Department of Tropical Public Health, Harvard School of Public Health, Boston, Mass., USA

An isoenzyme marker possibly associated with the susceptibility of *Biomphalaria glabrata* populations to *Schistosoma mansoni*

E. H. MICHELSON, L. DUBOIS

Summary

Nine laboratory populations and one field population of the snail host *Biomphalaria glabrata* were compared with respect to their electrophoretic patterns for acid phosphatase (AcP) and with their susceptibility to *Schistosoma mansoni* infection. A strong correlation (r = 0.98) was noted between the frequency of the isoenzymes AcP_{2-S} and AcP_{2-F} observed in the populations and the level of snail susceptibility as determined by bioassay. The isoenzyme AcP_{2-S} was associated with susceptibility, AcP_{2-F} with the refractory state. Breeding experiments between refractory and susceptible snails demonstrated that the refractory state was dominant and all F_1 snails exhibited the AcP_{2-F} isoenzyme and proved refractory to infection.

Key words: Biomphalaria glabrata, acid phosphatases of; snail susceptibility, enzyme marker of; snail susceptibility, inheritance of; gel electrophoresis.

Introduction

Variation in the susceptibility of the snail hosts of schistosomes to infection with allopatric strains of the parasite is a well documented phenomenon, first noted by Vogel (1940) and subsequently verified by numerous investigators. The resultant literature has been reviewed by Cram (1953) and by Basch (1976). Recent studies (Michelson and DuBois, 1978) suggest that the phenomenon is not restricted solely to distantly separated geographic snail strains, but may occur also in adjacent populations residing within a limited geographic region.

As a consequence of studies by Richards (1963, 1973) and Richards and Merritt (1972), snail susceptibility to schistosome infection is generally accepted

Correspondence: Dr. Edward H. Michelson, Department of Tropical Public Health, Harvard School of Public Health, 665 Huntington Avenue, Boston, Massachusetts 02115, USA

to be controlled by genetic factors. The nature of this genetic control appears to be quite complex, probably multi-factorial, and presently is not clearly understood. Detection of susceptibility, at the present time, depends upon a method of bioassay, in which snails of a given age and/or size are exposed to a specified number of miracidia under controlled laboratory conditions. Unfortunately, bioassays have not been universally standardized and it is frequently difficult to compare results of different studies. In addition, bioassays are time consuming and require several weeks to obtain an answer.

The present study reports the identification of an isoenzyme marker which appears to be associated with the susceptibility of laboratory populations of the snail *Biomphalaria glabrata* to *Schistosoma mansoni* infection. The electrophoretic technique employed has proven to be both sensitive and repeatable and provides answers in several hours instead of weeks.

Materials and methods

Snail populations

Nine laboratory populations of *B. glabrata* and one field population were used in the study. Strain designations, geographic origins, and period of laboratory maintenance are given in Table 1. Snails were reared and maintained at $26 \pm 1\,^{\circ}\text{C}$ in 5 or 10 gallon aquaria and fed a diet of romaine lettuce. The water was aerated and filtered and the snails were exposed to overhead fluorescent light for 12 h daily.

Bioassay

Each population analyzed by electrophoresis was tested for susceptibility in accordance with the procedure described by Michelson and DuBois (1978). Individual snails (5–7 mm in diameter) were exposed to 5 miracidia hatched from eggs obtained from the livers of mice infected with a Puerto Rican strain of *S. mansoni*. Each test consisted of 10 snails and a minimum of 4 tests were run for each population. The PR-1 strain was used as a control in all tests for miracidial quality, since in our laboratory this strain regularly has an infection rate in excess of 75%. Test snails were examined for infection by crushing 14 days post-exposure and the presence of sporocysts determined.

Table 1. Geographic origin, period of maintenance and strain designations of *Biomphalaria glabrata* populations

Strain	Geographic origin	Maintenance (yr)
PR-1	Puerto Rico	25
S-3	Lake Amaralina, Salvador, Bahia, Brazil	16
S-4	Salvador, Bahia, Brazil	1
RS-8	Riacho Seco, Castro Alves, Bahia, Brazil	3
RS-9	Riacho Seco, Castro Alves, Bahia, Brazil	3
G-2	Graviel, Castro Alves, Bahia, Brazil	6
B-1	Catinga do Moura, Bahia, Brazil	16
M-1	Morro do Afonso, Castro Alves, Bahia, Brazil	6
M-1′	Morro do Afonso, Castro Alves, Bahia, Brazil	1 month
BH	Belo Horizonte, Minas Gerais, Brazil	16

Preparation of extracts

Prior to the preparation of extracts, snails 12–15 mm in size were selected at random from stock colonies, blotted on paper toweling and air-dried for 30 min. The shell was removed and the ovotestis-digestive gland complex caudal to the flexure of the intestine was dissected free from the remainder of the body. Care was exercised to assure that all portions of the digestive tract were removed from the tissue sample. The tissue complex was rinsed briefly in distilled water, blotted, and homogenized manually in 2 ml glass tissue-grinders containing 0.5 ml of Tris-HCl buffer (pH 7.4) plus 5% sucrose. Homogenates were centrifuged ($733 \times g / 10$ min) and the supernatant fluid was mixed, prior to electrophoresis, with 40% sucrose (2:1 v/v). Samples were either used immediately or stored at $-20°$ C until required. Material which had been frozen for as long as one year produced patterns which were similar to fresh samples.

Polyacrylic gel electrophoresis (PAGE)

In the course of this study, tissue extracts were analyzed for a variety of enzyme systems including amylases, alkaline phosphatases, non-specific esterases, lactic dehydrogenase, isocitric dehydrogenase, hexokinase, ribonuclease, phosphoglucomutase, alcohol dehydrogenase, and acid phosphatase. Although differences were observed between susceptible and refractory populations with several of the systems tested, best results were consistently observed with respect to the mobility and pattern of the acid phosphatases (3.1.3.2). The present report deals, therefore, solely with this enzyme system. Several buffer and gel formulations were investigated, but only the technique which proved optimal is reported herein.

Samples (30 μl) were applied to vertical slabs (3 mm thickness) of 5% polyacrylamide gel prepared in 0.1 M tris-borate buffer (pH 8.9) containing 5% 0.1 M $MgCl_2$. A 0.1 M tris-borate buffer (pH 8.9) served as the electrolyte and gels were run at 350 V with a current of 50 to 90 mAMP until the bromophenol-blue dye front had migrated approximately 100 mm from the origin – requiring a time interval of 90 to 120 min. At completion of electrophoresis, the gels were incubated at $5°$ C for 20 min in 0.1 M sodium acetate, pH 5.0. Acid phosphatases (AcP) were visualized by incubating the gels ($37°$ C/30–60 min) in a stain-substrate mixture consisting of sodium alpha naphthyl phosphate (30 mg), 0.1 M $MgCl_2$ (0.25 ml), 10% $MnCl_2$ (0.25 ml), 20% NaCl (5 ml), Fast Blue BB (30 mg), and 0.1 M acetate buffer at pH 5.0 (50 ml). Gels were washed in distilled water, fixed in 12% acetic acid, and photographed. A minimum of 20 individual samples was run for each population.

R_f-values were determined by taking the mid-point of each band and comparing it to a bromphenol dye marker which was allowed to migrate a distance of 100 mm.

Mating experiments

Egg-masses obtained from the S-3 and PR-1 strains of *B. glabrata* were hatched and individual snails of each strain were isolated in plastic cups containing 200 ml of filtered aquarium water. The isolated snails were maintained until they reached sexual maturity (approximately 7.5 mm diam.), at which time they were paired with an individual of the other strain. Paired snails were allowed to remain in contact with one another for a period of 24 h and were then separated for up to three days to permit eggs to be laid. If eggs were not deposited in this interval, the same pair of snails were again placed together and the procedure continued until eggs were obtained. The egg-masses were then isolated and allowed to hatch so that we could obtain F_1 generation snails. A portion of the F_1 snails and their respective parents were analyzed by PAGE and another portion of the F_1 snails were tested for susceptibility. A limited number of F_2 snails were obtained and treated in a similar manner.

Results

Extracts prepared from strains of *B. glabrata* and stained for AcP exhibited two distinct regions of enzyme activity which have been designated AcP_1 and AcP_2. The more anodic region (AcP_1) consists of two bands which are not consistently visualized unless incubation in the substrate mixture is prolonged. They appear to be present in all strains tested and give no evidence of being
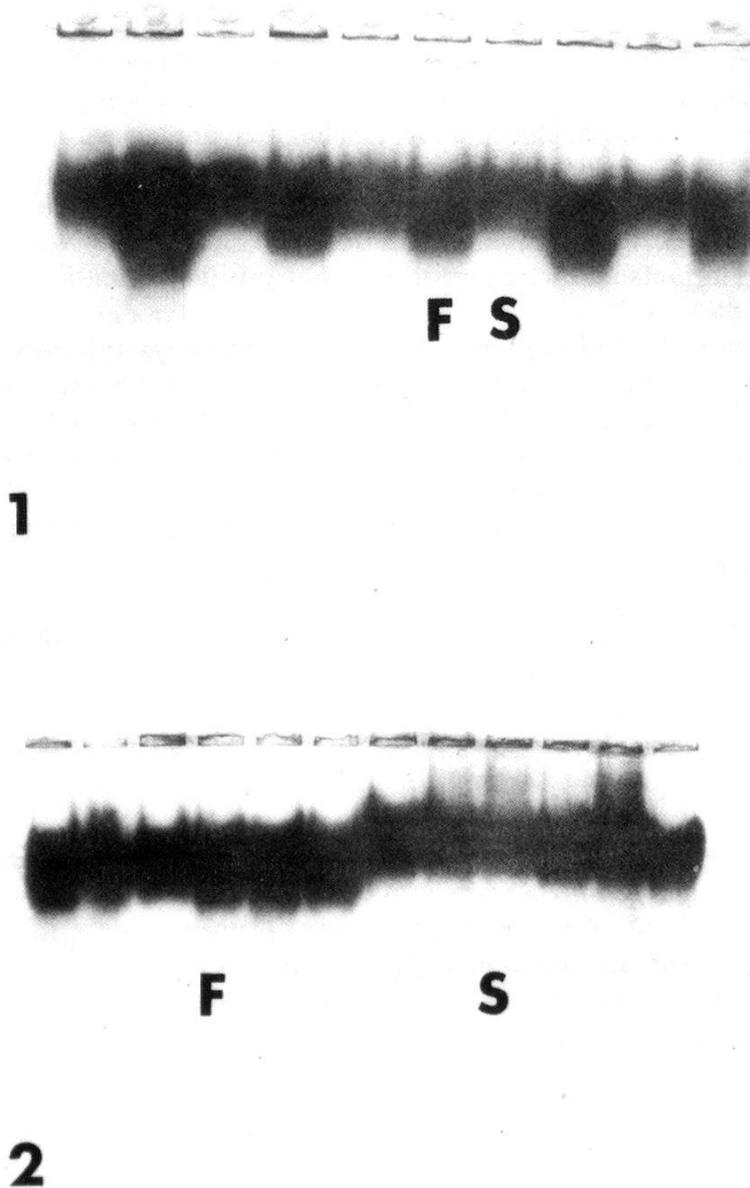

Fig. 1–2. Electrophoretic patterns of acid phosphatase isoenzymes (AcP_2) prepared from digestive gland-ovotestis extracts from two populations of *Biomphalaria glabrata*. Individuals from the refractory S-3 population have isoenzymes that migrate faster (F) than those of the susceptible PR-1 population (S). The two gels were prepared from population samples taken 3 months apart.

Table 2. Susceptibility of *Biomphalaria glabrata* populations and the frequency of the isoenzyme AcP_{2-F}

Population	Infection rate (%)	Frequency of AcP_{2-F}	
		Expected	Observed
PR-1	87.9	0.12	0.17
S-3	0	1.00	1.00
S-4	0	1.00	0.85
RS-8	0	1.00	0.85
RS-9	0	1.00	0.86
G-2	5.1	0.95	0.85
B-1	49.1	0.51	0.42
M-1	30.0	0.70	0.60
BH	87.5	0.12	0.16

Correlation coefficient Exp.: Obs. r = 0.98

polymorphic. The AcP_2 region consists of either a fast (AcP_{2-F}) or slow band (AcP_{2-S}) with one or the other being present (Figs. 1–2). No evidence was found that hybrids of the fast and slow bands occurred. Attempts to produce a hybrid pattern in vitro by mixing extracts of the two forms were not successful and analysis of these mixtures by PAGE revealed only a single broad band which was the additive combination of the two individual bands. The R_f-values of the fast and slow bands were 0.23 ± 0.001 and 0.20 ± 0.01, respectively.

Initial electrophoretic comparison of the refractory S-3 strain with the highly susceptible PR-1 strain suggested that the frequency of the isoenzymes AcP_{2-S} and AcP_{2-F} in a sample of the population was indicative of the level of susceptibility of that population to infection with *S. mansoni*. The AcP_{2-F} isoenzymes being associated with the refractory state and the AcP_{2-S} with susceptibility. In bioassays, the PR-1 and S-3 strains had infection rates of 87.9% and 0%, respectively, with a corresponding frequency of the AcP_{2-S} isoenzyme of 0.83 and 0. The results of bioassays and isoenzyme analysis on 7 additional populations of *B. glabrata* are given in Table 2. We compared samples of the M-1 and M-1' strains, both of which were obtained from the same pond, but at different times. The M-1 population was reared in the laboratory for 34 generations at the time of testing and exhibited an infection rate of 30% and the frequency of the AcP_{2-S} isoenzyme was 0.44. The M-1' population was tested on arrival from the field and exhibited an infection rate of 5% with the frequency of the AcP_{2-S} isoenzyme being 0.12.

In 15 crosses between S-3 and PR-1 snails, analysis by PAGE of 126 F_1 snails revealed that all the progeny, regardless of the genotype of the maternal parent, displayed the presence of the AcP_{2-F} isoenzyme. Progeny from three of the crosses were tested by bioassay and none of the snails were found suscep-

tible to infection whereas controls had infection rates which ranged from 88.9% to 100%. Electrophoretic analysis of 20 F_2 snails showed a frequency of 0.85 for AcP_{2-F} and 2 of 17 F_2 snails were found susceptible to infection. The occurrence of the susceptible F_2 snails cannot be explained with certainty; however, it is suggested that they may represent the progeny of self-fertilization by a susceptible, maternal parent. In a series of preliminary experiments in which albino and pigmented snails were mated, approximately 2.3% of 175 masses were found to contain both pigmented and albino snails. This suggests that self-fertilization and cross-fertilization may occur in the same individual and the employment of Mendelian-type ratios may be unreliable in assessing inheritance in hermaphroditic snails. Paraense (1956) was the first to note "that cross-fertilization never completely replaces self-fertilization," particularly in matings between individuals from geographically remote populations.

Discussion

Acid phosphatases are involved in the hydrolysis of a variety of orthophosphate esters and in transphosphorolation. The attributes, behavior, and distribution of these enzymes have been reviewed by Hollander (1971). The enzymes are widely distributed in nature and have been demonstrated to occur by biochemical, histochemical, and electrophoretic techniques, in the cells and tissues of several molluscan species (Cheng, 1964; Cheng and Rodrick, 1974; Coles, 1969; Gill, 1978; Wright et al., 1966; Wurzinger, 1979).

The role of AcP, with respect to specific physiologic or metabolic functions in the snail hosts of schistosomes, remains to be elucidated. In species of *Biomphalaria* and *Bulinus*, AcP has been found in the hemolymph (Rodrick and Cheng, 1974) and in a variety of cells and tissues (Muller, 1965; Wright et al., 1966). The detection of AcP in granular inclusions of *B. glabrata* amoebocytes (Muller, 1965; Jeong and Heyneman, 1976; Cheng and Garrabrant, 1977), in the cells associated with the encapsulation phenomenon (Harris and Cheng, 1975), as well as the elevation of the enzyme in the hemolymph of snails challenged with heat-killed bacteria (Cheng and Butler, 1979) suggests that this enzyme and other hydrolases might play an important role in the defense mechanisms of host snails. In this regard, it should be noted that in mammalian systems AcP is considered to be an enzyme marker for lysosomes (Novikoff, 1963; DeDuve, 1969). Although our studies demonstrate a correlation between the presence of a particular isoenzyme of AcP and susceptibility, there is no evidence which suggests that the enzyme is directly involved in the determination of susceptibility. Indeed, it is highly probable that our observations only reflect a close linkage of the genes controlling susceptibility and those of AcP.

The results of our breeding experiments with individual snails demonstrate that the refractory state is dominant to susceptibility in *B. glabrata* with respect

to *S. mansoni* infection. This suggests that *B. glabrata* populations in nature may consist largely of nonsusceptible snails; a hypothesis which gains a degree of support from published accounts of snail surveys conducted in areas in which schistosomiasis is endemic and in which low snail infection rates prevail. The hypothesis is not negated by the argument that infection exerts a selective pressure on the snail population, since neither snail mortality nor reduction of fecundity are immediate consequences of *S. mansoni* infection.

The AcP isoenzyme markers observed in the present study appear to be a valuable tool for studying the transmission potential of host snail populations and may contribute to our understanding of the epidemiology of schistosomiasis. It must be remembered, however, that the present study employed laboratory populations in which genetic diversity has been restricted as a consequence of in-breeding. It is of interest that our PR-1 and S-3 strains have been consistent with respect to bioassays, in spite of long periods of maintenance. Our studies on the M-1 population, however, demonstrate that gene frequencies may undergo significant alterations under laboratory conditions. The frequency of the AcP isoenzymes in natural populations and their correlation with susceptibility must be determined before a proper assessment of the present technique is possible. A study to clarify this problem is now in progress.

Basch P. F.: Intermediate host specificity in *Schistosoma mansoni.* Exp. Parasit. *39,* 150–169 (1976).

Cheng T. C.: Studies on phosphatase systems in hepatic pancreatic cells of the molluscan host of *Echino paryphium* sp. and in the rediae and cercariae of this trematode. Parasitology *54,* 73–79 (1964).

Cheng T. C.: The role of hemolytic hydrolases in the defense of molluscs against invading parasites. Haliotis *8,* 193–209 (1977).

Cheng T. C., Butler M. S.: Experimentally induced elevations in acid phosphatase activity in hemolymph of *Biomphalaria glabrata* (Mullosca). J. Invertebr. Path. *34,* 119–124 (1979).

Cheng T. C., Garrabrant T. A.: Acid phosphatase in granulocytic capsules formed in strains of *Biomphalaria glabrata* totally and partially resistant to *Schistosoma mansoni.* Int. J. Parasit. *7,* 467–472 (1977).

Cheng T. C., Rodrick G. E.: Lysosomal and other enzymes in the hemolymph of *Crassostrea virginica* and *Mercenaria mercenaria.* Comp. Biochem. Physiol. *52* B, 443–447 (1975).

Coles G. C.: Isoenzymes of snail livers – I. Hydrolysing enzymes and peroxidase. Comp. Biochem. Physiol. *29,* 403–411 (1969).

Cram E. B.: Evidence concerning geographic strains of human schistosomes and their molluscan hosts. Thapar Commeration Volume, University of Lucknow, p. 51–62 (1953).

DeDuve C.: The lysosome in retrospect. In: Lysosomes in biology and pathology, ed. by J. T. Dingle and H. B. Fell, vol. 1, p. 3–40. North-Holland, Amsterdam 1969.

Gill P. D.: Survey of isoenzymes in the snail *Cepaea nemoralis* using different buffer/gel systems in polyacrylamide disc gel electrophoresis: validity of camparison and effect of "Nothing Dehydrogenase" activity. Biochem. Genet. *16,* 531–540 (1978).

Harris K. A., Cheng T. C.: The encapsulation process in *Biomphalaria glabrata* experimentally infected with the metastrongylid *Angiostrongylus cantonensis:* enzyme histochemistry. J. Invertebr. Path. *26,* 367–374 (1975).

Hollander V. P.: Acid phosphatases. In: The enzymes, ed. by P. D. Boyer, vol. 4, p. 449–497. Academic Press, New York/London 1971.

Jeong K. H., Heyneman D.: Leukocytes of *Biomphalaria glabrata:* morphology and behavior of granulocytic cells in vitro. J. Invertebr. Path. *28,* 357–362 (1976).

Michelson E. H., DuBois L.: Susceptibility of Bahian populations of *Biomphalaria glabrata* to an allopatric strain of *Schistosoma mansoni.* Amer. J. trop. Med. Hyg. *27,* 782–786 (1978).

Muller R.: The histochemical localization of phosphatases and esterases in *Australorbis glabratus.* Proc. zool. Soc. London *144,* 229–237 (1965).

Novikoff A. B.: Lysosomes in the physiology and pathology of cells. Contributions of staining methods. In: Lysosomes, ed. by A.V.S. de Rueck and M. P. Cameron, p. 36–73. Little Brown & Co., Boston 1963.

Paraense W. L.: A genetic approach to the systematics of planorbid molluscs. Evolution *10,* 403–407 (1956).

Richards C. S.: Infectivity of *Schistosoma mansoni* for Puerto Rican mollusks, including a new potential intermediate host. Amer. J. trop. Med. Hyg. *12,* 26–33 (1963).

Richards C. S.: Genetics of a molluscan vector of schistosomiasis. Nature (Lond.) *227,* 806–810 (1973).

Richards C. S., Merritt J. W. jr.: Genetic factors in the susceptibility of juvenile *Biomphalaria glabrata* to *Schistosoma mansoni* infection. Amer. J. trop. Med. Hyg. *21,* 425–434 (1972).

Rodrick G. E., Cheng T. C.: Activities of selected hemolymph enzymes in *Biomphalaria glabrata* (Mollusca). J. Invertebr. Path. *24,* 374–375 (1974).

Vogel H.: Infektionsversuche an verschiedenen Bilharzia-Zwischenwirten mit einem einzelnen Mirazidium von *Bilharzia mansoni* und *B. japonica.* Zbl. Bakt. *148,* 29–35 (1941).

Wright C. A., File S. K., Ross G. C.: Studies on the enzyme systems of planorbid snails. Ann. trop. Med. Parasit. *60,* 522–525 (1966).

Wurzinger K.-H.: Allozymes of Ethiopian *Bulinus sericinus* and Egyptian *Bulinus truncatus.* Malacolog. Rev. *12,* 51–58 (1979).