronidazole-resistant strains of the protozoa.

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短　報

メトロニダゾールに低感受性の大腸アメーバの増殖に対するハロゲン化ビスフェノールの作用

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我々の教室でメトロニダゾールをくり返し投与された症例より分離培養した大腸アメーバ（Olivia II 株）のメトロニダゾールとハロゲン化ビスフェノールに対する感受性を細菌共棲株の赤痢アメーバ（HJ-2：KEIO）のそれと比較した．赤痢アメーバは 0.012 mM のメトロニダゾールで増殖が阻害されたが，Olivia II 株は 0.3 mM の濃度でも増殖は阻害されなかった．ハロゲン化ビスフェノールは 0.14～0.28 mM の濃度で HJ-2 株，Olivia II 株の何れをも24時間以内に殺滅した．これらのデータは最近問題となりつつあるメトロニダゾール耐性原虫についてハロゲン化ビスフェノールに対する感受性を更に詳しく調べる必要があることを示している．