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Alkaline phosphatase as marker of *Schistosoma mansoni* egg viability

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Abstract. A reliable assessment of the viability of schistosome eggs trapped in host tissues is difficult. The use of a coupling azo dye method for the detection of alkaline phosphatase (AlP) in *Schistosoma mansoni* ova was found to be a specific and sensitive method for differentiating between viable and dead eggs, and can be used in both immature and mature eggs. In fully developed miracidia within an egg, AlP activity was demonstrated in germ cells and in the sensory endings of the neural cells. The embryonating miracidia displayed AlP activity on the body surface and in von Lichtenberg's envelope. The alkaline phosphatase test for egg viability shows increased sensitivity when compared with the more conventional Oogram and Hatching tests.

The ability to assess the viability of schistosome eggs is of special interest in studies assessing the effects of drug or immune therapy on schistosome infections or following the rate of egg mortality over the course of a normal infection. Currently, the most frequently used methods for the measuring of egg viability are: (i) the hatching of miracidia (Richards et al. 1989), and (ii) the oogram method based on the assessment of egg morphology (Pellegrino et al. 1962). Both methods need relatively large amounts of material for light microscopy analysis not always readily available in cases of human schistosomiasis. A number of staining procedures, which are also used routinely to evaluate egg viability can result in diagnostic errors (Cheever 1986).

We were confronted with the problem of determining egg viability during our studies on both human schistosomiasis and in experimental *Schistosoma mansoni* infections in animals (Giboda et al. 1992a, b; Giboda and Smith 1994). In order to solve the problem we used alkaline phosphatase (AlP) staining of histological sections. This turned out to be a useful tool for the assessment of egg viability.

The histochemical detection of alkaline phosphatase in paraffin-embedded histological sections of schistosome eggs is of added interest, as Cesari et al. (1987), Pujol and Cesari (1990), and Lewis and Strand (1991) have since demonstrated the antigenic nature of alkaline phosphatase in adult *S. mansoni*.

MATERIALS AND METHODS

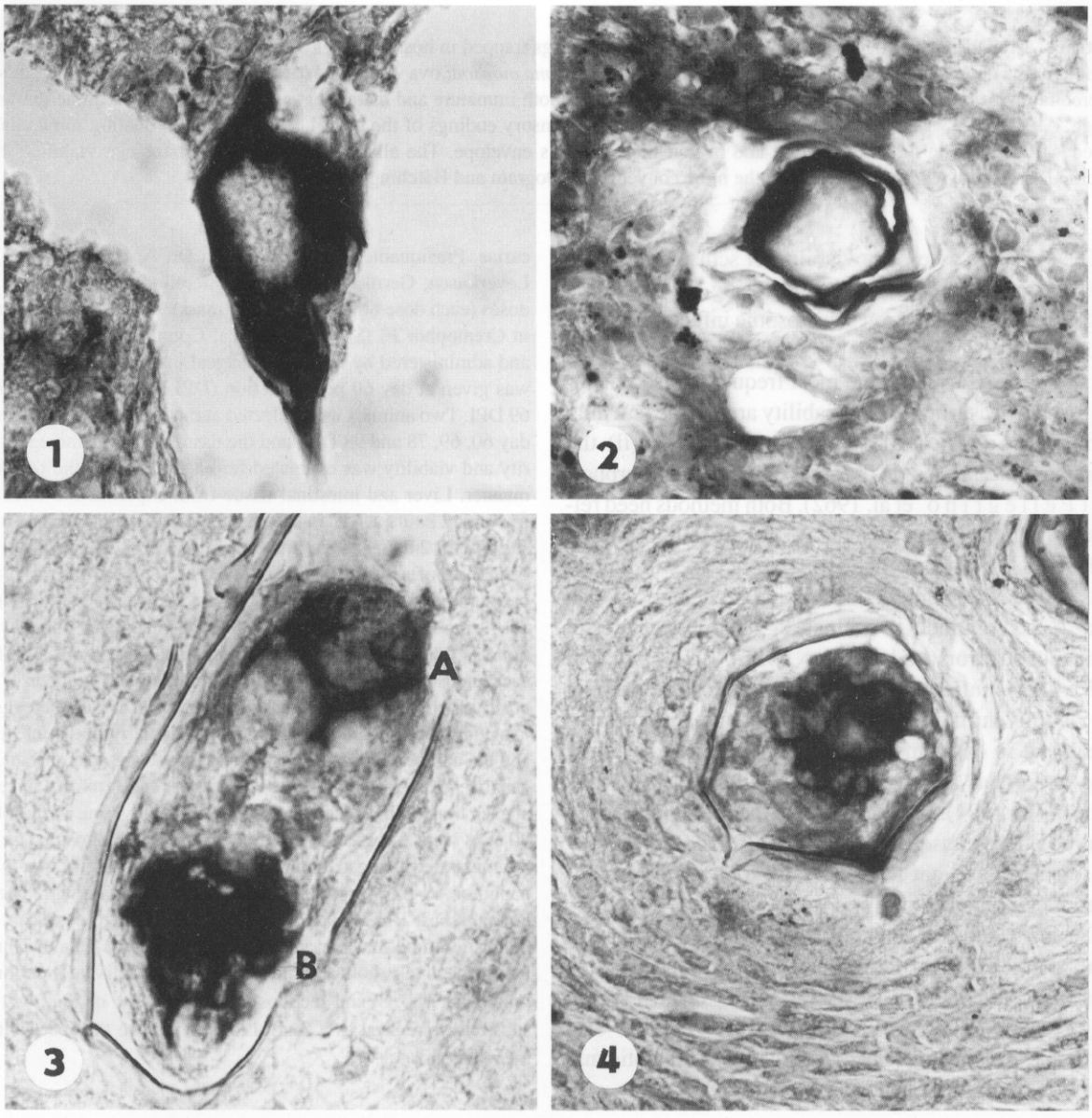
The detailed description of the experimental design is in a paper by Giboda and Smith 1994. Briefly, male outbred ICR mice were percutaneously infected with 200 *S.mansoni* cercariae. Praziquantel (PZQ) (gift from Dr. A. Harder, Bayer AG, Leverkusen, Germany) was administered to each mouse in two doses (each dose 800 mg kg^{-1} body mass) 9 days apart, suspended in Cremophor El (Sigma, Chemical Company, St. Louis, USA) and administered by intra-oesophageal intubation. The first dose was given at day 60 post infection (DPI), and the second one at 69 DPI. Two animals were selected at random from each group on day 60, 69, 78 and 98 DPI, and the tissue egg load and egg maturity and viability was estimated for each mouse in the following manner. Liver and intestinal tissue from experimental mice was fixed for 2 hours at 4°C in Baker's neutral formalin, washed in 5% sucrose for 2 hours at 4°C and transferred through ascending concentration of acetone (4°C) followed by benzene (at room temperature) and embedded in paraffin. The sections were cut at 8-10 µm. The AlP was detected histochemically in paraffin sections as follows: At least ten sections of each type of tissue from each mouse were microscopically examined and number of AlP positive and AlP negative eggs were recorded. Since the eggs are irregularly distributed in the tissues (particularly in the intestine), and the number of eggs per tissue and mouse are not constant, one cannot expect constant numbers of eggs per each group of mice and at each time point. In each group of mice at any time a total of 70-90 eggs were assessed. The presence of alkaline phosphatase was demonstrated using α-naphthyl phosphate, naphthol AS-D and naphthol AS-BI phosphate substrate in combination with diazonium salts Fast Blue BB and Fast Red TR (Pearse 1968). The incubation time for liver and intestinal tissue was 40 and 20 minutes respectively, both at room temperature. Inactivated sections (5 minutes at 100°C) and sections incubated in the medium without substrate were used as a control to demonstrate the specificity of the reaction.

RESULTS

Successive changes in the pattern of AlP localization were recorded during the development of the miracidium inside the egg. The miracidium, in the initial stage of embryonation, stained positive for AlP only on the

body surface and in von Lichtenberg's envelope (following the terminology of N e i l et al. 1988) (Figs. 1, 2). The site of AlP activity changed during the course of maturation of the miracidium. AlP was demonstrated in germ cells of the fully developed miracidium and in the sensory ending of the neural cells, located only at the anterior part of miracidium (terminology according to P a n 1980) (Figs. 3, 4.). In the liver where a black schistosomal pigment is present, coupling the azo dye with diazonium salt (Fast Red TR), stained AlP in the eggs red. This allowed for easier differentiation between hemozoin and positively stained AlP.

The viability of *S. mansoni* eggs in the liver of mice treated with two doses of PZQ is shown in Fig. 5. In PZQ treated mice a higher proportion of the eggs in the intestine were killed compared with those trapped in the liver. Nine days after the second dose of PZQ (18 days post treatment) nearly 29% of eggs in the liver and 14% in the intestine were positive for AlP. No viable eggs were found in the intestinal tissue 29 days after 2nd dose of PZQ whereas 6.3% of eggs in the liver displayed positive histochemical reaction for AlP.



Figs. 1-4. Activity of alkaline phosphatase in the egg of *Schistosoma mansoni*. **Figs. 1, 2.** Immature eggs with developing miracidium. Activity of alkaline phosphatase on the body surface layer and in von Lichtenberg's envelope. Fig. 1 - diagonal section (x 640); Fig. 2 - transversal section (x 500); both alpha-naphthyl phosphate + Fast Blue BB. **Figs. 3, 4.** Eggs with fully developed miracidium. Fig. 3 - longitudinal section (x 780); activity of alkaline phosphatase in sensory endings in the anterior part of miracidium. Fig. 4 - transversal section (x 780); alkaline phosphatase is bound with germ cells in the posterior part of miracicium. Both figures, naphtol AS-D phosphate + Fast Blue BB.

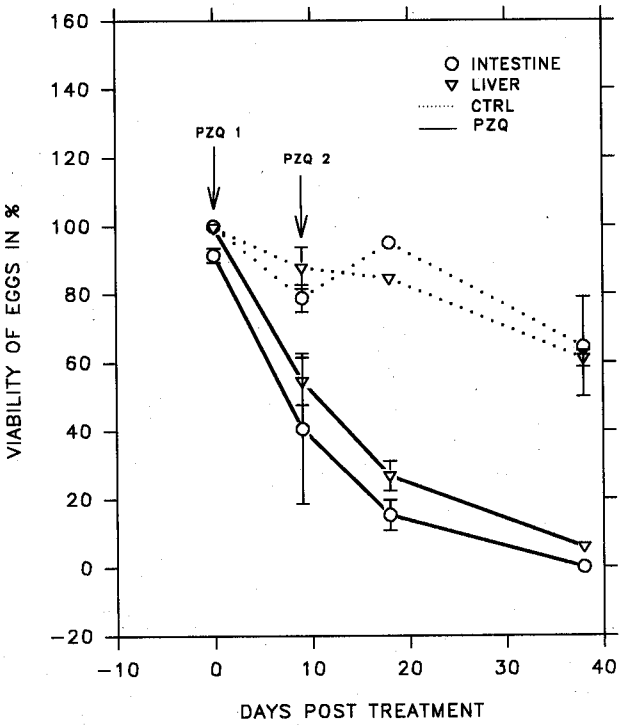


Fig. 5. Post treatment viability of *Schistosoma mansoni* eggs in mice treated with double oral dose of praziquantel at 60 DPI and at 69 DPI, and assessed by activity of alkaline phosphatase in both, immature and mature eggs. Each point on the graph represents the mean (± S. E.) of viable eggs calculated as the total number of AlP-positive: AlP-negative eggs from two mice and each type of tissues.

DISCUSSION

Coupling azo dye method for the detection of alkaline phosphatase in *S. mansoni* ova has proved to be a specific and sensitive method for differentiating between immature and mature dead and viable eggs. This contrasted with the other staining procedures that were also tested as possible indicators of *S. mansoni* egg viability: acid phosphatase, Ziehl Neelsen method, the periodic acid-Schiff (PAS) reaction, and Alcian blue-PAS. None of those turned out to be suitable for our purpose, because they either did not stain the morphological structures of the miracidium or could not differentiate between live and dead miracidia. Although the Oogram and Hatching tests were also used to assess viability in mature eggs, they did not provide an indication of the viability of immature eggs.

The presence of AlP in well preserved ova was reported by Dusanic (1959) and Andrade and Barka (1962), both using Gomori's method. In that test, calcified areas within the eggs also stained positive for AlP. However, in neither study was any evidence presented (e.g. controls for staining specificity) which showed that areas that stained positive for AlP could be differentiated from calcified areas. In our experiment, the calcified eggs were rapidly distinguished using von Kossa's stain (data not presented).

In our hands we consider the AlP test for egg viability to be more sensitive than either the Oogram or Hatching test. When oogram and hatching methods were compared with the AlP method, using material from the same experiment, no viable eggs were found in the liver and intestinal tissues 29 days after second dose of PZQ using the former methods (Giboda and Smith 1994). However, viability assessed by measuring AlP activity for the same time period revealed that 6.3% of the eggs trapped in the liver were viable but all eggs in the intestine were assessed as dead (Fig. 5).

Immunocytochemical study of soluble egg antigen (SEA) of the miracidium within the egg shell of *S. mansoni* demonstrated the SEA antigenic component to be in the area between the miracidium and the egg shell and in the membranous bodies of the epidermis (Bogitsh and Carter 1975). In addition, Hirata et al. (1987) demonstrated IgG fluorescence-positive substances in a restricted area, between the miracidium and the egg shell, of *Schistosoma haematobium* eggs. Localization of AlP in our study may correspond to the distribution of egg antigen as described by Bogitish and Carter (1975) and Hirata et al. (1987). In addition, alkaline phosphatase from adult *S. mansoni* has been demonstrated to be antigenic (Cesari et al. 1987, Pujol and Cesari 1990, Lewis and Strand 1991). Given the previous demonstration of the antigenic nature of this enzyme (AlP), which is a component of the schistosome egg, (Sung et. al. 1986, Hirata et al. 1987, Xu et al. 1988), its detection might well be used for serodiagnosis of an active infection or it may be exploited as an enzyme inhibitor.

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