JOURNAL OF INVERTEBRATE PATHOLOGY 33, 159–170 (1979)

Schistosome Sporocyst-Killing Amoebae Isolated from *Biomphalaria glabrata*

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Received January 30, 1978

Explants and swabs from the pericardium and mantle of three strains of *Biomphalaria glabrata*, two of them resistant to infection with *Schistosoma mansoni*, have yielded small amoebae, 3–5μm in diameter, in culture. These amoebae have been grown axenically through >50 passages to date. The amoebae form cysts in dense cultures. When mixed with *S. mansoni* mother sporocysts in vitro, the amoebae adhere to and kill the trematodes within several hours. For 1–2 days thereafter, the amoebae proliferate rapidly at a generation time of about 5 hr, then return to normal growth. Sonically disrupted sporocysts also induce proliferation. Live sporocysts do not attract the amoebae or emit soluble substances which influence amoebal growth. Amoebae also adhered to and killed *S. mansoni* daughter sporocysts and cells derived from *B. glabrata* embryos; however, they did not harm *S. mansoni* cercariae or rediae of other trematode species. The proportion of mantle explants yielding amoebae was significantly higher ($P<0.05$) in one of the resistant snail strains than in the susceptible strain; however, whether amoebae contribute to snail resistance is unknown. Exposure of snails to *S. mansoni* miracidia did not influence the proportion of snails yielding amoebae.

KEY WORDS: Schistosome; *Schistosoma mansoni;* sporocyst, amoebae; snail; *Biomphalaria glabrata*.

INTRODUCTION

Strains of the aquatic snail *Biomphalaria glabrata* differ greatly in their susceptibility to infection by individual strains of the blood fluke *Schistosoma mansoni* (Richards, 1975a; Basch, 1976). Conversely, strains of *S. mansoni* vary in infectivity for individual strains of *B. glabrata* (Richards, 1976). Snail susceptibility is genetically determined (Newton, 1953; Richards, 1975a) as is trematode infectivity (Wright, 1974). The genetic factors governing snail susceptibility exert their screening effects upon the mother sporocyst after its penetration of the snail as a miracidium. In resistant snails a vigorous host cellular response results in amoebocytic encapsulation of the sporocyst within 24–48 hr after snail penetration (Newton, 1952; Barbosa and Barreto, 1960; Lie et al., 1977). This innate cellular immunity is generally thought to be the "basic underlying mechanism of specificity" (Basch, 1976), but the possible involvement of soluble hemolymph factors (nutritional factors, agglutinins, growth inhibitors, etc.) and of other snail cells has not been determined (see review by Cheng, 1968). Moreover, whether encapsulation of sporocysts is the cause or the result of sporocyst death is unknown.

During attempts to culture cells of the amoebocyte-producing organ of Lie et al. (1975) and to study the response of these cells to mother sporocysts in vitro, we obtained from many explants of pericardium (and later mantle) excellent growth of a small amoeba which proved capable of adhering to and rapidly destroying sporocysts in vitro. In this paper we describe this amoeba and its destructive effects upon mother sporocysts of *S. mansoni*.

MATERIALS AND METHODS

Schistosomes. The schistosomes used were of a Puerto Rican (PR) strain (NIH-Sm-PR-1) and an Ethiopian strain of *S. mansoni*. Both were provided (in infected mice) by the U.S.–Japan Cooperative Med-

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ical Science Program, National Institute of Allergy and Infectious Diseases.

Culture of sporocysts. Miracidia were hatched in sterile artificial spring water (Ulmer, 1970) from eggs obtained from the homogenization in cold 1.7% saline of livers removed from 6- to 8-week-infected adult mice. After two washings in sterile spring water, using the phenomenon of miracidial phototaxis, miracidia were harvested, chilled, gently centrifuged, and then transferred into the culture medium. Ciliated epidermal plates would begin to slough off from the underlying sporocyst surface after about 3 hr in medium, and were usually fully shed by 18–24 hr. Sporocysts were always used after 1–3 days in the medium, in which they typically survive for 7–10 days.

Culture medium. The medium used for culture of miracidia (sporocysts), snail tissues, and amoebae was essentially that used by Voge and Seidel (1972); however, our medium contained only 5% fetal calf serum and 4.5 g/liter galactose. (Recently, we have substituted HEPES buffer, 25 mM, for the bicarbonate system, eliminating the need to gas cultures, and found it equally satisfactory.) Antibiotics included in the medium were either penicillin–streptomycin–Fungizone (PSF, Microbiological Associates, Inc.) at 1% (v/v), or Gentamicin (Microbiological Associates, Inc.) at 100 μg/ml. Phenol red was also added at 5 μg/ml as a *p*H indicator. Osmolality of the medium was about 140 MOSM. Culture vessels (see below) were gassed briefly with a mixture of 5% CO_2 and 95% air in order to maintain the *p*H at about 7.1 when bicarbonate buffer was used.

Snails. Three strains of *B. glabrata* were used: Puerto Rican (PR) albino, 10-R2 "blackeye" albino, and 13-16-1 albino. PR albino snails were provided through the U.S.–Japan Cooperative Medical Science Program. 10-R2 and 13-16-1 strains were provided by Dr. C. S. Richards of the Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases.

We have found juveniles of the PR albino strain to be 94–100% susceptible to infection with PR strain *S. mansoni* miracidia (exposure of five miracidia/snail in 1 ml spring water) but totally refractory to infection with the Ethiopian strain of parasite; adult snails of this strain are refractory to infection with both strains of *S. mansoni*. Juveniles and adults of the 10-R2 and 13-16-1 strains are totally refractory to various strains of *S. mansoni* (Richards, 1975b).

Snails of each strain were maintained separately and allowed to inbreed in large aquaria with a water temperature of 25°C and were fed iceberg or romaine lettuce and chalk.

Culture of tissue explants, mantle swabs, and amoebae. Originally, as discussed above (see Introduction), explants of pericardial sacs from our three snail strains were cultured in an attempt to obtain outgrowths of amoebocytes or of their hemopoietic precursors. Later, when it was realized that the small cells which did grow were in fact amoebae and not snail cells, explants and swabs of the mantle cavity were also cultured in an effort to (1) pinpoint the foci of the amoebae in or on the snails, and (2) to determine what proportion of the snails of each strain were infested or infected with the amoeba. Mantle explants were removed from the region located from 2 to 5 mm anterior to the anterior end of the pericardium and to the right and left of the kidney (renal ridge). Swabs were made of the same region of the mantle cavity using small cotton swabs attached to the end of toothpicks. After about 5 sec of very gentle swabbing, the swab was jiggled for about 15 sec in 1 ml of medium in a Leighton tube, which was then incubated at 27°C. Snails were always placed in distilled water containing 2% (v/v) PSF for at least 1 hr before explants were removed or swabs made. The shell diameter of the snails used was 13–17 mm.

After excision from the snail, explants were rinsed three times (10–15 min each

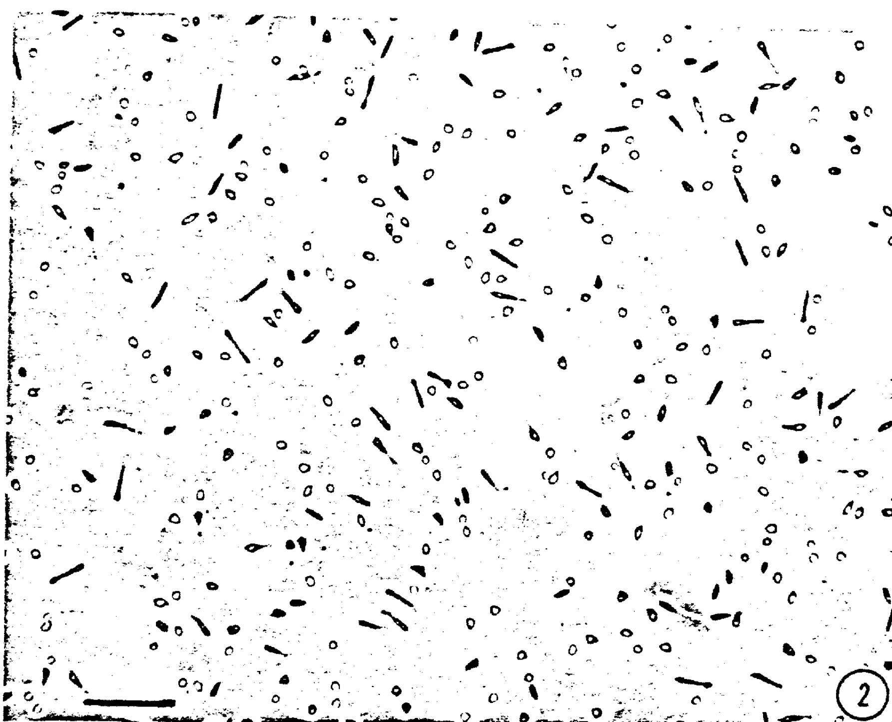

FIG. 2. Amoebae growing axenically in a ring-slide chamber after isolation from pericardial explant; note occurrence of both rounded and elongated forms. Bar = 40 μm.

and control sporocysts were prepared for scanning and transmission electron microscopy (SEM and TEM) by fixation in 3% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2, 380 mOsM) for 1.5 hr. Material for TEM was postfixed in 1% osmium tetroxide for 1 hr, dehydrated in acetone, and embedded in Epon. Sections were stained with lead citrate and uranyl acetate, then examined in an RCA EMU-3h electron microscope. After glutaraldehyde fixation, material for SEM was dehydrated with acetone, treated with trichlorotriflouromethane, critical point-dried, and then examined with an ISI (International Scientific Instruments) Mini-SEM.

RESULTS

Appearance, Size and Behavior of Amoebae

Amoebae migrating out of a pericardial explant are shown in Figure 1. When crowded, most of the cells are round and refractile; however, in less dense and in rapidly proliferating cultures (Fig. 2) nearly half of the cells may be flattened and elongated. The latter form is often observed rounding up in 1–2 sec. The cells measure 3–5 μm in diameter when round and 2–3 x 6–12 μm when stretched out (excluding filopods; see below). The diameter of the nucleus in the round amoebae is one-third to one-half that of the entire cell. The

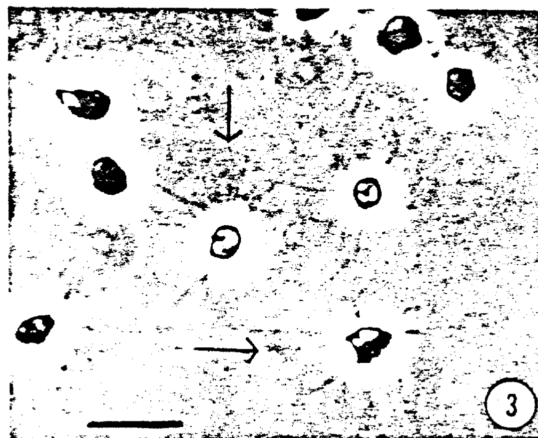

FIG. 3. Amoebae *in vitro*, showing numerous filopodia (arrows) which attach them to the substratum. Bar = 10 μm.

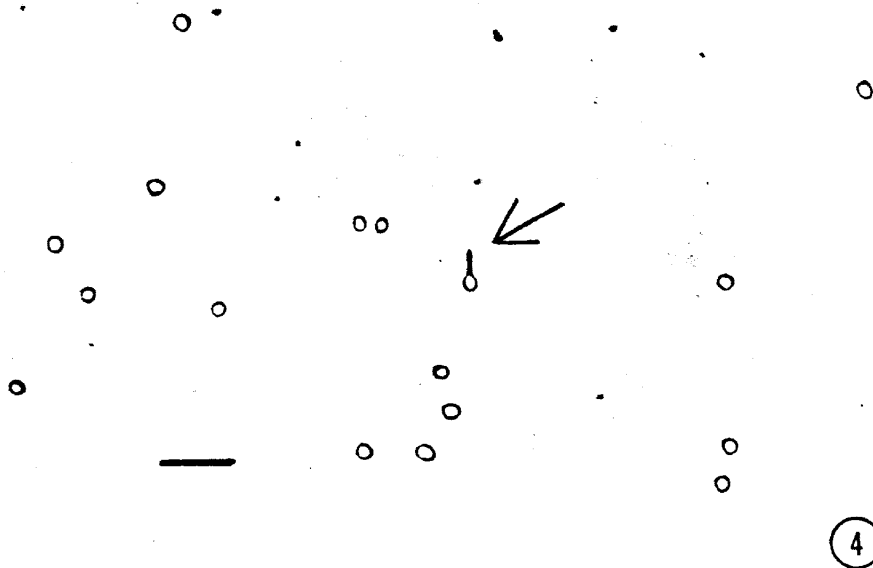

FIG. 4. Amoeba with long, straight pseudopod (arrow). Bar = 20 μm.

rounded cell form is the motile one: It moves by extending and retracting long filopodia (Fig. 3) and changes direction frequently. It occasionally extends long, straight pseudopodia (Fig. 4) which break off and lyse instantly. During cell division (Fig. 5), two daughter cells of approximately equal size are formed; cytokinesis is rapid, often occurring in as short a time as 30–45 sec. Contractile vacuoles have not been seen in hanging-drop preparations of amoebae, even when the medium has been diluted 1:1 with spring water or replaced by pure spring water.

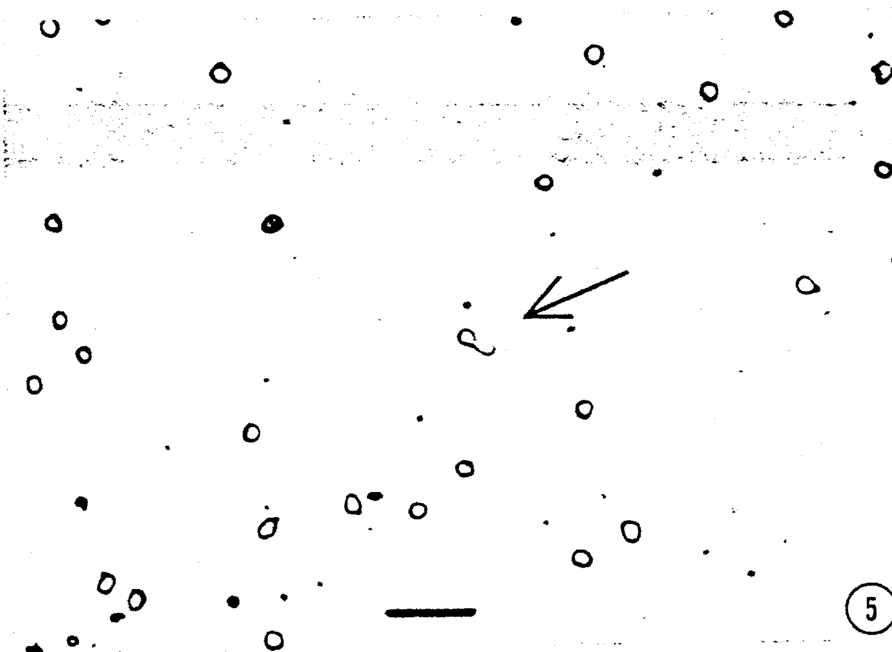

FIG. 5. Dividing amoeba (arrow). Bar = 20 μm.

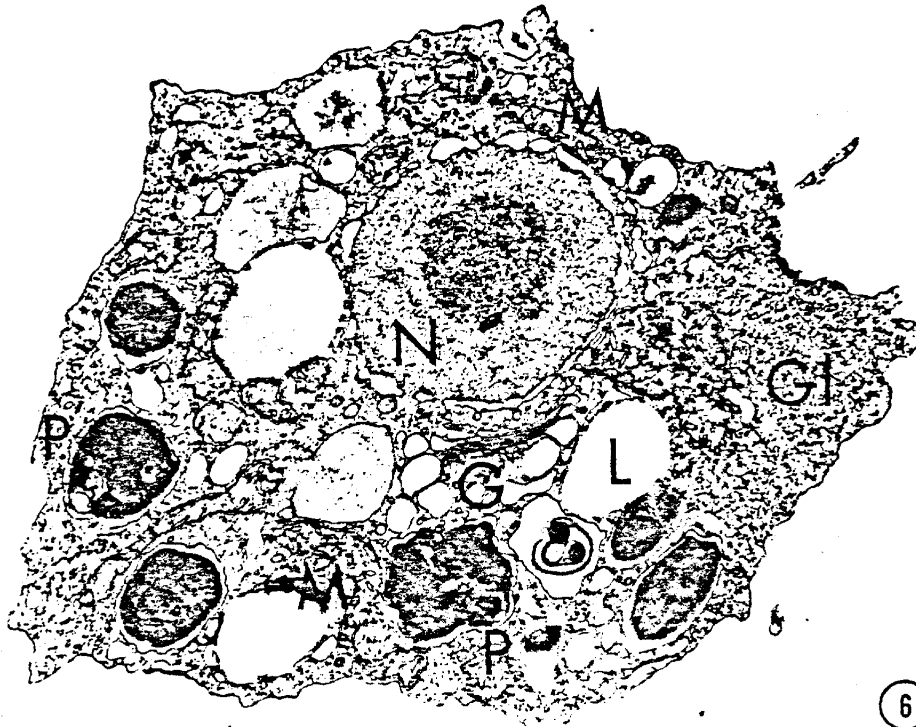

FIG. 6. Electron micrograph of the amoeba in its trophozoite form. N, Nucleus with central nucleolus; G, Golgi complex; Gl, glycogen; P, phagosome; M, mitochondrion; L, lipid vacuole. × 15,000.

The ultrastructure of the trophozoite form of this amoeba is seen in Figure 6. The eccentrically situated nucleus contains a centrally located nucleolus and has large pores leading to the cytoplasm. Mitochondria, phagosomes, lipid vacuoles, and glycogen granules are common features of the cytoplasm. A Golgi apparatus is occasionally seen; however, rough and smooth endoplasmic reticulum are rarely encountered.

In dense cultures (2–5 x 10^6 cells/ml), the amoebae may encyst (Fig. 7). The cyst wall consists of two layers: a thin, irregular, loosely attached outer wall and a thicker, smooth, round inner wall. Pores or ostioles in the cyst wall have not yet been observed. Between the inner and outer cyst walls clumps of membranous material are frequently seen in two or three places. The plasmalemma is often deeply crenulated. All cysts observed so far have been uninucleate.

The amoebae derived from each of our snail strains were morphologically identical and affected schistosome sporocysts in the same manner (see below); hence they probably all belong to one species.

Frequency of Amoeba Outgrowth from Snail Tissues

Among naive snails of our three strains, the proportion of pericardial explants which yielded amoebae in culture was as follows: 13-16-1 strain snails, 10 of 31; 10-R2 strain, 1 of 12; and PR albino strain, 2 of 16. Student's t test revealed no significant differences ($P<0.05$) between any of these proportions. Moreover, in no case did prior exposure of snails to miracidia significantly alter amoeba yields from cultured pericardia.

Mantle swabs did yield amoebae from 2 of 6 naive snails of the 13-16-1 strain, 2 of 6 of the PR albino strain, but from 0 of 6 of

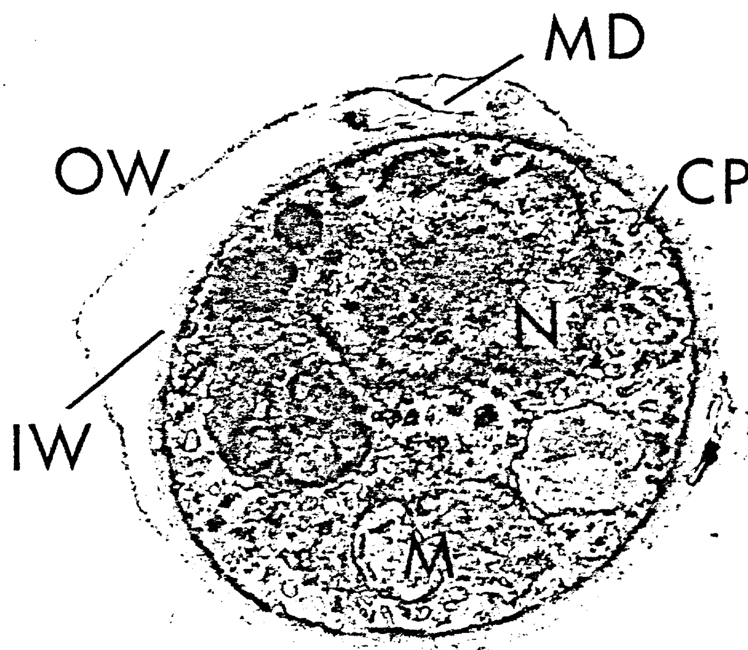

FIG. 7. Encysted amoeba. OW, Outer cyst wall; IW, inner cyst wall; MD, membranous debris within outer cyst wall; CP, crenulated plasmalemma; M, mitochondrion; N, nucleus. × 28,300.

the 10-R2 strain. Differences were not statistically significant.

Mantle explants produced amoebae in 8 of 17 naive snails of the 13-16-1 strain, 2 of 4 of the 10-R2 strain, and 2 of 13 of the PR albino strain. The one difference that was statistically significant ($P<0.05$) was that between the 13-16-1 and PR albino strains.

Growth of Amoebae in Vitro

The amoebae grow well in our medium using all types of culture vessels tested. Normal generation time is 24–48 hr when cells are growing at log phase (generally when cell density is 5–10 x 10^5/ml). Growth usually ceases after the cells have attained a concentration of 3–5 x 10^6/ml; at this concentration the amoebae encyst. Surprisingly, growth is not affected by colchicine (5–1000 μg/ml), demecolcine (*N*-desacetyl-*N*-methyl-colchicine, (5–500 μg/ml), or vinblastin (8–40 μg/ml); nor is it inhibited by certain antiprotozoal drugs such as metronidazole (Flagyl, 10 μg/ml) or fumagillin (Fumadil B; 10 μg/ml). The amoebae grow best at 20–27°C, and they grow well at a *p*H at least as low as 6.0. When all oxygen is removed from the medium by placing the loosely capped Leighton tubes in a Gas-Pak chamber (BBL, BioQuest), growth ceases. The amoebae grow well when the medium is mixed 1:1 with spring water. They do not, however, grow in pure spring water or in filtered aquarium water, yet they do survive for at least 5 days in these waters, probably by encysting. To date, two separate amoeba cultures have been maintained through more than 50 passages over a 15-month period.

Killing of Sporocysts

When cultured sporocysts are introduced into either a ring-slide chamber or a Leighton tube containing settled amoebae at concentrations in the range of 600–6000 cells/mm^2 of substratum, amoebae begin to

FIG. 8. Mother sporocysts of PR-strain *Schistosoma mansoni*, approximately 1 hr after mixing with amoebae. Note numerous attachments (arrows) of amoebae to sporocysts. Bar = 60 μm.

FIG. 9. Scanning electron micrograph of amoebae-coated mother sporocyst exhibiting shrinkage at one end: about 2 hr after mixing with amoebae. × 1000.

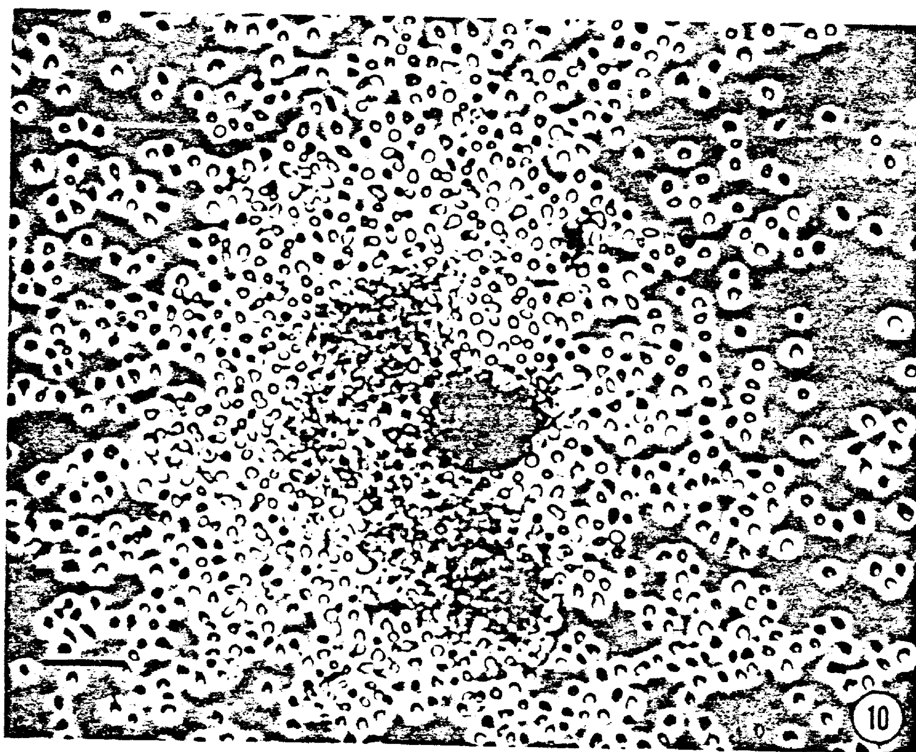

FIG. 10. Amoebae proliferatering around killed sporocyst. Bar = 40 μm.

attach immediately to the sporocyst tegument (Fig. 8). Attachment appears to be random, aided by collisions effected by the vigorous stretching movements of the sporocysts and by the movements of amoebae. Time-lapse cinematography has provided no evidence of attraction of cells to sporocysts until the latter have been damaged, i.e., a "leaky" sporocyst attracts amoebae.

Within 1 hr after mixing, sporocyst-bound amoebae begin to damage, deform, and degrade the beleaguered sporocysts. The first sign of damage is a shrinkage or deflation of one end of the sporocyst (Fig. 9). During the next 2–3 hr, the sporocyst shrinks even further, and its contents begin to leak out through ruptures in the tegument. After 24–36 hr, few sporocysts can be found or recognized in cultures with amoebae, whereas control cultures contain healthy, active sporocysts. The attachment of only a few amoebae, perhaps only one, suffices to cause sporocyst death. After killing the sporocyst, amoebae proliferate rapidly around the dead sporocyst (Fig. 10). Experiments involving separation of amoebae and sporocysts by a Nucleopore membrane (0.5-μm pore diameter) have revealed that physical contact between amoebae and parasites is necessary for rapid sporocyst killing to occur. The ultrastructural aspects of the killing process will be described elsewhere.

Absence of Strain Specificity in Amoeba Attachment and Killing

Amoebae isolated from any of our three snail strains attach to and kill *S. mansoni* sporocysts of both strains equally well (based on subjective observations), regardless of whether the snail source is naive or "sensitized" to miracidia. Moreover, the amoebae adhere to and destroy *S. mansoni* daughter sporocysts, the latter obtained by teasing apart mother sporocysts located in the head–foot portion of infected PR albino strain snails. The amoebae do not attach to

S. mansoni cercariae, to rediae of other trematodes (*Cercaria gorgonocephala* and *Cercaria macrocauda*) teased out of the digestive gland of *Oxytrema silicula* obtained from a local coastal river or to *Fasciola hepatica* rediae obtained from infected *Lymnaea columella*. The amoebae do adhere to and kill cells of the "Bge" cell line established from embryonic cells of *B. glabrata* (Hansen, 1976).

Postsporophagy Proliferation (PSP)

At 12–24 hr after being mixed with cultured sporocysts at an amoebae:sporocyst ratio of between 200:1 and 1000:1, the amoeba growth rate increases dramatically (Fig. 11). Generation time decreases to as short as 5 hr. This growth spurt starts at approximately the time when most sporocysts have just reached a state of total decomposition caused by penetration and phagocytosis by amoebae, a process we call "sporophagy." Postsporophagy proliferation (PSP) continues for 1–2 days, after which the growth rate returns to normal. PSP is not enhanced by previous exposure of the amoebae to sporocysts.

Experiments involving separation of amoebae and sporocysts by a Nucleopore membrane (0.5-μm pore diameter) indicated that intact, healthy sporocysts do not liberate any soluble substances in vitro which can induce proliferation of amoebae. However, sonically disrupted sporocysts induce rapid proliferation of amoebae as effectively as live ones when mixed with amoebae; the sonicate is even more effective after being immersed in boiling water for 10 min. When the sonicate is centrifuged at 2100*g* for 30 min, most of the proliferation-inducing material is associated with the pellet. Beyond this, nothing is known of the chemical nature of the sporocyst substance(s) inducing PSP.

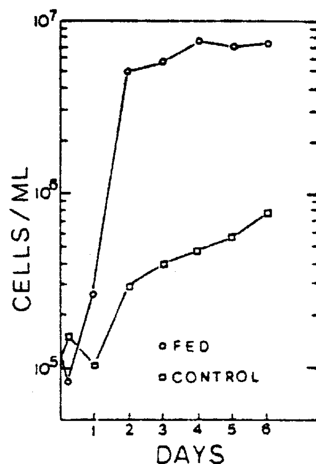

FIG. 11. Comparison of the growth rates of control amoebae (□) vs amoebae which have been "fed" mother sporocysts (○) at zero time. Cells were grown in Leighton tubes at 27°C: counts were made with a hemacytometer.

DISCUSSION

This is not the first report of an isolation of amoebae from *Biomphalaria*. Richards (1968) found two species of amoebae as intracellular parasites of the mantle collar, tentacles, foot, and intestinal wall of laboratory-reared *B. glabrata* and other species of *Biomphalaria* and *Bulinus*. In general, these amoebae could be transmitted to naive snails, of the same or of another species, by simple exposure; little success at culturing the amoebae in vitro was achieved. The size of the smaller of these amoebae is comparable (rounded, 5 μm in diameter) to our amoeba, but the shape of the motile trophozoites differs somewhat from that of our amoeba. However, this morphological dissimilarity could be due to differences in the media used. Therefore, it is possible that our amoeba is identical with the smaller of Richards' amoebae. Taxonomic classification of our amoeba awaits further morphological and serological studies; but it appears that the amoeba belongs to the Hartmannella–Acanthamoeba group of small limax amoebae.

It is not clear whether our amoebae infect snail cells or tissues, or whether they simply reside on the external surface of the mantle or pericardial epithelium. Our results with swabs suggest the latter condition; however, the mantle tissue is delicate and our swabs may have ruptured it, releasing amoebae located within it. Amoebae

which arose from cultured pericardia may not have been growing in (or on) the pericardium, but instead could have been contaminants acquired from the posterior end of the mantle cavity, which abuts against the pericardium. Whether this amoeba can be found in other tissues of the snail is unknown; however, in some preliminary studies we were unable to obtain them in cultures of explants from tentacle and foot. How the amoebae are transmitted from snail to snail is also unknown.

The amoebae do not appear to be pathogenic for the snail, i.e., we have not noticed any obvious ill health or pathologic conditions in snails from which we have isolated the amoebae. However, it is possible that the amoebae affect the growth and fecundity of the snails, therefore the potential usefulness of this amoeba in biological control of the snail in endemic areas needs to be explored.

Despite the fact that the proportion of mantle explants yielding amoebae was higher among 13-16-1 strain, *S. mansoni*-resistant, snails than among the susceptible PR albino strain snails, it is doubtful whether the amoebae contribute to the resistance of the 13-16-1 strain, or conversely, whether the relative scarcity of amoebae in the PR albino strain is responsible for its susceptibility. While the PR albino strain is susceptible to Puerto Rican strain *S. mansoni*, it is resistant to the Ethiopian strain of the parasite; if the absence of amoebae dictated susceptibility, one would expect the PR albino snail strain to be susceptible to all strains of *S. mansoni*. Furthermore, snails of the 10-R2 strain are *S. mansoni*-resistant, yet their tissues did not yield amoebae at a significantly higher rate than did those of the PR albino strain.

Although perhaps unrelated to snail resistance, the destructive effects of the amoebae on mother and daughter sporocysts of *S. mansoni* are interesting in their own right and bear a striking resemblance to the action of peritoneal macrophages, neutrophils, and eosinophils upon schistosomula of *S. mansoni* (Perez and Smithers, 1977; Dean et al., 1974; MacKenzie et al., 1977; McLaren et al., 1977) and of neutrophils upon larvae of *Ascaris suum* (Thompson et al, 1977). It may be that all of these cell types, including the amoeba, damage their target helminths by a common method. The ultrastructure of the adhesion to and penetration of mother sporocysts by the amoeba will be reported in a subsequent paper. However, at present we still know nothing about the biochemical events occurring during the amoeba-mediated rupture of the sporocyst tegument. Further studies with this amoeba may provide insight into the ways in which other parasitic amoebae, e.g., *Entamoeba histolytica* (see Takeuchi and Phillips, 1975; Deas and Miller, 1977), and pathogenic free-living amoebae, e.g., *Naegleria fowleri* (see Martinez et al., 1973; Cursons et al., 1978), penetrate their respective target tissues.

ACKNOWLEDGMENTS

This research was supported by Grant AI-13295 from the National Institute of Allergy and Infectious Diseases, NIH. Transmission electron microscopy for this study was done by Mr. Thomas Gallenstein and Dr. Patricia Buckley of the Department of Zoology, Oregon State University; scanning electron microscopy was done by Mr. Alfred Soeldner of the Department of Botany and Plant Pathology.

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