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Cytotoxicity Induced by Bismuth Subcitrate in *Giardia lamblia* Trophozoites

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Abstract—The cytotoxicity of colloidal bismuth subcitrate (CBS) was investigated in cultured *Giardia lamblia* trophozoites on the basis of cell attachment, morphology and viability studies. The effects on cell membrane integrity were evaluated by the permeability to trypan blue, and the morphological alterations were studied by phase-contrast microscopy and transmission electron microscopy. Our data show that although CBS induced loss of cellular viability (morphological and regrowth studies), the cell membrane permeability was not altered. The attachment of *G. lamblia* trophozoites to culture vials was rapidly disrupted by CBS. Ultrastructural observations revealed that this drug promoted modifications on the cell shape, displacement of nucleus and of cytoskeletal structures, pronounced cytoplasmic vacuolization, dorsal and ventral protuberance of cytoplasmic membrane and heavy deposition of electron-dense precipitates in adhesive disc, nuclear membrane and cytoplasmic components. In contrast, membranes and microtubules were apparently undamaged. Some of these results suggest that the cytotoxicity of CBS to *G. lamblia* cultures is the result of its binding to cytoskeletal components. As far as we know, this work is the first demonstration that bismuth subcitrate could kill a human protozoan parasite suggesting its potential in the therapy of giardiasis.

Keywords: *Giardia lamblia*; bismuth subcitrate; ultrastructure; viability; adherence.

Abbreviations: CBS = bismuth subcitrate; HBSS = Hanks' balanced salt solution.

INTRODUCTION

Giardia lamblia is a binucleated flagellated protozoan that causes infection of the small intestine that may give rise to diarrhoea with or without malabsorption. This parasite is endemic throughout the world and it is one of the most frequently found intestinal parasites in children living in developing countries. Although various drugs have been available for several decades to treat this infection, none is entirely satisfactory due to the high incidence of undesirable side-effects, frequent relapses and potential carcinogenic effects. Therefore, there is an obvious need for alternative antigiardial agents.

Recent studies have shown the occurrence of gastric giardiasis concomitant with intestinal giardiasis and a significant association of *G. lamblia* with *Helicobacter pylori* in gastric biopsies (Sanad *et al*., 1996). It is well known that *H. pylori* is a Gram-negative bacterium causing gastric mucosal infection and that is susceptible to a wide range of antimicrobial agents, namely bismuth compounds.

These findings prompted us to study the interaction of colloidal bismuth subcitrate (CBS), used for the treatment of gastric and duodenal ulcers (Lambert *et al*., 1992), with *G. lamblia* trophozoites. The bismuth antimicrobial action is complex and still poorly understood (Domenico *et al*., 1996, Lambert and Midolo, 1997). Thus, in this work we studied the *in vitro* effects of CBS on the viability, adherence and morphology of the parasite to evaluate its potential value in the chemotherapy of giardiasis and to investigate the drug action mechanism(s).

MATERIALS AND METHODS

Chemicals

Bile bovine, L-cysteine, L-ascorbic acid and ferric ammonium citrate were obtained from Sigma Chemical Co.; casitone and yeast extract were obtained from Difco Laboratories and bovine serum and antibiotic solution were obtained from Biochrom K.G. We are indebted to Yamanouchi Pharma for the gift of bismuth subcitrate.

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Parasites and cultures

Giardia lamblia (WB strain, originally from a patient with chronic diarrhoea) was obtained from The American Type Collection (Rockville, MD, USA) (ATCC 30957). Trophozoites were routinely culture in 10 ml of Diamond's TYI-S-33 medium modified by Keister (1983) and supplemented with a commercial penicillin–streptomycin–amphotericin solution. After 2 days, the cultures were harvested by cooling the culture vials, at 4°C, and centrifugation at 1500 rpm for 10 min. Trophozoites were washed three times in Hanks' balanced salt solution (HBSS, pH 7.1) and cell count was performed in a haemocytometer (Neubaeur cell-counter chamber). These cells were used as inoculum for studying the effects of CBS on viability, morphology and adherence of *G. lamblia* trophozoites.

CBS toxicity studies

An inoculum of 10^6 trophozoites was exposed to CBS (1–10 mg/ml) in HBSS (pH 7.1) for 2 hr, at 37°C, using 10-ml polystyrene screw-capped vials. Control experiments were performed in similar experimental conditions without the drug, in the presence only of the drug solvent (HBSS, pH 7.1). After incubation, the vials were cooled at 4°C and the suspension centrifuged at 1500 rpm, for 10 min. Trophozoites were washed three times in cold HBSS and the cell pellets were resuspended in HBSS. Trophozoites viability were directly determined by phase-contrast microscopy, counting the live and dead cells in a haemocytometer (Hill *et al*., 1986). Parasites were considered viable if they had a characteristic pear-shaped structure, flagellar motility, normal architecture of ventral disc and refractory quality. To assess cell membrane integrity, trophozoite suspensions were incubated on ice with an equal volume of 0.4% trypan blue, and loaded into a haemocytometer for viable cell counting (Aley *et al*., 1994). The regrowth assay, based on the ability of viable *G. lamblia* trophozoites to multiply and growth in fresh culture medium after being exposed to a lethal agent, has been extensively used for the determination of parasite viability (Cedillo-Reviera and Muñoz, 1992; Farbey *et al*., 1995; Hempill *et al*., 1996; Hill *et al*., 1996). The reduction of the number of attached trophozoites, after regrowth, as an index of reduction of viability. Thus, 30 μl of each incubation were subcultivated for 48 hr, at 37°C, in 10 ml of fresh TYI-S-33 medium supplemented with antibiotics. Subsequently, the medium was removed by vacuum aspiration and the adherent trophozoites were detached by incubation for 10 min, at 4°C, in ice-cold HBSS. Finally, the number of adherent trophozoites was determined microscopically using a haemocytometer. The experiments were performed three times and the results were expressed as a percentage of viability and of number of attached cells after 48 hr.

Adherence study

The direct effect of CBS on adherence of *G. lamblia* trophozoites was evaluated as previously described by Edlind and Hang (1990). An inoculum of 5×10^5 cells were distributed into vials (10 ml) and were incubated for 2 hr, at 37°C, in TYI-S-33 medium without antibiotics. Preliminary studies showed that the maximum number of cells was attached at this time. The medium with unattached cells was discarded and replaced with prewarmed medium, with and without CBS (1–10 mg/ml), and the cultures were incubated for 2 hr, at 37°C. Adherent cells were dislodged by a 10-min incubation in ice-cold HBSS, and the number of attached cells were determined microscopically using a haemocytometer. The experiments were performed three times and the results were expressed as number of attached cells.

Transmission electron microscopy

Samples were treated as reported previously (Chávez *et al*., 1986). Cell pellets were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.0), post-fixed in 1% osmium tetroxide and uranyl acetate, dehydrated in ethanol and in propylene oxide and embedded in Epon 812 (TAAB 812) resin. Ultra-thin sections were stained with lead citrate and uranyl acetate and observed in a JOEL 100 S electron microscope.

RESULTS

Viability studies

CBS induces loss of cellular viability but does not change the membrane integrity, as evaluated by the permeability of trophozoites to trypan blue (Fig. 1a). The dose–response curve indicates that

Plate 1. (a–f) Transmission electron micrographs of *Giardia lamblia* trophozoites (a) Untreated parasites (20000×); (b–f) Trophozoites exposed to CBS for 2 hr, at 37°C. (b) Note the grotesque modification of cell shape with dorsal protuberance (arrows), displacement of nucleus and fragmentation and displacement of adhesive disc (16000×). (c) Various dorsal protuberances (arrows) and displacement of axonemal microtubules (11500×). (d) Some cells show absence of adhesive disc, displacement of axonemal microtubule with the cytoplasm less electron-dense (23000×). (e) Note electron-dense precipitates on adhesive disc and on nuclear membrane (arrows) (68700×). (f) Pronounced vesiculation (V), blebbing (B) and electron-dense precipitates are also found on adhesive disc (arrows) (11500×). (N = nucleus; AD = adhesive disc; FA = axonemal microtubules).

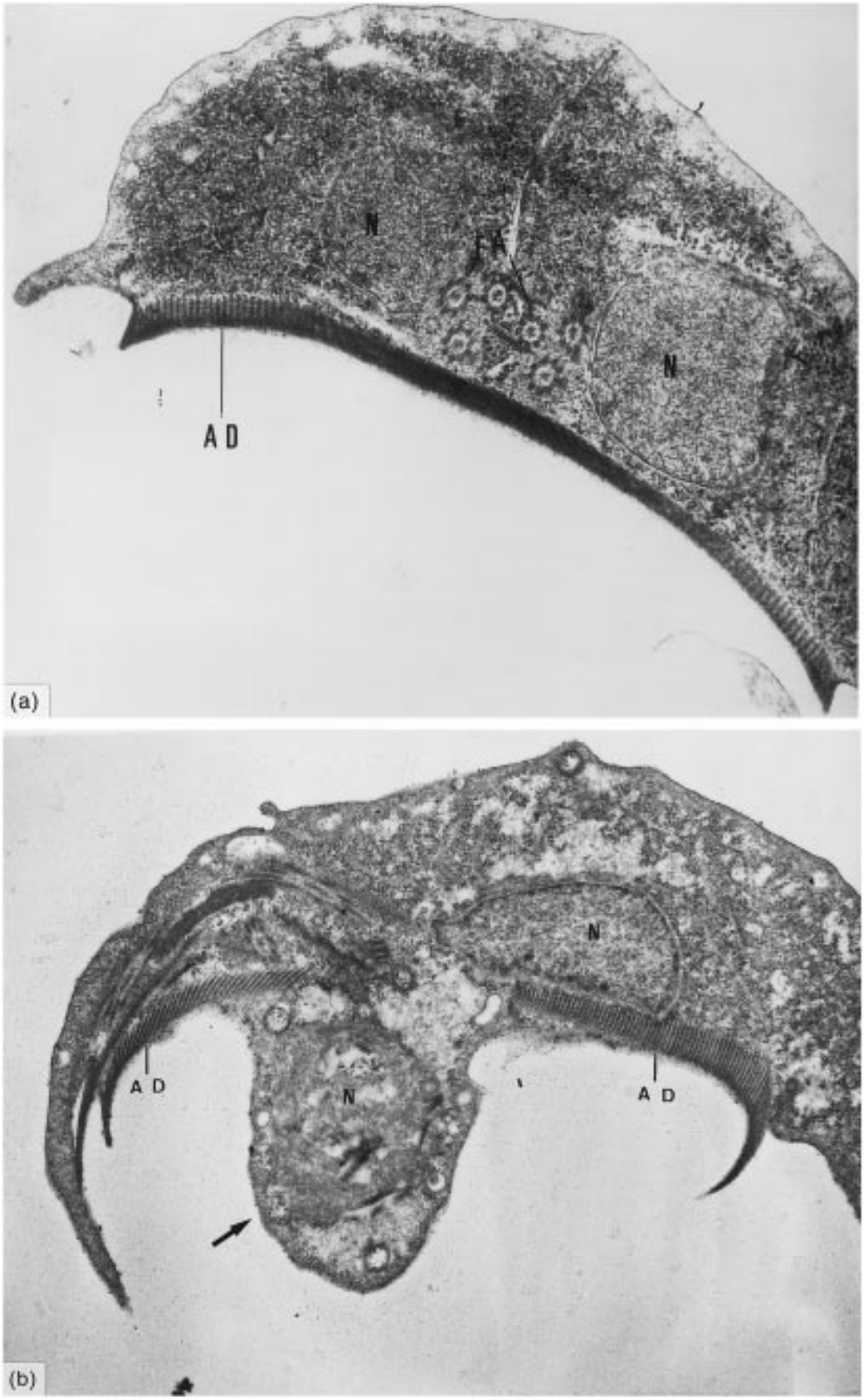
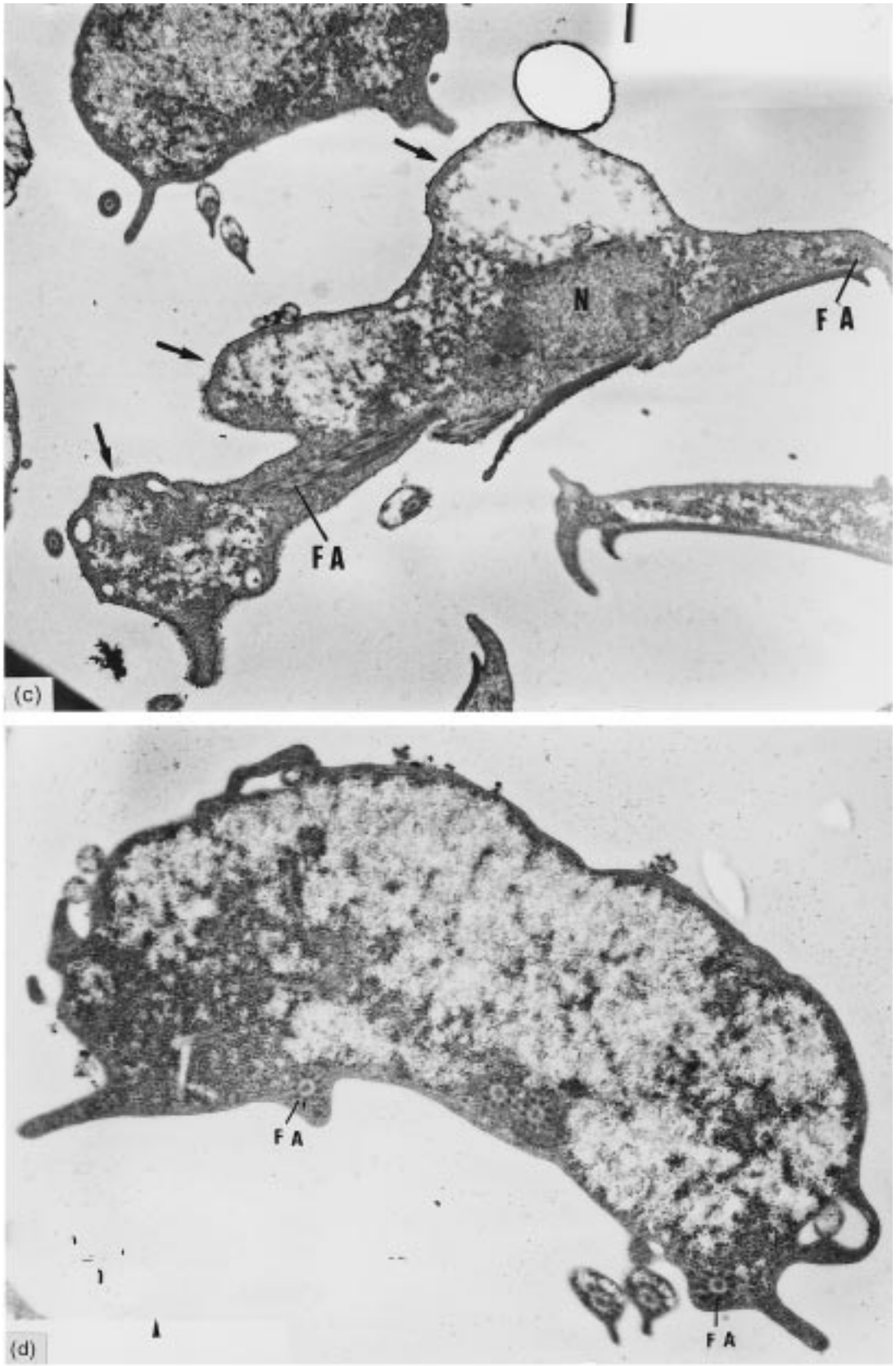


Plate 1 [*continued overleaf*]



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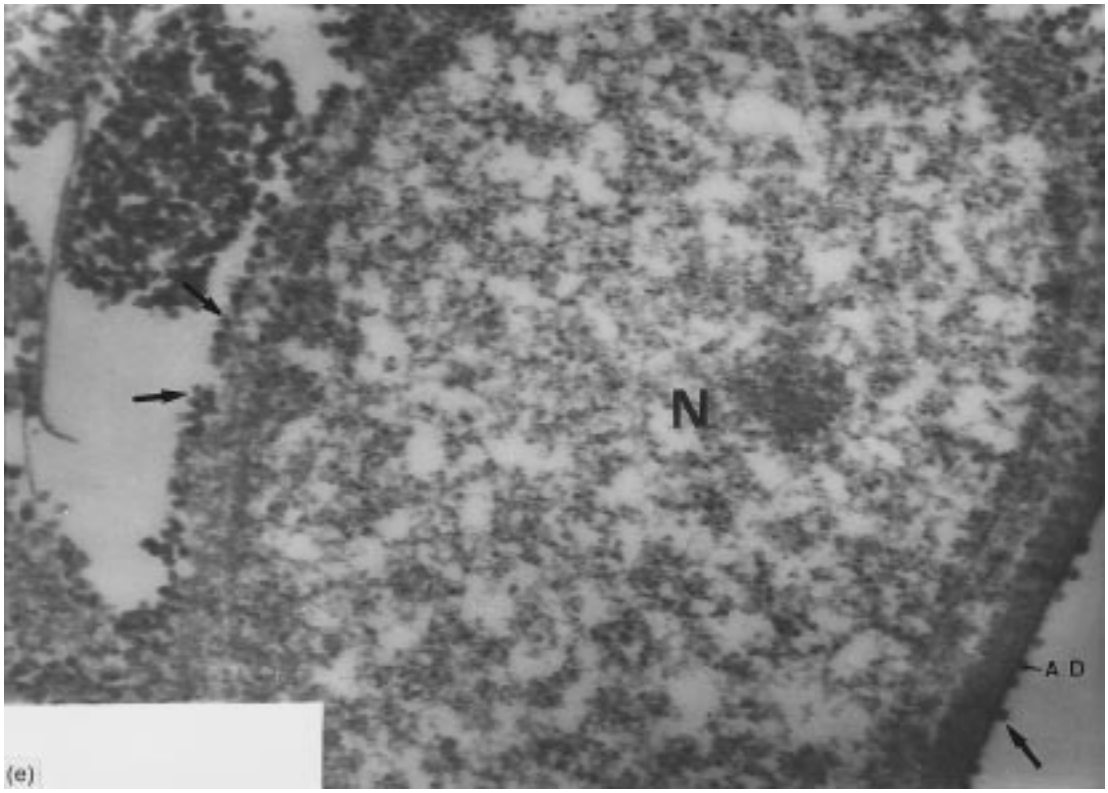

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CBS (1–10 mg/ml) induced the death of 11–90% of *G. lamblia* trophozoites as evaluated by the morphological criteria (Fig. 1a). The killed parasites show, by phase contrast microscopy, a typical pear-shaped structure, refractory quality and loss of flagellar motility (not shown).

The results, obtained by regrowth assays, point to a 22–100% decreases in the number of adherent cells over 48 hr, after exposure to CBS (1–10 mg/ml) (Fig. 1a). These results confirm the lethal effect of CBS and are correlate very well with those obtained by morphological criteria.

Adherence inhibition

G. lamblia trophozoites can attach to *in vitro* cultured cells as well as to glass and to a variety of artificial substrates (Kulda and Nohynková, 1995). Considering that inhibition of its attachment may have therapeutic potential, it was of interest to study the ability of CBS to promote the *G. lamblia* detachment. Figure 1(b) shows that *G. lamblia* attachment to plastic culture vials is strongly inhibited by CBS.

Ultrastructural effects of CBS on trophozoites

Transmission electron microscopy of untreated *G. lamblia* trophozoites shows that these cells have a typical cytoskeleton with four pairs of flagella and adhesive disc, nucleus, and cytoplasmic membrane (Plate 1a). CBS induces morphological changes in cells after exposure for 2 hr, at 37°C. Trophozoites show modifications of cell shape, dorsal and ventral protuberance, displacement of nucleus and of cytoskeletal structures and fragmentation of adhesive disc (Plate 1b,c). Frequently, flagella appear intact but irregularly dispersed in the cytoplasm (Plate 1d). Moreover, electron dense deposits are much more evident on adhesive disc, nuclear membrane and other cytoplasmic components in trophozoites treated with CBS (Plate 1e,f). In some cells we can see pronounced cytoplasmic vesiculation and vacuolization as well as surface protrusions (blebs) (Plate 1f).

DISCUSSION

Bismuth salts have been used in the treatment of gastric and duodenal ulcers, and were the first antibacterial drugs used in the eradication of *H. pylori* infection (Coghlan *et al.*, 1987). Considering the occurrence of *G. lamblia* in the gastric mucosa and the association of *G. lamblia* with *H. pylori* in gastric biopsies, we studied the cytotoxicity of bismuth

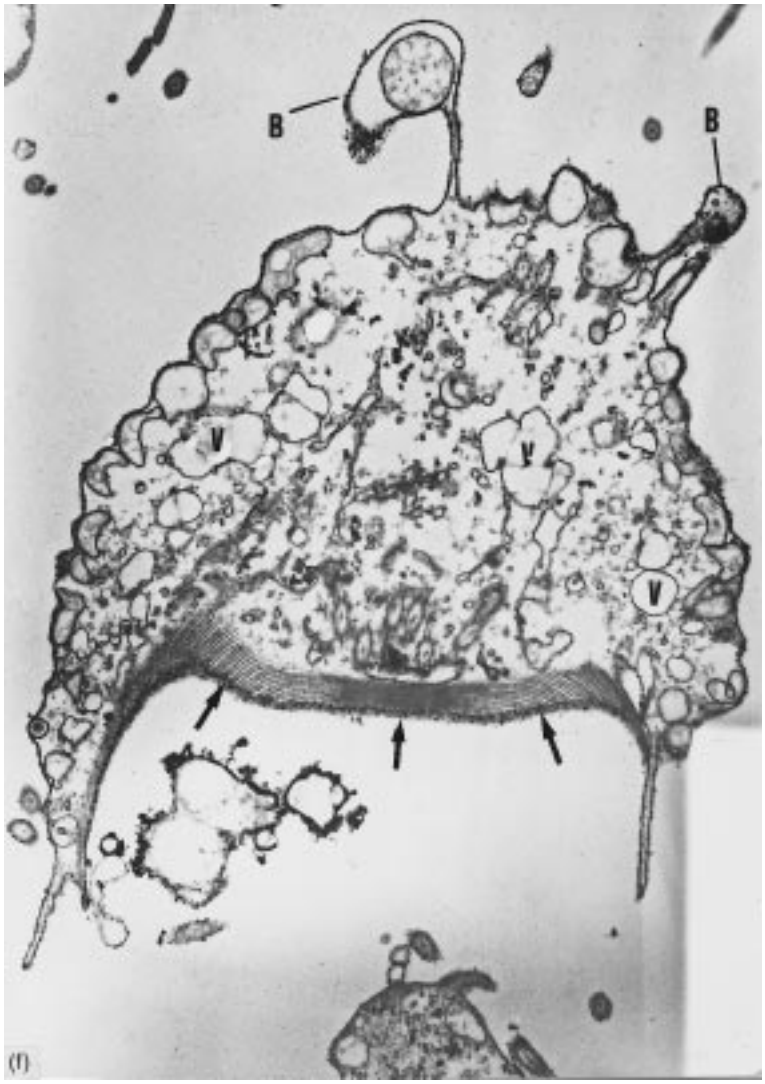

Plate 1 [*continued*]

subcitrate in *G. lamblia* trophozoites to evaluate its potential use in therapy of giardiasis.

Adherence of *G. lamblia* trophozoites to enterocytes is essential for colonization and is considered to play a crucial role in the pathogenesis of giardiasis (Farthing *et al.*, 1997). Therefore, inhibition of parasite attachment may have therapeutic potential. The present study demonstrates that CBS inhibits the *G. lamblia* trophozoites attachment *in vitro* and induces loss of cell viability. Although the permeability of cells treated with CBS does not change, the lethal effect of CBS was demonstrated by the regrowth assay. Our data have additional implications regarding the clarification of drug action mechanism(s). Considering that microtubules are the major components of the adhesive disc, which mediates the cell attachment, and of the cytoskeleton, which is essential for maintaining the cell structure (Kulda and Nohyhová, 1995), our results suggest an interaction of bismuth subcitrate with *G. lamblia* microtubules that could be the basis of its antigiardial action. This is supported by ultrastructural observations and adherence studies. Actually, trophozoites treated with CBS show modifications of cell shape, fragmentation and displacement of adhesive disc components, electron-dense deposits in adhesive disc, irregular dispersion of axonemal microtubules in the cytoplasm and detachment of *G. lamblia* cells.

It has been reported that the important feature of antigiardial chemotherapy may be the concentration at the site of infection rather than the drug plasma levels (Reynoldson *et al.*, 1992), and that bismuth subcitrate is a safe drug which exerts local effects

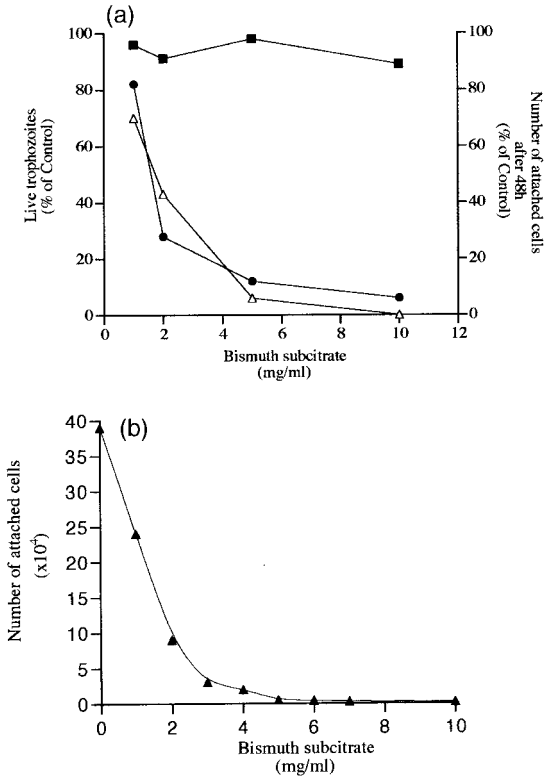

Fig. 1. (a) Killing of *Giardia lamblia* trophozoites by bismuth subcitrate. Trophozoites were incubated with various concentrations of CBS for 2 hr at 37°C, and trophozoite viability was determined by trypan blue exclusion (■), morphology (●) and regrowth assay (number of attached cells after 48 hr) (△). (b) Effect of bismuth subcitrate in adherence of *G. lamblia* trophozoites to the culture vessel after 2 hr of drug exposure. These dose–response curves are typical of three independent experiments and error bars are not indicated, since for the most part, they are encompassed by the size of the symbols.

on gastroduodenal mucosa. In therapy regimens for the treatment of gastric and duodenal ulcer the usual daily dose of CBS is 480 mg and the treatment period is usually 2 wk (Reynolds, 1996; Robinson *et al*., 1997). Considering these findings, our data suggest that CBS can be effective in the treatment of giardiasis and may be particularly useful in associated *G. lamblia* and *H. pylori* infections.

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