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Biliary Lipids Support Serum-Free Growth of *Giardia lamblia*

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***Giardia lamblia* has been grown in vitro only in media containing serum or serum fractions. How this pathogen can grow in the human small intestinal lumen without serum is not known. We found that samples of human hepatic or gall bladder bile maintained *G. lamblia* survival for 24 to 48 h in medium without serum but did not support growth. By contrast, an artificial biliary lipid dispersion containing six bile salts, phosphatidylcholine (PC), and cholesterol, in the ratios characteristic of human bile, supported parasite growth in medium without serum or serum fractions. To define the requirements, we showed that 1-palmitoyl-2-linoleoyl-PC or 1-palmitoyl-2-oleoyl-PC (which predominate in human bile) satisfied the requirement for PC. Moreover, either glycocholate or glycodeoxycholate could be substituted for the bile salt mixture. The finding that biliary lipids can support serum-free growth of *G. lamblia* may help explain why this parasite colonizes the upper small intestine.**

Giardia lamblia is an important cause of waterborne intestinal disease in the United States, as well as in less-developed countries (2). The flagellated trophozoite form of this parasite specifically colonizes the upper small intestine of humans by attaching to mucosal epithelial cells or "swimming" in the intestinal fluid. Thus, trophozoites are exposed to complex and changing mixtures of host nutrients, digestive enzymes, and bile. The harshness of this environment is emphasized by the fact that the normal bacterial flora of the duodenum and jejunum is relatively sparse ($<10^5$/ml [7]).

We are interested in the physiological and biochemical adaptation of *G. lamblia* to the small intestinal environment. Since *G. lamblia* has been grown in vitro only in complex media with either serum (4, 5, 16, 17) or a lipoprotein-cholesterol serum fraction (25), we now ask how *G. lamblia* can grow in the intestinal lumen without serum.

Jarroll et al. suggested that serum might supply lipids to *G. lamblia*, since these parasites appear to have a limited capacity for de novo lipid biosynthesis (15). In 1937, Hegner and Eskridge proposed that bile might favor growth of *Giardia* sp. in vivo (12). Farthing et al. (5, 6) proposed that *G. lamblia* might obtain its lipids from bile in the small intestine. Recent studies have shown that in the presence of serum, growth of *G. lamblia* is stimulated by bovine (6, 16), porcine, human, and guinea pig bile, thereby reducing the generation time by up to 30% (6). Furthermore, bovine bile and taurocholate stimulate the uptake of lecithin from medium containing serum (5). The studies presented here were designed to test the hypothesis that bile or biliary components might provide *G. lamblia* with some of the same nutrients supplied by serum in vitro. In light of the studies of Jarroll et al. (15) and Farthing et al. (5), biliary lipids (BL) were prime nutrient candidates for *Giardia* growth.

The major BL are bile salts and phospholipids, which stimulate the excretion of cholesterol in mixed micelles (1, 14). Biliary phospholipids are interesting in that their composition is relatively homogeneous and constant among the mammalian species studied. The biliary phospholipids are virtually all (>97%) phosphatidylcholine (PC), with little phosphatidylethanolamine. Moreover, biliary PCs have a restricted fatty acid composition, with palmitic acid in position 1 (~70%) and oleic acid (~15%) or linoleic acid (~30%) predominating in position 2 (18).

We now report for the first time that defined mixtures of biliary lipids containing bile salts, synthetically prepared pure phosphatidylcholines (PC) and cholesterol (C), in the proportions characteristic of human bile, support growth of *G. lamblia* in medium free of serum or serum fractions.

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MATERIALS AND METHODS

Parasites and cultivation. *G. lamblia* WB (ATCC 30957) was isolated from the duodenal fluid of a 27-year-old male with symptomatic giardiasis of 2.5 years duration (22). Trophozoites were grown in filter-sterilized Diamond TYI-S-33 medium (4) with 10% adult bovine serum and 1% fetal calf serum, modified by adding bovine bile (500 μg/ml, bacteriological use; Sigma Chemical Co., St. Louis, Mo.) as described before (6, 16), doubling the cysteine concentration (16), and omitting the ferric ammonium citrate, vitamin mixture, and antibiotics (10). Parasites were grown at 37°C, subcultured twice weekly, and enumerated with a hemacytometer.

Trophozoites in late log phase were used in all experiments. Nonattached parasites were discarded with the growth medium. Attached trophozoites were rinsed once with Hanks balanced salt solution supplemented with 5 mM cysteine hydrochloride, pH 7.0, and were then detached by chilling, washed twice by centrifugation, and resuspended in this buffer.

Experimental media and procedures. The basal experimental medium was adapted from growth medium without bile to resemble duodenal fluid more closely. First, the serum was eliminated and the phosphate buffer was replaced by bicar-

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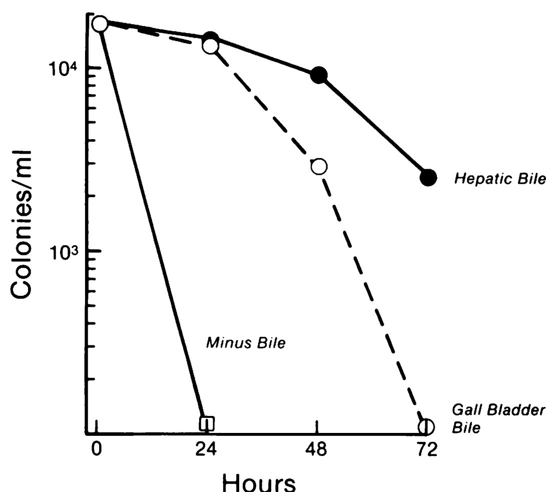

FIG. 1. Effect of human bile on survival of *G. lamblia* in serum-free medium. In this experiment, the final concentration of bile salts from hepatic bile was 0.21 mM, and from gallbladder bile was 0.27 mM; concentrations of cholesterol were 11 and 9 μM, and those of PC were 86 and 50 μM, respectively. Symbols: □, no bile added; ○, gall bladder bile; ●, hepatic bile.

bonate (10 mM), which neutralizes the duodenal fluid in vivo (3). Second, since the concentration of nutrients varies greatly, we decreased the concentrations of Biosate peptone (BBL Microbiology Systems, Cockeysville, Md.) twofold (to 1.5% [wt/vol]) and reduced the glucose concentration to 0.6% (33 mM). Since the growth medium is somewhat hypertonic, we eliminated the NaCl and prepared the experimental medium in Hanks balanced salt solution with 5 mM L-cysteine and 5.5 mM ascorbic acid as reducing agents. The final pH was 7.0. Twentyfold concentrates of Biosate, glucose, and ascorbic acid were brought to pH 7.0 with NaOH, filter sterilized, and stored frozen. Cysteine was dissolved in Hanks salts without HCO_3 and neutralized for each experiment. $NaHCO_3$ was prepared for each experiment. Piperacillin (500 μg/ml; Lederle Laboratories, Pearl River, N.Y.) and amikacin (125 μg/ml; Bristol Laboratories, Syracuse, N.Y.), which do not affect parasite growth (8), were added to prevent bacterial growth, since filtration might remove essential bile components. Parasites (2×10^4 to 5×10^4/ml, final concentration) were added to 1-dram (4.7 ml) borosilicate glass screw-cap vials containing experimental medium, bile, or BL as specified in a final volume of 4 ml.

The vials were capped tightly and incubated upright at 37°C. Cultures were assessed with an inverted microscope, and their viability was determined by colony assay at 24-h intervals (9). This assay was used because the ability of a cell to multiply is a more stringent criterion for viability than microscopic or electronic counting in the present context. Vials were chilled on ice and mixed to detach parasites. If necessary, because of high cell concentration, parasites were diluted in cold medium. Single-cell suspensions were added to duplicate tubes of complete TYI-S-33 growth medium (with antibiotics, bovine serum, and bile) containing 0.15% melted agarose. After mixing, the medium was solidified by chilling on ice. Individual viable parasites grew into visible colonies after 5 to 6 days of incubation at 37°C. The data are given as number of colonies per milliliter (mean number of colonies per tube divided by the volume). Duplicates usually varied by <20%. All experiments were repeated at least twice.

Bile and BL. Hepatic bile came from a postcholecystectomy patient with an externally draining T tube, and postmortem gallbladder bile was from a neonate who had died of cardiac arrest. Optimum dilutions of each bile sample were determined in preliminary experiments.

All lipids were from Sigma Chemical Co., unless otherwise stated. For concentrated stock BL preparations, cholesterol and PC (crude egg yolk or pure synthetic) in hexane were added to glass vials and the solvent was evaporated under N_2. Bile salt solution (144 mM, final concentration) was added to disperse the PC (42 mM, final concentration) and C (14 mM, final concentration) with vortexing and then rocking for 24 h. BL (5 mM) (see below) contained 3.6 mM bile salts (1.08 mM each glycocholate and glycochenodeoxycholate, 0.54 mM glycodeoxycholate, 0.36 mM taurocholate and taurochenodeoxycholate, 0.18 mM taurodeoxycholate), 1.05 mM PC, and 0.35 mM cholesterol.

Purity of bile salts was checked by thin-layer chromatography and was always >95% (usually 98%). Since many bile salts are hygroscopic, their concentrations in solutions and bile were determined by the 3-alpha hydroxysteroid dehydrogenase assay with cholate as the standard (24). PC and cholesterol determinations were done according to published methods (11, 19).

RESULTS

Effect of human bile on survival of *G. lamblia*. More than 99% of trophozoites died in <24 h in experimental medium

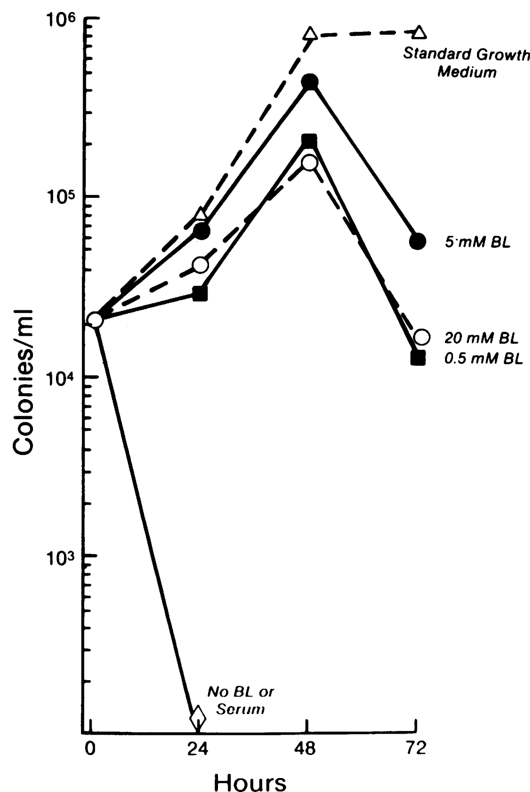

FIG. 2. BL support growth of *G. lamblia* in serum-free medium. Parasite growth in experimental medium with various concentrations of BL (containing crude PC) was compared with that in standard growth medium with bovine bile (Sigma) and serum. Symbols: (◇, no BL or serum; ■, 0.5 mM BL; ○, 20 mM BL; ●, 5mM BL; △, standard growth medium.

without serum or bile (Fig. 1). The addition of human gallbladder bile or hepatic bile prolonged survival for 24 to 48 h, but did not support parasite multiplication. Moreover, subsequent samples of hepatic bile from the same subject appeared toxic to the parasites (not shown).

Effects of BL on serum-free growth of *G. lamblia*. To identify the components of whole bile which prolonged survival and may in addition promote parasite growth, we tested a BL mixture based on the dominant lipid composition of human bile (1, 22). The ratio of primary to secondary bile salts was 2:1 and 3:1 for glyco-conjugated to tauro-conjugated bile salts (as described in Materials and Methods).

In initial experiments, BL not only prolonged parasite survival, but supported multiplication for 48 h (Fig. 2). The number of parasites increased with BL concentration from 0.5 to ~5 mM (total lipid added) and decreased at BL concentrations of 20 mM and above. At 5 mM BL, the initial doubling time (8.8 h) was close to that in standard growth medium containing serum and bovine bile (7.5 h), although the maximum yield of cells was approximately half. In 10 experiments, the number of colonies per milliliter increased from 2×10^4 to 2.7×10^4, to 1.3×10^5 to 4.7×10^5 in 48 to 72 h with 5 mM BL. Cell number increased by an average of 9.6-fold (range, 4.4- to 23.5-fold). After the peak of growth, viability tended to decrease (Fig. 2). However, in five other experiments in which the initial inoculum of cells was halved, this decrease was much less pronounced (see Fig. 4). Colony number increased from 10^4/ml to 8×10^4 to 28×10^4/ml, with an average 16.6-fold increase in cell number (range, 8 to 28-fold).

Lipids extracted from *G. lamblia* grown in medium with serum included phosphatidylethanolamine and sphingomyelin, as well as PC (15). Moreover, the neutral lipid fraction contained both esterified and nonesterified sterols (15). Bile has mainly free C, while serum C is esterified. Addition of each of these lipids to complete BL medium neither increased the rate or extent of growth nor delayed the decline in viability (Fig. 3). The only exception was a slight (~20%) stimulation by sphingomyelin, which was consistent from one experiment to the next.

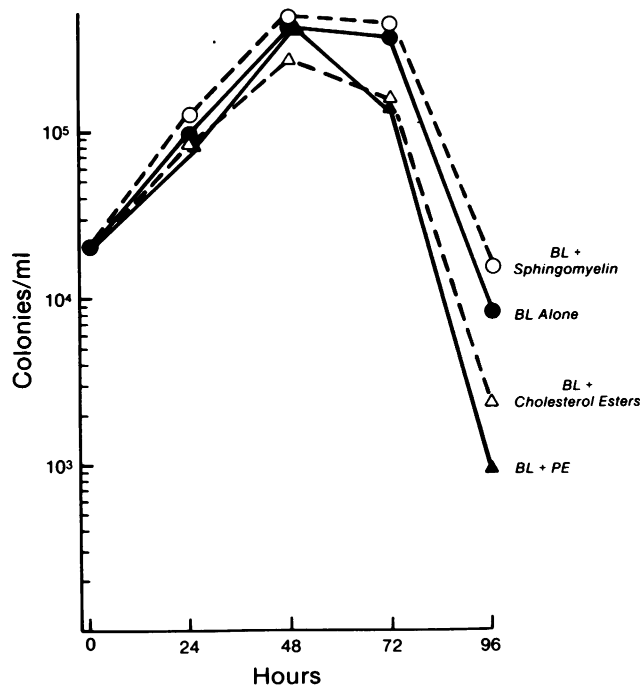

FIG. 3. Effect of cholesterol esters and additional phospholipids on *G. lamblia* growth in BL medium. Supplementation of BL medium with crude PC (●) with sphingomyelin (○, 0.18 mM), crude phosphatidylethanolamines (▲, 0.18 mM), or cholesterol esters (△, cholesterol palmitate and oleate, 0.5 mM each), did not stimulate parasite growth. The additional lipids were dispersed in the concentrated BL, as described in the text.

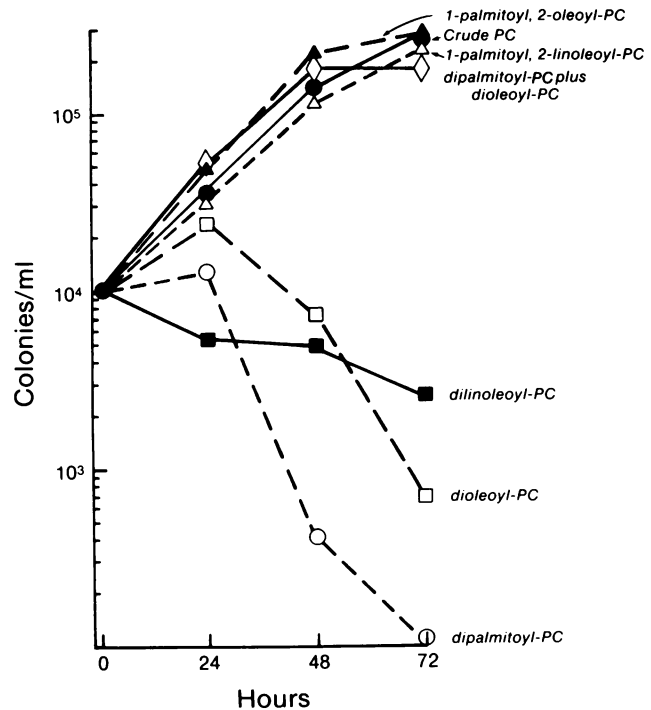

FIG. 4. Defined synthetic PCs support growth of *G. lamblia* in serum-free medium. Stock BL dispersions were prepared with the indicated synthetic PC (purity, ~99% by thin-layer chromatography), singly or in combination, substituting for the crude egg yolk PC. In each case, the final PC concentration was 1.05 mM. Symbols: ●, crude PC; ○, dipalmitoyl-PC; □, dioleoyl-PC; ■, dilinoleoyl-PC, △, 1-palmitoyl-2-linoleoyl-PC; ▲, 1-palmitoyl-2-oleoyl-PC, ◇, dipalmitoyl-PC plus dioleoyl-PC (1:1).

Defining the requirements for BL. Since undefined PC was used in the initial experiments, we explored the phospholipid requirements of the BL medium by testing the ability of pure, synthetic PC to support growth of *G. lamblia*. The species of PC tested were those that predominate in human bile.

The compound 1-palmitoyl-2-oleoyl-PC or 1-palmitoyl-2-linoleoyl-PC (or a 2:1 combination, not shown) supported *Giardia* growth as well as did crude PC (Fig. 4). Moreover, mixtures of dipalmitoyl-PC with either dioleoyl-PC or dilinoleoyl-PC or both (not shown) were also as effective as the foregoing compounds (Fig. 4). By contrast, parasite viability decreased in BL prepared with dipalmitoyl-, dioleoyl-, or dilinoleoyl-PC alone. Evidently, *G. lamblia* needs PC with both saturated and unsaturated fatty acids. It is interesting that these fatty acids need not both be on the same molecule.

To define further the requirements for the bile salt components of BL, we substituted individual bile salts at 3.6 mM, the total concentration of the six bile salts in the standard mixture (at 5 mM). Bile salts are amphipaths which

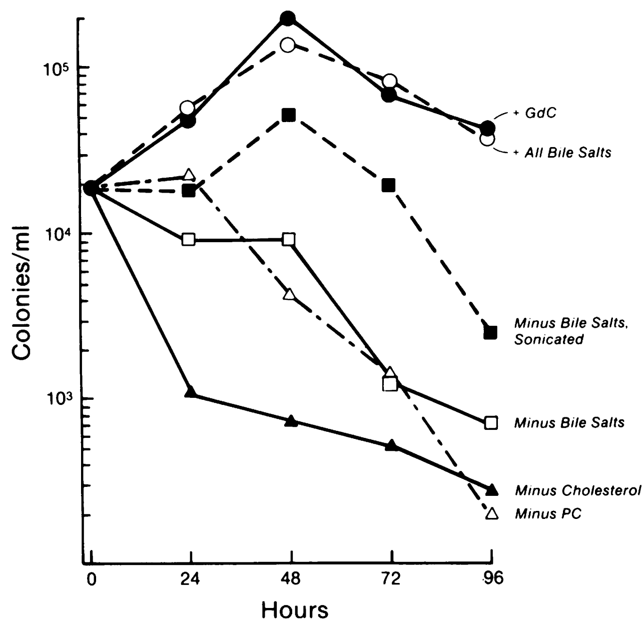

FIG. 5. Dependence of serum-free growth upon bile salts, PC, and cholesterol. Survival and growth of *G. lamblia* trophozoites were compared in experimental medium with glycodeoxycholate (GdC) versus six bile salts. In addition, the effect of omitting bile salts, PC, or cholesterol was assessed. Pure dipalmitoyl-PC plus dioleoyl-PC (0.52 mM each) were used in this experiment. Symbols: ○, all bile salts; ●, GdC only; □, minus bile salts; ■, minus bile salts and sonicated; △, minus PC; ▲, minus cholesterol.

undergo concentration-dependent self-aggregation (20) and have remarkable abilities to disperse phospholipids in micellar form (1, 14). The CMC (critical micellar concentration or midpoint of the concentration range in which micellization is important) of glycocholate is relatively high (10 mM in 0.15 M NaCl), while that of glycodeoxycholate is low (2 mM [20]). The data in Fig. 5 show that glycodeoxycholate alone supported growth as well as did the "physiological" mixture of bile salts. In other experiments (not shown), glycocholate alone was as effective as the bile salt mixture. If bile salts were omitted, the parasites survived without multiplication for 24 h, but then died (Fig. 5). However, if the PC and cholesterol were sonicated (1 min, 70% setting, 4°C; Bronwill sonifier), survival was prolonged and some parasite multiplication was observed, indicating that dispersal of PC and cholesterol was an important function of the bile salts (Fig. 5). In the same experiment, the trophozoites died if PC or cholesterol or both (not shown) were omitted from the BL (Fig. 5).

The sterol requirement for *Giardia* growth in BL medium could be satisfied by β-sitosterol (24-ethylcholesterol) or stigmasterol (3β-hydroxy-24-ethyl-5,22-cholestadiene) and (less well) by cholesterol palmitate (Fig. 6).

DISCUSSION

These studies have shown that defined mixtures of pure BL support growth of *G. lamblia* in serum-free medium. The growth requirements were satisfied by one or more natural bile salts, cholesterol, and PC containing palmitic and either oleic or linoleic acid.

It is likely that the cholesterol and PC are essential for biosynthesis of trophozoite membranes (15). We do not know whether the bile salts are metabolized by *G. lamblia*, although uptake of taurocholate has been reported (5). It seems likely that a major role of bile salts is to disperse cholesterol and PC in micellar form, thereby promoting uptake of these lipids by *G. lamblia*, since sonication increased growth in media with PC and cholesterol but lacking bile salts. The parasite does not deconjugate bile salts (24), and is extremely tolerant of conjugated bile salts (50% lethal dose, >40 mM [unpublished data]). A 5 mM concentration of BL contains 3.6 mM bile salts. The total bile salt concentration in the human small intestine decreases with distance along the small intestine from ~20 to 2.5 mM (22).

Notably, both glycodeoxycholate (CMC, ~2 mM [20]) and glycocholate (CMC, ~10 mM) supported growth in BL medium. Despite the high CMC of pure glycocholate, it is likely that it does help disperse the PC and C for the following reasons. First, phospholipids tend to lower the CMC of bile salts, and second, bile salts aggregate over a much wider concentration range than most other detergents (14, 20).

Because they form micelles, bile salts may favor growth of *G. lamblia* in the duodenum in another way. Products of lipolysis such as unsaturated free fatty acids are toxic to trophozoites in vitro (13; Reiner, Gillin, and Zenian, Am. J. Trop. Med. Hyg., abstr. no. 144, 1984; Reiner and Gillin, J. Infect. Dis., in press), yet *G. lamblia* flourishes in the upper small intestine. We have recently shown that bile salts, above their CMC, tend to protect trophozoites from killing by oleic acid (Reiner and Gillin, in press).

By subculturing trophozoites at more frequent intervals (48 to 72 h), we maintained them in BL medium for 24 serial subcultures. Growth was variable; the increase in cell num-

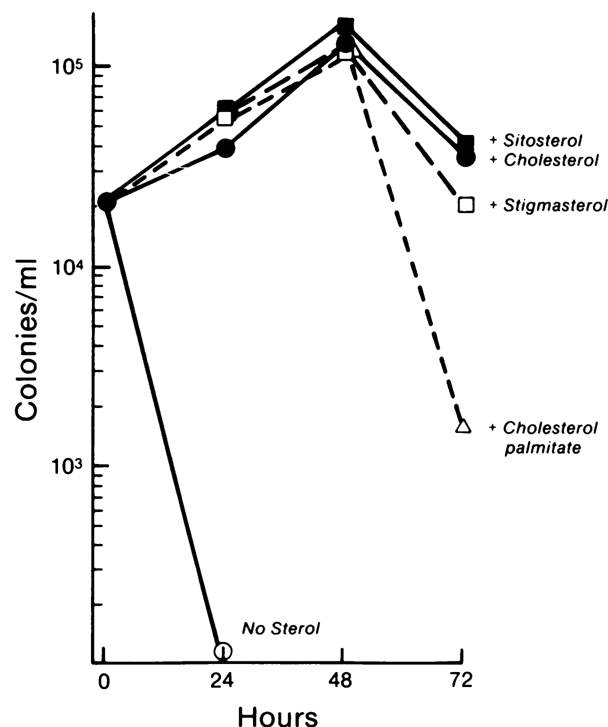

FIG. 6. Ability of sterols and sterol esters to support growth of *G. lamblia*. Parasites were grown in defined BL medium without added sterol (○) or with cholesterol (●), cholesterol palmitate (△), sitosterol (■), or stigmasterol (□) at 0.35 mM. The BL contained dioleoyl- and dipalmitoyl-PC, 0.52 mM each.

ber ranged from none to sevenfold. This indicates that BL do not fulfill all the functions of serum. In complementary studies (Gillin et al., Am. J. Trop. Med. Hyg., abstr. no. 252, 1985), we found that other factors, such as iron (which is excreted in bile) and duodenal mucus, increased growth of *G. lamblia* in serum-free BL medium in both initial and serial subcultures. Mucus increased the cell number 1.5- to 5-fold and also ameliorated the postpeak decline in cell number.

Previous studies of growth stimulation by whole bile or bile components (5, 6, 16) were performed in medium containing serum. In another recent report (25), trophozoites were grown with a lipoprotein-cholesterol serum fraction and albumin.

Our studies are the first to show multiplication of *G. lamblia* in the absence of serum or serum fractions. They were undertaken to increase understanding of how *G. lamblia* colonizes a specific serum-free milieu, rather than to devise a serum substitute for routine culture. The data strongly support the idea (5, 12) that biliary lipids may be important for the growth of *G. lamblia* in vivo, and may help to determine the specific colonization of the small intestine by this pathogen.

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