PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 146, 1101–1105 (1974)

# Sulfhydryl Compounds Under Controlled Gas in Culture of *Schistosoma mansoni* Sporocysts (38253)

EDWARD J. BUECHER,[1] GLADYS PEREZ-MENDEZ, EDER L. HANSEN, AND EVANGELINE YARWOOD

*Clinical Pharmacology Research Institute, 2030 Haste Street, Berkeley, California 94704*

*Schistosoma mansoni*, a trematode causing schistosomiasis in man, has a complete mode of asexual reproduction in the intermediate host, the snail *Biomphalaria glabrata*. In the snail an invading miracidium develops into a mother sporocyst which produces daughter sporocysts. These migrate to the digestive gland where they produce a new generation of daughter sporocysts (progeny-daughters) or cercariae, the more highly organized stage invasive to man. We have cultured daughter sporocysts to permit further understanding of the host-parasite relationship and the conditions leading to the eventual production of cercariae. Sporocysts cultured monoxenically in association with *Aedes albopictus* cells gave rise to progeny-daughters (1); however, medium conditioned by cell cultures supported only limited development of the daughter sporocysts (2). In fresh medium sporocysts degenerated within a few days.

We now report that the requirement for either conditioned medium or associated cells has been obviated by addition of sulfhydryl compounds to the medium and control of the gas phase of the cultures; under these conditions multiplication of daughter sporocysts occurred, with progeny-daughters being liberated into the medium.

*Materials and Methods*. Albino *B. glabrata* were infected with *S. mansoni*. Five groups of snails infected over a period of 6 mo were used in the present studies. The methods for surgical release of daughter sporocysts from mother sporocysts and their subsequent axenization, inoculation, and culture have been described (1, 2). Daughter sporocysts were selected for uniformity of size (150–200 μm), motility, and morphology; 10–27 were inoculated in duplicate into Nuclepore chambers (1, 2) which were partially immersed in 2 ml of medium contained in 3 cm glass petri dishes. Cultures were held in a humidified atmosphere in glass containers. The required gas phase was obtained by passing the appropriate gas mixture through the containers at a flow rate of 200 ml/min (3); after 60 min the inlet and outlet were sealed and the gas flow discontinued. The gas mixtures consisted of 0.5% $CO_2$ with 20, 9, 3 or approximately 1% $O_2$, or 0.04% $CO_2$ with 9, 3, or approximately 1% $O_2$, the remainder in each case being $N_2$. No $O_2$ was included in the gas mixtures indicated as containing approximately 1%; however, an entrapment of air has been reported for a similar system (4). The gas phase was reestablished periodically and after each observation. Control cultures were incubated in humidified air (0.04% $CO_2$–20% $O_2$). All cultures were incubated in the dark at 27°.

The medium contained 30% Schneider's *Drosophila* Medium (Grand Island Biological Co., Grand Island, NY [GIBCO] No. 172), 2 g/liter galactose, and 10% fetal calf serum inactivated at 56° for 30 min; phenol red was included at 10 mg/liter. The osmolality was 163 mOsm; the pH was 7.2 in air and gas mixtures containing 0.04% $CO_2$, and 7.0 in those with

[1] Present address: Colorado Women's College, Department of Biology, Denver, Colorado 80220.



Copyright © 1974 by the Society for Experimental Biology and Medicine
All rights reserved.

*Printed in U.S.A.*

0.5% $CO_2$. Conditioned medium was prepared by exposing rapidly growing cultures of *A. albopictus* to a mixture of 75% Schneider's *Drosophila* Medium and 25% inactivated fetal calf serum as previously described (2). Before use with sporocyst cultures it was filtered, diluted to 30% Schneider's *Drosophila* Medium–10% fetal calf serum, and galactose and phenol red were added. Some of the conditioned medium was stored at 4° for up to 1 year. Sulfhydryl compounds were added from concentrated stock solutions which were stored as frozen aliquots. Dithiothreitol (Cleland's Reagent) was prepared at 32 m*M* and used at 0.1 m*M* unless otherwise noted. A combination of cysteine (free base) and glutathione (reduced) was prepared at 60 m*M* and 30 m*M* respectively, and used unless otherwise noted, at 1 m*M* and 0.5 m*M*. This addition decreased the pH of the medium by 0.2 unit. In some cultures sulfhydryl-containing media were replenished, either daily or after 3 days.

In some cases 2.5% Ficoll (Pharmacia, Uppsala, Sweden) or 0.25% Methocel (Dow Chemical Co., Midland, MI), either 15 or 1500 cps, were included in the medium. Additional supplements tested were: 5% *B. glabrata* hemolymph, fresh or inactivated 10 min at 56°; BME vitamins (GIBCO No. 104); 10% chick embryo extract (GIBCO No. 511); 200 mg/l fucose, 30 mg/l sodium pyruvate; 30 μg/l DEAE-Dextran (Pharmacia); 50 mg/l horse hemoglobin.

Observations were made with a Letiz inverted microscope. After 7 days the sporocysts were counted and their size, morphology, and development assessed; subsequent observations were made at 1–3 day intervals until the cultures were terminated. After 7–18 days they were examnied in fresh mounts or fixed in acetic sublimate or glutaraldehyde and stained by Feulgen's technique. One 9-day culture was exposed to $^3H$-thymidine (methyl $^3H$; sp act 24.5 Ci/mmole, 25 μCi/ml medium; The Radiochemical Centre, Amersham, England) for 18 hr and examined for synthesis of deoxyribonucleic acid by autoradiographic techniques.

The presence of progeny-daughters in the cultures could sometimes be confirmed by an increase over the number of sporocysts inoculated. In other cultures, degeneration and partial disintegration of one or more of the originally inoculated daughters made confirmation by count impractical. In these cases the progeny-daughters were readily distinguished from the original daughters by their size, number of cells, and morphology.

*Results.* In fresh medium in air without sulfhydryl compounds sporocysts degenerated within 7 days and had little or no germinal development. Neither controlled gas phase nor sulfhydryl compounds alone improved the response. When used together, however, the response was markedly improved (Table I). With sulfhydryl compounds used at optimal levels (see below), all gas mixtures which contained either decreased $O_2$ or increased $CO_2$ were effective. Decreased $O_2$ appeared to be more effective than increased $CO_2$, probably because the lower $O_2$ level was necessary to protect the sulfhydryls. Progeny-daughters were produced after 7–10 days in culture, and at least 90% of the original sporocysts showed marked increase in growth, longevity, and embryonic development. Growth was particularly notable in 0.5% $CO_2$–ca. 1% $O_2$; sporocysts increased up to seven-fold in length, motile ones measuring up to 1300 μm. Progeny-daughter production, however, was not greater in this gas mixture.

The sulfhydryl compounds were effective within a narrow concentration range; they

TABLE I. Emergence of Progeny-Daughter Sporocysts of *S. mansoni* in Axenic Culture with Sulfhydryl[a] Compounds and Varying Levels of $CO_2$ and $O_2$.

| | $CO_2$% | |
|---|---|---|
| $O_2$% | 0.04 | 0.5 |
| ca. 1 | progeny-daughters | progeny-daughters |
| 3 | progeny-daughters | progeny-daughters |
| 9 | progeny-daughters | progeny-daughters |
| 20 | degenerate[b] | embryos |

[a] Either 0.1 m*M* dithiothreitol or 1.5 m*M* total cysteine and glutathione.

[b] Gas phase, air.

TABLE II. Development of Daughter Sporocysts of *S. mansoni* in Axenic Culture in 0.5% $CO_2$–9% $O_2$ with Varying Levels of Sulfhydryl Compounds.

| Sulfhydryl | m*M* | Sporocyst development |
|---|---|---|
| Dithiothreitol | 0 | degenerate |
| | 0.02 | degenerate |
| | 0.03 | large embryos |
| | 0.05 | large embryos |
| | 0.10 | progeny-daughters |
| | 0.20 | small embryos |
| | 0.30 | degenerate |
| Cysteine + glutathione | 0 | degenerate |
| | 0.75 | large embryos |
| | 1.50 | progeny-daughters |
| | 3.00 | degenerate |

were ineffective at the lowest concentrations and toxic at 2–3 times the optimal levels (Table II) when tested with a single effective gas phase. Replenishment of sulfhydryl-containing media was detrimental to the cultures.

Conditioned medium supported limited development of the sporocysts, but the degree of embryonic development was insufficient to lead to production of progeny-daughters. The stimulatory effect of conditioned medium was not diminished by storage at 4°; results with conditioned medium stored up to 1 year were similar to those with freshly harvested conditioned medium. The response to gas phase and sulfhydryl was not improved by using conditioned medium in place of fresh medium.

Embryonic development was still going on in cultures terminated up to 14 days, as shown by the many mitotic divisions in fixed and stained specimens and by uptake of $^3$H-thymidine in autoradiographs of the 9-day culutre. Most sporocysts had increased two- to five-fold in length and contained up to 800 cells, increasing from the initial size, when fixed and stained, of 20 × 145 $\mu$m containing about 175 cells. Sporocysts fixed during the course of the experiment suggested that development was proceeding either in the direction of numerous small embryos, up to 30 counted, or toward a few large embryos having morphology of potential progeny-daughters.

Progeny-daughters emerged in 23 of the cultures in fresh meduim held at least 7 days. Though initially motile and displaying the typical morphology of small daughter sporocysts, they were fragile and usually degenerated within a few days. Some, however, grew in culture; one was still motile 9 days after it was first observed. Three fixed and stained progeny-daughters measured 21 × 80 $\mu$m, 28 × 92 $\mu$m, and 62 × 80 $\mu$m; they contained 43, 57, and 110 cells, respectively.

The characteristics of cultures with sulfhydryl are compared in Table III with those of axenic cultures in conditioned medium and monoxenic cultures. Under optimal axenic conditions the sporocysts grew larger and progeny-daughters appeared earlier, although they were not as numerous as in monoxenic cultures.

Among the supplements tested, the best response was obtained with Ficoll or Methocel, 1500 cps. Vitamins, fucose, DEAE-Dextran, and Methocel, 15 cps, were well tolerated, but no progeny-daughters were produced in media containing them. Hemolymph, chick embryo extract, sodium pyruvate, and horse hemoglobin exhibited some degree of toxicity.

*Discussion.* Although *in vivo* the daughter sporocyst migrates from the peripheral tissues to a new location, usually the digestive gland, before asexual reproduction begins, there are no evident morphological changes. The daughter sporocyst already contains germinal cells which, in the new location, multiply and organize to produce more daughter sporocysts or cercariae.

Support for the development of daughter sporocysts was provided *in vitro* either by associated tissue culture cells or in axenic medium under specific conditions, that is, lower oxygen tensions and addition of sulfhydryl compounds or, to a limited degree, by addition of conditioned medium. In the monoxenic culture system the growth environment was provided by the metabolic activities of the tissue culture cells, and in an axenic medium by lowering of $O_2$ to 9% or less and addition of sulfhydryls. These could provide proper redox values which might be needed by the organism for the functioning of important adenosine triphosphate-producing pathways, or for maintaining enzymes in the

TABLE III. Characteristics of *S. mansoni* Sporocysts Cultured Axenically in Fresh Medium with Sulfhydryl Compounds Compared to Conditioned Medium (2) and Monoxenic Cultures (1).

| | Axenic | | |
|---|---|---|---|
| | Fresh[a] | Conditioned[b] | Monoxenic |
| Live cultures: | | | |
| Longevity,[c] days | 18 | 13 | 22 |
| Progeny-daughters/100 inoculated | 2.2 | — | 6.7 |
| Initial appearance of progeny, days | 7 | — | 9 |
| Fixed and stained specimens[c]: | | | |
| Length, μm | 792 | 400 | 524 |
| Embryo size, μm | 40 × 46 | 16 × 18 | 54 × 54 |
| Number of cells in embryo | 50 | 10 | 50 |

[a] $O_2$ 9% or less.
[b] Air.
[c] Maximum observed.

configuration needed for maximum activity. An $O_2$ level of 9% has been reported for snail hemolymph (5). There apparently is no need for increase in $CO_2$, as the response was not enhanced with concentrations above 0.04%. Determination of specific redox values are now being undertaken with the use of redox dyes in the medium.

The sulfhydryl level was found to be critical; only a single concentration in each system proved satisfactory. The sulfhydryl concentration cannot be expected to remain stable, even with decreased $O_2$. Attempts to maintain a satisfactory sulfhydryl level by replacing the medium were unsuccessful; possibly irreversible cell damage occurred from interruption of the gas phase by replenishment of the medium during the early growth period.

Little is known about the basis of the conditioning effect in this (2) or other (6) culture systems, but it undoubtedly represents a biochemical alteration of the medium, in our case a stable one. The monoxenic system has the advantage that the growing cell cultures simultaneously condition the medium and maintain an appropriate gas phase. The greater importance of the latter is suggested by the fact that reproduction can occur in fresh medium under favorable redox conditions but does not occur in conditioned medium in air. In our experiments there was no additive effect with the use of sulfhydryl in conditioned medium.

Studies will be continued regarding the exact effect of our physiochemical conditions on sporocyst development and to determine whether these conditions can be altered to initiate cercarial development.

*Summary.* Daughter sporocysts of *S. mansoni* cultured axenically in the presence of sulfhydryl compounds in a controlled gas environment underwent growth and development, in some cases culminating in the production of a new generation of daughter sporocysts. The effect occurred within a narrow range of concentrations of either dithiothreitol or a combination of cysteine and glutathione, together with lowering the oxygen level, but not when sulfhydryl or decreased oxygen were used alone. Sporocyst development was equivalent to that obtained monoxenically in association with cell cultures and greater than occurred in medium conditioned by cell cultures.

We wish to thank Dr. Kirsten Walen for the autoradiography and Dr. Donald Heyneman, George Williams Hooper Foundation, University of California, San Francisco, for his generous cooperation in providing us with infected snails. The excellent technical assistance of Barbara David is greatly appreciated. This work was supported by NIAID Grant No. A1-07359.

1. Hansen, E. L., Perez-Mendez, G., Long, S., and Yarwood, E., Exp. Parasitol. 33, 486 (1973).

2. Hansen, E. L., Perez-Mendez, G., and Yarwood, E., Exp. Parasitol., in press.

3. Berntzen, A. K., Ann. N. Y. Acad. Sci. **139**, 176 (1966).

4. Richter, A., Sanford, K. K., and Evans, V. J., J. Nat. Cancer Inst. **49**, 1705 (1972).

5. Cheng, T. C., and Lee, F. O., Exp. Parasitol., **30**, 393 (1971).

6. Temin, H. M., Pierson, R. W. Jr., and Dulack, N. C. *in* "Growth, Nutrition, and Metabolism of Cells in Culture" (G. H. Rothblatt and V. J. Cristofalo, eds.), Vol. 1, p. 74. Academic Press, New York (1972).

---

Received Apr. 17, 1974. P.S.E.B.M., 1974, Vol. 146.