

AN ABSTRACT OF THE THESIS OF

Carl A. Boswell for the degree of Doctor of Philosophy in Zoology  
presented on August 1, 1985.

Title: Immunological Perspectives of a Host-Parasite System: Studies  
on the Humoral and Cellular Components of *Biomphalaria glabrata*, New  
World Host for *Schistosoma mansoni*.

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Abstract approved: \_\_\_\_\_

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The host snail *Biomphalaria glabrata* and the parasitic trematode *Schistosoma mansoni* are valuable subjects for studies evaluating and defining immunological parameters that determine the outcome of individual host-parasite encounters. In this thesis, both soluble and cellular components of the snail's hemolymph were examined to gain insight into the basis for resistance and susceptibility to the parasite. Because agglutinins have antibody-like activity (e.g. agglutinate particles with appropriate surface ligands, some have been found on cell surfaces) they are considered by many to be immunoglobulin analogs. An agglutinin was isolated from snail plasma, its physicochemical properties defined, and its possible role in the snail-parasite interaction examined. The presence of additional agglutinins is inferred from cross-adsorption experiments.

It is known that the plant lectin concanavalin A binds to the surface of both hemocytes and sporocysts. I therefore tested the hypothesis that bringing hemocytes from susceptible (compatible) snails into contact with sporocysts in the presence of a cross-linking, or

"bridging", reagent would result in sporocyst killing. Data are presented showing that indeed sporocyst death increased dramatically when such an encounter occurred. While hemocyte cytotoxicity is induced by Con A treatment of sporocysts, further investigation revealed that bridging between the target and effector does not appear to be the inducing mechanism. It is suggested that Con A treatment of sporocysts causes them to reveal surface markers that normally benign hemocytes recognize as foreign, which then triggers a cytotoxic reaction by the hemocytes. These results are discussed in the context of lectin-dependent cell-mediated cytotoxicity reported for murine cytotoxic T-lymphocytes.

Studies of this type will lead to greater understanding of host-parasite immunobiology and of the evolutionary origins of the vertebrate immune system.

Immunological Perspectives of a Host-Parasite System: Studies on the  
Humoral and Cellular Components of Biomphalaria glabrata, New  
World Host for Schistosoma mansoni

by

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A THESIS  
submitted to  
Oregon State University

in partial fulfillment of  
the requirements for the  
degree of

Doctor of Philosophy

Completed August 1, 1985  
Commencement June, 1986

APPROVED:

Redacted for privacy

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Date thesis is presented August 1, 1985

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## ACKNOWLEDGEMENTS

This part of the thesis is the one I most looked forward to writing, and most dreaded, because this is the opportunity to recognize those who gave so much support, yet it will be impossible to adequately relate what that support meant.

I still marvel at my major professor, Dr. Christopher J. Bayne's, patience, enthusiasm, knowledge of the literature, and willingness to listen and help. From him I grew to appreciate the importance of conscientious study and lab work, and the value of maintaining one's scientific and personal integrity. His tutelage in scientific writing and critical thinking has provided me with a solid foundation upon which I can confidently build scientific credibility. I can only hope that during the six years of my graduate training he has found satisfaction in something I have done. It was mainly through his inspiration and ever present enthusiasm that I persevered through the low times to reach the high ones.

I also express my gratitude to the Zoology Department, Oregon State University, Sigma Xi Foundation, and the Zoology Department, University of Oklahoma, for their support, financial and otherwise.

I was graced by a cohort of graduate students that were unrivaled in personal and intellectual compatibility. I especially wish to acknowledge my association with: (Mother) Mary Yui, with whom I spent more time camping, soaking in hot springs, and eating Big Macs than has her boyfriend; Sandy "On the Verge" Petersen, whom I admire for being so successful despite the pitfalls both extrinsic and

self-inflicted, she encountered; and a man who is a sterling outfielder (day games only), and my equal in political cynicism and the appreciation of good beer, Tom Zoeller. With out these people, and many others of the same mold, e.g. Dave Herbst, John Scott, Jill Meisenhelder, graduate life would not have been crazy enough to remain sane.

I dedicate this thesis to my parents, Clifford E., and Marjorie M. Boswell. Their encouragement and occasional financial support I am very grateful for. I am also grateful for their patience during the periodic bouts in my earlier years with chronic Lack of Direction. The ol' homestead was a place I could depend on as a sort of refuge, with no expectations, no conditions, and an immediate familiarity. Thanks folks.

I also wish to acknowledge the support of Patty Estes. Her warmth and acceptance has filled a void I was not totally aware was there, and made the final push eminately more bearable. I look forward to returning the favor and to many more years to come.

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## CHAPTER 1

### IMMUNOLOGICAL PERSPECTIVES OF A HOST-PARASITE SYSTEM:

#### STUDIES ON THE HUMORAL AND CELLULAR COMPONENTS OF

#### BIOMPHALARIA GLABRATA, NEW WORLD HOST FOR

#### SCHISTOSOMA MANSONI

### INTRODUCTION

From the time of Methchnikoff's report (1905) that ameboid cells within a starfish embryo accumulated around a foreign object (in this case a rose thorn), investigators have been searching for and describing immunologically active cells and substances in invertebrates. Much effort has been expended in the search for pre-vertebrate immunoglobulin, and the general consensus is that it is absent from invertebrates (Warr, 1981). This perhaps points out a widespread preconception that if a vertebrate-type immune system (specific antibody, anamnestic response, lymphocytes) cannot be discerned in an organism, that organism does not have an immune system. Yet the fact remains that the world abounds with invertebrates, the vast majority of which are apparently quite healthy, despite living in an environment rich with pathogens and parasites. This observation suggests that invertebrates must possess efficient systems for internal surveillance and clearance of foreign

materials from their internal milieu.

It is clear that immunoglobulin is not a component of the invertebrate immune response repertoire, yet the so-called superfamily of vertebrate immunoglobulin-related compounds is composed of molecules with amino acid compositions similar to those found within invertebrates (Williams and Gagnon, 1982; Vasta and Marchalonis, 1985). Some of these substances from invertebrates are lectins (carbohydrate-binding proteins), and their sugar specificity suggests that they could act as recognition molecules in tasks including tissue and organ differentiation, transport of nutrients or metabolites, or recognition and marking of foreign material (Lackie, 1980). Lectins have been found in association with the membranes of phagocytes from several molluscs (Vasta et al, 1982; Renwrautz and Stahmer, 1983; van der Knaap et al, 1983). With their antibody-like agglutinating and opsonizing activity, these lectins are prime candidates for pre-vertebrate recognition molecules, i.e. immunoglobulin analogs.

In addition to agglutinins and other humoral factors of putative immunologic importance such as bactericidins, lysins (Cooper and Lemmi, 1981), and phenoloxidase (Soderhall, 1982), all metazoan animals possess amoeboid phagocytic cells (Ratcliffe and Rowley, 1981). In simpler animals such as sponges and cnidarians, these cells act predominantly to transport nutrients from the sites of ingestion to peripheral regions of the body. In organisms with some form of circulatory system, nutrient and waste transport is accomplished via the body fluids, and motile phagocytes (hemocytes) take on more specialized roles. These activities include sealing and repairing of

wounds, clearance of necrotic tissues and invading microbes, and walling off (encapsulation) and destruction of larger bodies, e.g. metazoan parasites. These phenomena appear to be ubiquitous throughout the metazoan phyla, yet the stimuli that induce these reactions, and the biochemical processes underlying them are poorly understood.

The research on which this thesis is based was undertaken to gain insight into what comprises an invertebrate immune system, and how it functions when called upon to protect the organism. The work was accomplished using a model consisting of an invertebrate host, the pulmonate snail Biomphalaria glabrata, and the human blood fluke Schistosoma mansoni. B. glabrata is the New World vector and intermediate host for this parasite, which infects an estimated 200 million people world wide (Noble and Noble, 1982).

Snail strains have been developed that are genetically defined in terms of their resistance or susceptibility to a specific strain of the parasite (Richards, 1975; 1984). In these studies the strains used predominantly were the M-line, which is susceptible, and the 13-16-R1, which is resistant to the PR-1 strain of S. mansoni.

The difference between susceptibility and resistance in this host-parasite system is clearly based on the activity of the snail's hemocytes (Basch, 1976; Lie et al, 1980). There are still numerous unanswered questions regarding the mechanisms employed by these cells in both recognition and killing processes, and humoral factors appear to be involved in certain circumstances (Bayne et al, 1980a; Loker and Bayne, 1982; Granath and Yoshino, 1984). One potential class of

opsonic humoral factors could be the agglutinins found in snail hemolymph. It may be that susceptibility is due to the absence of an agglutinin found only in resistant snail hemolymph. The fact that hemocytes from resistant snails are cytotoxic in vitro in the absence of any soluble snail components (Bayne et al, 1980a) may simply mean that this putative recognition factor is also carried on the surface of the hemocytes, as suggested for macrophages (Sharon, 1984), and may even be synthesized by them.

The first part of this thesis reports on the search for and characterization of a soluble factor in the hemolymph of B. glabrata that could have an immunologic function. The remainder of the research focused on the cellular components of the snail's immune system. In particular, an in vitro cytotoxic reaction induced by the plant lectin Concanavalin A, and mediated by susceptible snail hemocytes was studied. Lectin-dependent cell-mediated cytotoxicity studies were initiated based on the idea that if the resistance factor supposedly missing from susceptible hemolymph was an agglutinin, an exogenous agglutinin or lectin may mimic its function.

## REFERENCES

- Basch, P.F. 1976. Intermediate host specificity in Schistosoma mansoni. Exp. Parasitol. 39:150-169.
- Bayne, C.J., Buckley, P.M. and DeWan, P.C. 1980a. Schistosoma mansoni: Cytotoxicity of hemocytes from susceptible snail hosts for sporocysts in plasma from resistant Biomphalaria glabrata. Exp. Parasit. 50, 409-416.
- Cooper, E.L. and Lemmi, C.A.E. 1981. Invertebrate humoral immunity. Dev. Comp. Immunol. 5:3-21.
- Granath, W.O. and Yoshino, T.P. 1984. Schistosoma mansoni: Passive transfer of resistance by serum in the vector snail, Biomphalaria glabrata. Exp. Parasit. 58:188-193.
- Lackie, A.M. 1980. Invertebrate Immunity. Parasitology. 80:393-412.
- Lie, D.J., Jeong, K.H. and Heyneman, D. 1980. Tissue reactions induced by Schistosoma mansoni in Biomphalaria glabrata. Ann. Trop. Med. Parasitol. 74:157-166.
- Loker, E.S. and Bayne, C.J. 1982. In vitro encounters between Schistosoma mansoni primary sporocysts and hemolymph components of susceptible and resistant strains of Biomphalaria glabrata. Am. J. Trop. Med. Hyg. 31:999-1005.
- Metchnikoff, E. Immunity in Infective Diseases. 1905. Reprinted by Johnson Reprint Corp., New York, 1968. 591 pp.
- Noble, E.R. and Noble, G.A. 1982. Parasitology. The Biology of Animal Parasites. Lea and Febiger, Philadelphia. 522pp.
- Ratcliffe, N.A. and Rowley, A.F. (Eds) 1981. Invertebrate Blood Cells, vol. I and II. Academic Press, New York. 641 pp.
- Renwrantz, L. and Stahmer, A. 1983. Opsonizing properties of an isolated hemolymph agglutinin and demonstration of lectin-like recognition molecules at the surface of hemocytes from Mytilus edulis. J. Comp. Physiol. 149:535-546.
- Richards, C.S. 1975. Genetic factors in susceptibility of Biomphalaria glabrata for different strains of Schistosoma mansoni. Parasitology. 70:231-241.
- Richards, C.S. 1984. Influence of snail age on genetic variation of Biomphalaria glabrata for infection with Schistosoma mansoni. Malacologia. 25:493-503.

Sharon, N. 1984. Surface carbohydrates and surface lectins are recognition determinants in phagocytosis. *Immunol. Today*. 5:143-147.

Soderhall, K. 1982. Prophenoloxidase activating system and melanization - a recognition mechanism of arthropods? A review. *Dev. Comp. Immunol.* 6:601-611.

van der Knaap, W.P.W., Boots, A.M.H. and Sminia, T. 1983. Immunorecognition in Lymnaea stagnalis. *Dev. Comp. Immunol.* 7:645-648.

Vasta, G.R. and Marchalonis, J.J. Invertebrate agglutinins and the evolution of humoral recognition factors. 1985. In: *Cell Receptors and Cell Communication in Invertebrates*, (B.A. Cinader, ed.). Marcel Dekker, New York. In press.

Vasta, G.R., Sullivan, J.T. Cheng, T.C. Marchalonis, J.J. and Warr, G.W. 1982. A cell membrane-associated lectin of the oyster hemocyte. *J. Invert. Pathol.* 40:367-377.

Warr, G.W. 1981. Immunity in invertebrates. *J. Invert. Pathol.* 38:311-314.

Williams, A.F. and Gagnon, J. 1982. Neuronal cell Thy-1 glycoprotein: Homology with immunoglobulin. *Science*. 216:696-703.

## CHAPTER 2

ISOLATION, CHARACTERIZATION AND FUNCTIONAL ASSESSMENT  
OF A HEMAGGLUTININ FROM THE PLASMA OF BIOMPHALARIA  
GLABRATA, INTERMEDIATE HOST OF SCHISTOSOMA MANSONI<sup>1</sup>

Carl A. Boswell and Christopher J. Bayne

## ABSTRACT

An agglutinin from the plasma of Biomphalaria glabrata, vector of Schistosoma mansoni, has been isolated and partially characterized. Its isolation is a simple two-step process, yielding an essentially pure preparation that can then be manipulated free from other plasma components. It is a large glycoprotein ( $10^6$  daltons), found in four of the five snail strains tested, and possesses affinity for galactose-type sugar residues. Based upon criteria such as molecular weight, carbohydrate and erythrocyte specificity, physical stability and anatomical source, this agglutinin is considered to be distinct from others reported from this mollusc. Involvement of the agglutinin in the host-parasite interaction is discussed.

<sup>1</sup> Slightly modified from Dev. Comp. Immunol., 1984, 32:322-327.

## INTRODUCTION

Invertebrate immune responses are characterized as being predominantly cell mediated; circulating hemocytes phagocytose small foreign particles, or encapsulate larger bodies. The mechanism(s) whereby these cells recognize a surface as "non-self" remain to be determined (Bayne, 1983); one possibility is that substances soluble in hemolymph may "mark" (opsonize) a parasite, for instance, as foreign, and thereby elicit the proper immune response from hemocytes. Plasmas from certain gastropods do contain opsonic substances (Sminia et al, 1979; Renwraantz et al, 1981). Agglutinins, widespread in invertebrate body fluids, may be such soluble recognition factors. In some molluscs, opsonization is due to soluble agglutinins (Pauley et al, 1971; Renwraantz and Mohr, 1978; van der Knaap et al, 1982; Renwraantz and Stahmer, 1983). In others, agglutinins are both humoral and intrinsic components of hemocyte surfaces (Vasta et al, 1982; Renwraantz and Stahmer, 1983).

Hemocytes of the planorbid snail Biomphalaria glabrata, New World vector for the human blood fluke Schistosoma mansoni, phagocytose erythrocytes of some species without a need for opsonization (Abdul-Salam and Michelson, 1980). Yet earlier work from this lab suggests that snail plasma factors may be important in the initial encounter between schistosome sporocysts and host hemocytes, and may in some cases determine the outcome of this encounter (Bayne et al, 1980a). With this in mind we have searched for soluble factors

that might play a role in host hemocyte-parasite interactions.

### MATERIALS AND METHODS

Animals. All snails were maintained at 26 C in Ulmer's artificial spring water (Ulmer, 1980) and fed lettuce and chalk ad libitum. The strains tested for the presence of agglutinin were developed by Dr. C.S. Richards at NIH (Richards, 1975; 1984), and included L311, MRLc, M-line, 10-R2, and 13-16-R1. The first three are susceptible to our PR-1 strain of Schistosoma mansoni, and the last two are resistant. The parasite cycle is maintained in our lab using Swiss-Webster mice as definitive host and the M-line strain of snail as the intermediate host (Stibbs et al, 1979).

Hemolymph Collection. This was as previously described (Bayne et al, 1980a), and the plasma was cleared of all cells and debris by centrifugation at 400 xg for 10 min at 4 C. Initial agglutination assays, screening for erythrocyte and sugar specificities, were done with freshly collected plasma. At least twelve snails (10-15 mm shell dia) were bled for each batch. All other characterizations were done with agglutinin isolated as described below.

Reagents. DEAE-Sephadex A-50 and all sugars and glycoproteins used in inhibition assays were purchased from Sigma Chemical Co. Glycoproteins were desialyzed by incubating 10 mg ml<sup>-1</sup> in 0.1 N H<sub>2</sub>SO<sub>4</sub>, 0.85% NaCl for 1 hr at 80 C. The desialyzed glycoprotein was then returned to physiological conditions by dialysis with hemagglutination buffer (HB, 6.6 mM phosphate buffer, pH 7.3 with

.85% NaCl and .02% sodium azide; Wheeler et al, 1950) overnight at 4 C. Galactogen was prepared by homogenizing the albumen glands from five adult Lymnaea stagnalis in 7 ml ice-cold 2.5% perchloric acid. To 1 vol. of the 39,000 xg supernatant was added 1.5 vol. of 95% ethanol, and the galactogen was allowed to precipitate for 2.5 hr at 4 C. This was then centrifuged at 4,500 xg for 10 min, the pellet redissolved in distilled water (dw), and reprecipitated with ethanol overnight. The resulting pellet was dissolved in dw, lyophilized, then taken to 5 ug ml<sup>-1</sup> with HB.

Agglutination Assays. The following erythrocytes were obtained locally: human types A<sub>1</sub>, A<sub>2</sub>, B, O; horse; cat; dog; goat; pig; cow; rabbit; mouse; rat; calf; lyophilized and glutaraldehyde-fixed sheep, rabbit, goat, pig; trypsinized and glutaraldehyde-fixed calf. The assays were performed in round-bottom micro-titer plates, in which 25 ul of test solution in two-fold serial dilutions in wells 1-11 was mixed with 25 ul of a 2% suspension of erythrocytes thrice washed in HB. The twelfth well contained only buffer and erythrocytes. The plate was agitated by hand for ten seconds, then allowed to develop for one hour at room temperature. The plate was scored for agglutinin titer, the cells resuspended by gently tapping the edge of the plate, then incubated at 4 C overnight and scored again. The agglutinin titer, defined as the reciprocal of the greatest dilution at which agglutination occurred, was consistently higher upon reading the second time, yet the relative end points between tests remained unchanged.

Inhibition Assays. Initial inhibition studies were performed

using sugars at 500 mM and glycoproteins at 40 mg ml<sup>-1</sup> final concentrations. Sugars and glycoproteins were dissolved in HB at 4 times the stated concentration, then added to the series of agglutinin dilutions at 1/4 the final volume. Rabbit erythrocytes (RaRBC) were then added, the plate was gently agitated by hand for 10 sec., then agglutination was allowed to develop. When a sugar or glycoprotein was seen to be inhibitory at the original level, additional runs were done to determine the lowest amount that reduced the apparent agglutinin titer by at least two dilutions.

Agglutinin Isolation. The agglutinin was isolated in a two-step process. Snail plasma (cell-free hemolymph), dialyzed for two hours at 4 C against HB, was passed over a column of DEAE-Sephadex A-50 equilibrated with HB. Whereas most plasma proteins bound to the matrix, agglutinating activity was recovered in the unbound fraction. This fraction was then taken to 10% saturation with crystalline ammonium sulfate and allowed to precipitate overnight at 4 C. The precipitate was resuspended by brief vortexing, then pelleted at 8,000 xg for 30 min. The pellet was dissolved in distilled water, dialyzed against distilled water overnight at 4 C, lyophilized, then reconstituted in HB, usually to one-half the volume of the original plasma from which it had been isolated.

Stability Experiments. Freeze-thaw experiments were carried out using a dry ice:ethanol bath. Once a 50 ul sample was frozen solid, it was removed from the bath and held in the hand until thawed. It was then left at room temperature for five minutes before being returned to the freezing bath. Isolated agglutinin was heat treated

by incubating separate 100 ul volumes in constant temperature (30, 40, or 50 C) water baths for one hour. Stability at various pH's was tested by dialyzing the agglutinin against the following 0.1 M buffers for 24 hr at 4 C: glycine-HCl, pH 2; Na acetate-acetic acid, pH 4; Na-K phosphate, pH 6 and 8; glycine-NaOH, pH 10 and 12 (Hale, 1965). The agglutinin solutions were then reequilibrated with HB by similar dialysis, then tested for activity. Since only small volumes were available, special dialysis chambers were used, based on the design of Armstrong and Tate (1980).

Sodium meta-periodate oxidation of carbohydrate moieties was performed according to the method of Fuke and Sugai (1972).

Ouchterlony double diffusion was utilized to confirm the presence of carbohydrate moieties on the isolated agglutinin. Concanavalin A (Con A), lentil lectin, peanut lectin, and Ricinus lectin (Boehringer Mannheim Biochemicals) were tested at 1 and 2 mg ml<sup>-1</sup> for their ability to form a precipitate with the agglutinin.

Determination of Molecular Weight. Polyacrylamide gel electrophoresis was based on the Laemmli method (1970), and the separated proteins were visualized by the silver stain of Merrill et al (1981). The high molecular weight marker kit from Sigma was used to estimate MW. Octopus dofleini hemocyanin (subunit MW  $3.5 \times 10^5$  daltons) was included for the electrophoresis runs in non-reducing conditions. Sedimentation velocity ultracentrifugation was performed on a Beckman Model E analytical ultracentrifuge. Isolated agglutinin dissolved in HB was sedimented at 40,000 RPM in a 7 cm radius rotor and the migrating front was monitored at 280 nm.

Function of the Agglutinin. The ability of the agglutinin to affect sporocyst agglutination was tested according to methods developed in this lab (Loker et al, 1984). The sporocyst agglutination assay was performed in siliconized 500 ul test tubes. Sporocysts (n = 150), transformed in vitro from mouse liver-derived miracidia (Stibbs et al, 1979), were placed in the tubes, washed free of culture medium, then agglutinin was added at approximately original concentration in Medium F (Stibbs et al, 1979) with 5% BSA. After 24 hours, agglutination was scored by rapidly twirling the tube to resuspend the parasites, then looking for aggregated sporocysts under a dissecting microscope.

### RESULTS

Of the 12 erythrocyte types screened, only fresh, untreated rabbit cells were agglutinated by B. glabrata plasma. All snail strains except the 10-R2 possessed this agglutinating activity. Titers varied from one batch of plasma to another, ranging from 16 to 256. When whole snail plasma was used, hemolysis of RaRBC was occasionally seen, but this was not a consistent phenomenon.

Agglutinin activity was significantly reduced by carbohydrates with galactose-type structure. The most potent inhibitors, in decreasing order of effectiveness, and the concentrations at which they reduced the apparent titer by two dilutions were:

N-acetyl-D-galactosamine (14.3 mM), desialized bovine mucin (.625 mg ml<sup>-1</sup>) > raffinose (100 mM) > 3-o-galacto-D-arabinose (125 mM) >

galactogen ( $1.25 \text{ mg ml}^{-1}$ ) > bovine mucin ( $1.25 \text{ mg ml}^{-1}$ ).

The agglutinin was insensitive to rapid freezing and thawing, retaining its original activity after ten cycles. When it was maintained at 50 C for one hour, all activity was lost whereas no decrease in activity was observed after one hour at 40 C.

Agglutinating activity was relatively stable to pH shifts, being irreversibly reduced only when the pH was altered radically from neutrality (Fig. 2.I). The agglutinin is independent of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ions; neither dialysis against 100 mM EDTA for 24 hours nor the inclusion of EDTA in HB changed the agglutinin titer.

The agglutinin was inactivated when treated with 20 mM sodium periodate.

Of the four lectins tested, the agglutinin formed precipitin lines only with Con A and lentil lectin.

The purifications obtained during isolation procedures are illustrated in Fig. 2.II. Clearly the agglutinin is a minor component of the total plasma solute (lane 1). It is one of only a few proteins which are obtained in the DEAE Sephadex flow-through (lane 2). Since the silver stain employed is approximately 50 times more sensitive than Coomassie blue (Merril et al, 1981), the isolated agglutinin represented as a single band (10% ammonium sulfate precipitate) in lane 3 can be considered to be essentially pure. Though not all of the agglutinin was precipitated by 10% ammonium sulfate (lane 4), higher concentrations began to precipitate the contaminants. Because of its low resolution on SDS-PAGE, the estimated molecular weight under reducing conditions is given as a range of 89-105 kilodaltons

(Kd).

Sedimentation velocity ultracentrifugation of the native, isolated agglutinin resulted in an  $S_{20,w}$  value of 24.98. Assuming a globular configuration for the molecule, this corresponds to an approximate MW of  $0.8-1.2 \times 10^6$  Kd.

The question of what role disulfide linkages between subunits might play in maintaining molecular integrity was explored using SDS-PAGE in the absence of reducing agent. Lane 2 in Figure 2.III reveals three diffuse bands that correspond to approximately 2, 3, and 4x multiples of the fully reduced agglutinin (Fig. 2.III, lane 1).

Repeated attempts to demonstrate agglutinating activity toward S. mansoni sporocysts gave negative results. Although live sporocysts agglutinate in plasma from all snail strains tested in our lab (Loker et al, 1984), this molecule does not appear to be the active species.

### DISCUSSION

It is evident from the ten-fold greater molecular weight with sedimentation velocity ultracentrifugation than SDS-PAGE that the agglutinin described here is a large glycoprotein comprised of approximately 10 subunits. The "ladder" effect seen in Fig. 2.III (lane 2) occurs when subunits that are associated non-covalently are denatured in SDS, and normally hidden (cryptic) -SH groups are exposed. If no exogenous reducing agent is present, these groups can react with one another, forming disulfide-linked multimers (Fuller et

al, 1973; Francis and Becker, 1984). Hence we envisage this hemagglutinin to be a decamer of  $10^5$  dalton subunits, which are non-covalently associated. Each subunit may contain internal cysteine residues which, in the presence of SDS, can establish covalent linkages between subunits.

The diffuse appearance of the agglutinin on PAGE suggested that it may be a glycoprotein. The presence of carbohydrate on the molecule was confirmed by its sensitivity to sodium periodate oxidation (see next paragraph) and by its precipitation by Con A and lentil lectin. The molecule probably contains some distal mannose-type moieties. Con A is not glycosylated, so the precipitate could not be due to any lectin-like activity of the agglutinin. Lacking competing sugar studies, the possibility of hydrophobic interactions can not be ruled out.

The active agglutinin required intact carbohydrate constituents, since it was inactive after periodate oxidation. This finding implies either that sugar moieties are important in maintaining the molecule's tertiary or quaternary structure, or that sugar residues may be a primary component of the ligand-binding site, or both.

The two-step isolation process resulted in an essentially pure agglutinin preparation. When, after stability experiments, activity of whole plasma was compared with isolated agglutinin, there was no indication that cofactors or divalent cations are important.

The agglutinin described here appears to be distinct from other agglutinins reported from B. glabrata (Gilbertson and Etges, 1977; Michelson and Dubois, 1977; Stein and Basch, 1979; Jeong et al, 1981).

This distinction is based on criteria such as MW, sugar and erythrocyte specificity, stability under various physical conditions, and divalent cation requirements (Table 2.I). The agglutinin for glutaraldehyde-fixed, trypsinized calf erythrocytes (GTC) reported by Jeong et al (1981) has not been extensively characterized, so judgements concerning similarities based on physical or chemical parameters must await further analyses. Cross-absorption studies in this lab using GTC cells and RaRBC indicate no cross-reactivity by our isolated agglutinin. Nor have we observed an increase in titers of this agglutinin against either cell type in snails infected with Echinostoma lindoense as reported by Jeong et al (1981). These results should be viewed with caution, however, since our experiments were done using erythrocytes from a single calf, and the apparent titer of GTC agglutinin is greatly influenced by the particular source of cells (Jeong, pers. comm.).

Compatibility between strains of snails and parasites may depend upon acquisition of host antigens onto the parasite surface, thereby masking its own antigens. This has been shown unequivocally in the adult stages (McLaren and Terry, 1982; reviewed by Dean, 1983). We reasoned that if the agglutinin was a putative masking agent, one should see sporocyst aggregates in its presence. Results of the sporocyst agglutination assay gave no indication of agglutinin binding to sporocysts. These results, along with the observation that one resistant strain of snail possesses the agglutinin while the other does not, speak against the agglutinin being important in the interplay between host and parasite. However, our results do not

preclude the possibility that sporocysts mimic the agglutinin. Sporocysts cultured in the complete absence of snail-derived molecules display surface antigens that cross-react with antibodies directed against snail plasma components (Yoshino and Bayne, 1983). When an agglutinin-specific probe is developed, the question of agglutinin participation at the host-parasite interface will become resolvable.

#### ACKNOWLEDGMENTS

We are indebted to Drs. Kensel Van Holde and Karen Miller for their help in performing and interpreting the ultracentrifugation experiment and for the gift of purified Octopus hemocyanin. Thanks also to Mary Yui and Tom Zoeller for critical comments on the manuscript. This study was supported by grants from Sigma Xi, The Scientific Society, and by NIH grant AI-16137 to C.J.B.

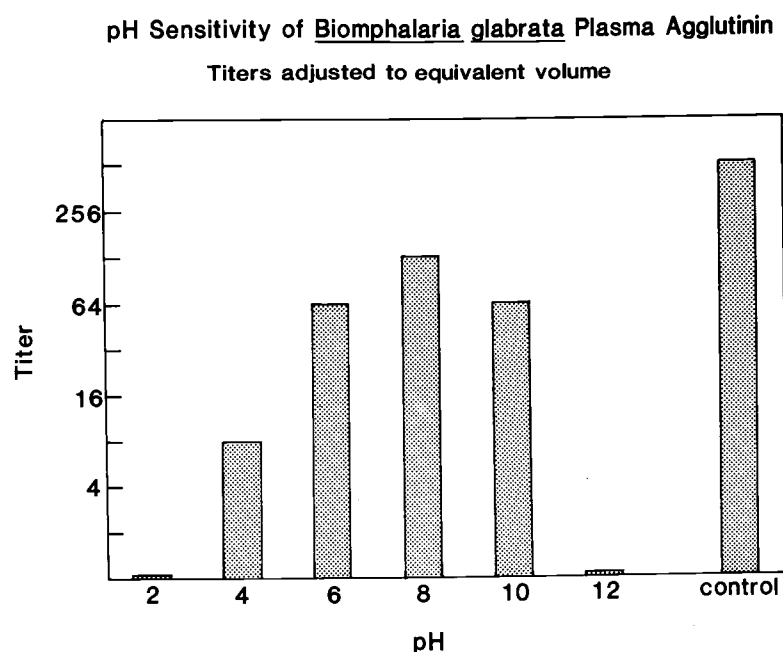


Figure 2.I. Agglutinin titers after dialysis against buffers of different pH, and readjustment to physiological pH. The values were adjusted to compensate for changes in volume. The control was not dialyzed.

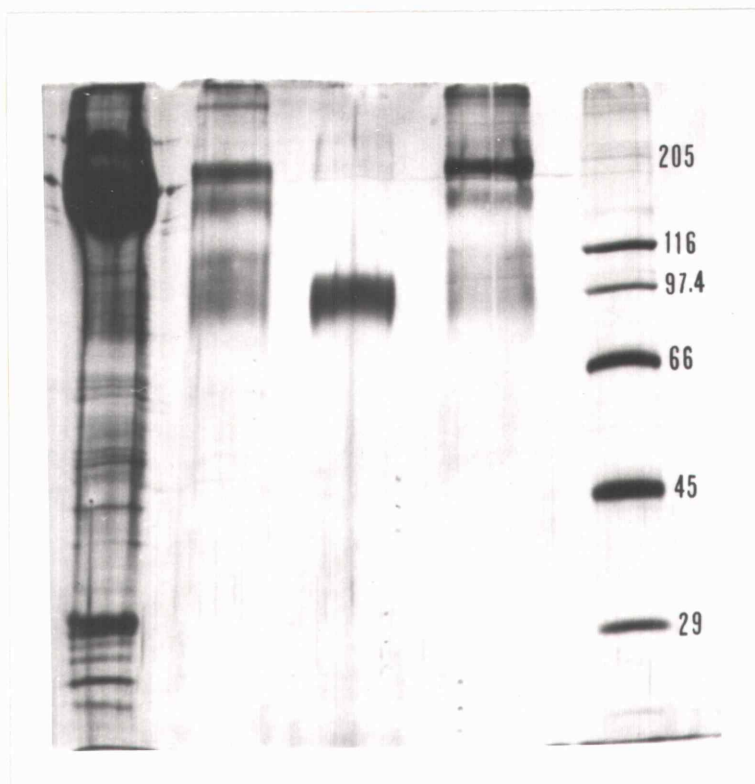


Figure 2.II. SDS-PAGE illustrating the steps of agglutinin isolation. The running gel was 7.5% acrylamide, in .375 M Tris buffer with 0.1% SDS. All samples were treated with 10% 2-mercaptoethanol. Lane 1: whole snail plasma, diluted 1:4. Lane 2: ion exchange chromatography void volume. Lane 3: precipitate from 10% ammonium sulfate fractionation of the same sample as Lane 2. Lane 4: 10% ammonium sulfate fractionation supernatant. Lane 5: molecular weight standards,  $\times 10^{-3}$  daltons.

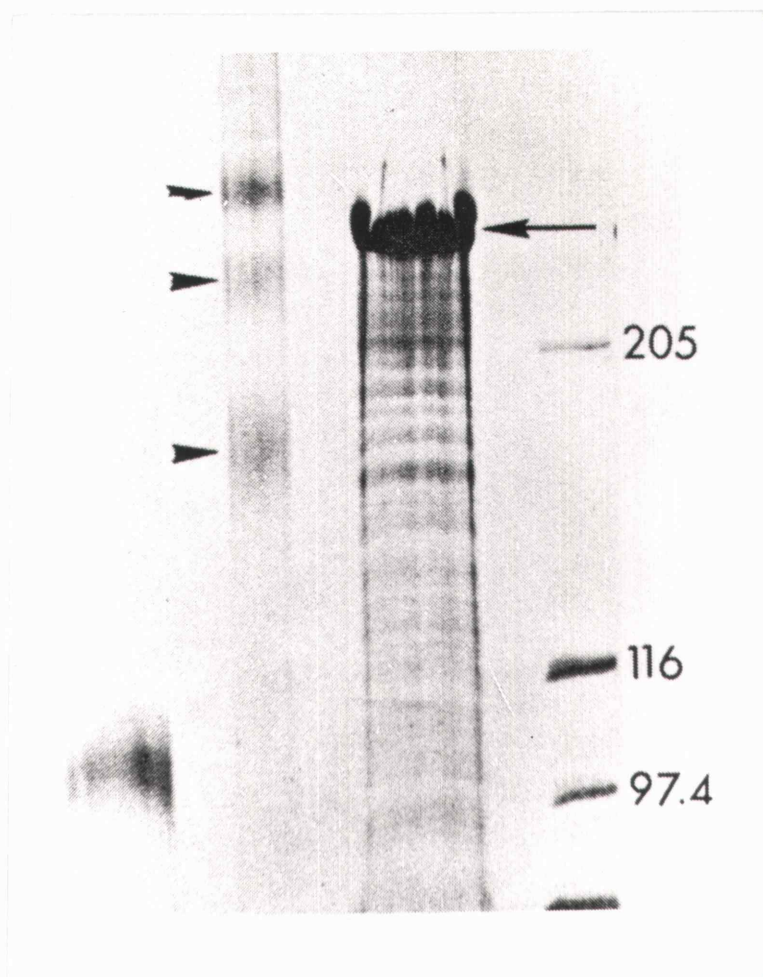


Figure 2.III. SDS-PAGE demonstrating "ladder" effect when agglutinin was not treated with a reducing agent. The running gel was 5% acrylamide, in .375 M Tris and 0.1% SDS. All samples were treated with 2% SDS and 5% 2-mercaptoethanol, except lane 2. Lane 1: isolated agglutinin. Lane 2: isolated agglutinin (arrow heads) in 2% SDS, no 2-mercaptoethanol. Lane 3: *Octopus dofleini* hemocyanin (arrow), MW 350 Kd. Lane 4: molecular weight standards,  $\times 10^{-3}$  daltons.

TABLE 2.I  
Comparison of Agglutinins Reported from Biomphalaria glabrata<sup>1</sup>

Source	Erythrocytes Agglutinated	Sugar Specificity	Heat Stability	Freeze/Thaw	Divalent Cations Required	MW
Hemolymph (Bahian strain) <sup>2</sup>	Human, rabbit	not done	inactivated @ 56°C, 30'	labile	yes	not done
Hemolymph, albumen gland, egg masses (many strains) <sup>3</sup>	Human A <sub>1</sub> , B, O	glucose	56°C, 30' no effect	no effect (10X)	not done	non-dialyzable
Hemolymph, albumen gland, egg masses. (PR-B strain) <sup>4</sup>	Human A <sub>1</sub> , A <sub>2</sub>	not done	not done	labile	yes	55,000
Hemolymph (4 strains) <sup>5</sup>	rabbit	Gal-NAC	inactivated @50°C, 60'	no effect (10X)	no	ca. 1x10 <sup>6</sup>

<sup>1</sup> In Stanislawski, Renwranz and Becker (1976) agglutinins for human A, B and O erythrocytes were not characterized.

<sup>2</sup> Gilbertson and Etges, 1967.

<sup>3</sup> Michelson and Dubois, 1977.

<sup>4</sup> Stein and Basch, 1979.

<sup>5</sup> This report.

# REFERENCES

- Abdul-Salam, J.A. and Michelson, E.H. 1980. Phagocytosis by amoebocytes of Biomphalaria glabrata: Absence of opsonic factor. *Malacological Rev.* 13:81-83.
- Armstrong, I.L. and Tate, W.P. 1980. A simple device for the dialysis of small volumes. *Anal. Biochem.* 106:469-470.
- Bayne, C.J., *Molluscan Immunobiology*. 1983. In: *The Mollusca*, vol. 5, pt. 2. (A.S.M. Saleuddin and K.M. Wilbur, eds.). Academic Press, New York. pp. 407-486.
- Bayne, C.J., Buckley, P.M. and DeWan, P.C. 1980a. Schistosoma mansoni: Cytotoxicity of hemocytes from susceptible snail hosts for sporocysts in plasma from resistant Biomphalaria glabrata. *Exp. Parasitol.* 50:409-416.
- Bayne, C.J., Loker, E.S., Yui, M.A., and Stephens, J.A. 1984. Immune-recognition of Schistosoma mansoni primary sporocysts may require specific receptors on Biomphalaria glabrata hemocytes. *Parasite Immunol.* 6:519-528.
- Dean, D.A. 1983. A review. Schistosoma and related genera: Acquired resistance in mice. *Exp. Parasitol.* 55, 1-104.
- Francis, R.T. and Becker, R.R. 1984. Specific indication of hemoproteins in polyacrylamide gels using a double-staining technique. *Anal. Biochem.* 136:509-514.
- Fuke, M.T. and Sugai, T. 1972. Studies on the naturally occurring hemagglutinin in the coelomic fluid of an ascidian. *Biol. Bull.* 143:140-149.
- Fuller, G.M., Raseo, M.A., McCombs, M.L., Barnett, D.R. and Bowlan, B.H. 1973. Subunit composition of haptoglobin 2-2 polymers. *Biochemistry.* 12:253-258.
- Gilbertson, D.E., and Etges, F.J. 1967. Haemagglutinins in the haemolymph of planorbid snails. *Ann. Trop. Med. Parasitol.* 61:144-147.
- Hale, L.J. *Biological Laboratory Data*. London: Methuen and Co. Ltd., 1965.
- Jeong, K.H., Sussman, S., Rosen, S.D., Lie, K.H. and Heyneman, D. 1981. Distribution and variation of hemagglutinating activity in the hemolymph of Biomphalaria glabrata. *J. Invert. Pathol.* 38:256-263.

- Laemmli, V.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
- Loker, E.S., Yui, M.A. and Bayne, C.J. 1984. Schistosoma mansoni: Agglutination of sporocysts, and formation of gels on miracidia transforming in plasma of Biomphalaria glabrata. *Exper. Parasitol.* 58:56-62.
- McLaren, D.J. and Terry, R.J. 1982. The protective role of acquired host antigens during schistosome maturation. *Parasite Immunol.* 4:129-148.
- Merril, C.R., Goldman, P., Sedman, S.A. and Ebert, M.H. 1981. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* 211:1437-1438.
- Michelson, E.H., and Dubois, L. 1977. Agglutinins and lysins in the molluscan family Planorbidae: A survey of hemolymph, egg-masses, and albumen-gland extracts. *Biol. Bull.* 153:219-227.
- Pauley, G.B., Krassner, S.M. and Chapman, F.A. 1971. Bacterial clearance in the California sea hare, Aplysia californica. *J. Invert. Pathol.* 18:227-239.
- Renwrantz, L. and Mohr, W. 1978. Opsonizing effect of serum and albumen gland extracts on the elimination of human erythrocytes from the circulation of Helix pomatia. *J. Invert. Pathol.* 31:164-170.
- Renwrantz, L., Schancke, W., Harm, H., Erl, H., Liebsch, H. and Gercken, J. 1981. Discriminative ability and function of the immunobiological recognition system of the snail, Helix pomatia. *J. Comp. Physiol.* 141:477-488.
- Renwrantz, L. and Stahmer, A. 1983. Opsonizing properties of an isolated hemolymph agglutinin and demonstration of lectin-like recognition molecules at the surface of hemocytes from Mytilus edulis. *J. Comp. Physiol.* 149:535-546.
- Richards, C.S. 1975. Genetic factors in susceptibility of Biomphalaria glabrata for different strains of Schistosoma mansoni. *Parasitology* 70:231-241.
- Richards, C.S. 1984. Influence of snail age on genetic variation of Biomphalaria glabrata for infection with Schistosoma mansoni. *Malacologia.* 25:493-503.
- Sminia, T., Van Der Knapp, W.P.W. and Edelenbosch, P. 1979. The role of serum factors in phagocytosis of foreign particles by blood cells of the freshwater snail, Lymnaea stagnalis. *Dev. Comp. Immunol.* 3:37-44.

Stanislawski, E., Renwrantz, L., and Becker, W. 1976. Soluble blood group reactive substances in the hemolymph of Biomphalarai glabrata (Mollusca). J. Invert. Pathol. 28:301-308.

Stein, P.C., and Basch, P.F. 1979. Purification and binding properties of hemagglutinin from Biomphalaria glabrata. J. Invert. Pathol. 33:10-18.

Stibbs, H.H., Owczarzak, A., Bayne, C.J. and DeWan, P. 1979. Schistosome sporocyst-killing amoebae isolated from Biomphalaria glabrata. J. Invert. Pathol. 33:159-170.

Ulmer, M.J. Notes on the rearing of snails in the laboratory. 1970. In: Experiments and Techniques in Parasitology. (A.J. MacInnis and M. Voge, eds), pp. 143-144. Freeman, San Francisco.

van der Knaap, W.P.W., Doderer, A., Boerrigter-Barendsen, L.H. and Sminia, T. 1982. Some properties of an agglutinin in the haemolymph of the pond snail Lymnaea stagnalis. Biol. Bull. 162:404-412.

Vasta, G.R., Sullivan, J.T., Cheng, T.C., Marchalonis, J.J. and Warr, G.W. 1982. A cell membrane-associated lectin of the oyster hemocyte. J. Invert. Pathol. 40:367-377.

Wheeler, W.E., Lubby, A.L. and Scholl, M.L.L. 1950. The action of enzymes in hemagglutinating systems. II. Agglutinating properties of trypsin-modified red blood cells with anti-Rh sera. J. Immunol. 65:39-46.

Yoshino, T.P. and Bayne, C.J. 1983. Mimicry of snail host antigens by miracidia and primary sporocysts of Schistosoma mansoni. Parasite Immunol. 5:317-328.

## CHAPTER 3

AGGLUTININS IN BIOMPHALARIA GLABRATA: SPECIFICITY, AND  
ACTIVITY AGAINST ENZYME-TREATED ERYTHROCYTES

The Biomphalaria glabrata agglutinin described in Chapter 2 has been further characterized. Both its specificity and its relationship to other possible plasma hemagglutinins have been examined. An attempt was also made to quantify the degree of purification at each step of its isolation protocol by Specific Activity analysis.

The isolated agglutinin and whole snail plasma both failed to agglutinate human erythrocytes (HRBC) of any of the ABO blood types. To determine if this lack of activity was due to the absence of relevant carbohydrate structures on the erythrocytes, these cells were exposed to pronase, a general protease, or to neuraminidase, a specific carbohydrase. Both treatments can cause the exposure of carbohydrate structures not normally available for agglutinin binding.

To determine if there was more than one hemagglutinin in the plasma of M-line B. glabrata, plasma from which agglutinating activity to one erythrocyte type had been removed was assayed for residual activity to a different type.

## MATERIALS AND METHODS

Pronase Treatment. Freshly drawn human A, B, and O, and rabbit erythrocytes were washed as described in Chapter 1, then pelleted at 400 xg for 6 min. To each volume of packed cells, 2 volumes of 0.1% pronase (Sigma Chemical Co. type XIV in TBS; 0.05M Tris, 0.85% NaCl,

10mM CaCl<sub>2</sub>, 10mM MgCl<sub>2</sub>, 0.02% Na azide, pH 7.3) was added. The packed cells were resuspended in this solution and incubated for 30 min at 37°C with occasional agitation. Cells were then washed 3x with TBS before use in agglutination assays.

Neuraminidase Treatment. Ten units of Clostridium perfringens neuraminidase (Sigma type V) in 0.1 ml of TBS were added to 1 ml of a 10% suspension of cells. The mixture was incubated for 30 min at 37°C, washed 3x with TBS, then used in agglutination assays. Untreated cells were included in parallel assays as controls.

Cross-adsorption Experiments. B. glabrata plasma, obtained as stated (Bayne et al, 1980), was diluted 1:1 with TBS to prevent osmotic lysis of the mammalian erythrocytes, and mixed with an equal volume of packed cells. These were incubated at 4°C for 1 hr with periodic mixing, then the plasma was removed, after centrifugation, and tested for agglutinating activity against different cell types. Controls included the same cell type that was used for the adsorption.

Specific Activity. The degree of purification achieved by each step in the isolation of the agglutinin (Chapter 1) was quantified by calculating the ratio:

$$\frac{\text{Titer}}{\text{Protein (mg ml}^{-1}\text{)}} = \text{Specific Activity (SA)}.$$

Protein determinations were according to Hartree's modification of Lowry (Hartree, 1972), and were made, in parallel with titer determinations, for whole snail plasma, the DEAE-Sephadex flow-through fraction of that plasma, and the dialyzed, lyophilized and

reconstituted precipitate from the 10% ammonium sulfate cut of the ion-exchange fraction.

## RESULTS

Agglutination titers of snail plasma against enzyme treated human or rabbit erythrocytes are given in Table 3.I.

When isolated agglutinin was assayed for activity against pronase-treated HRBC, the titers against types A, B, and O were 128, 256, and >1024, respectively.

When snail plasma was adsorbed with RaRBC all activity to this cell type was lost. However, activity against pronase-treated HRBC was retained, though at a lower level than when unadsorbed plasma was used. Thus the titers of RaRBC-adsorbed plasma against types A, B, and O HRBC were 32, 4, and 16, respectively.

The results from three separate experiments to obtain the specific activity (SA) for each isolation step are given in Table 3.II.

## DISCUSSION

The alteration in HRBC susceptibility to agglutination after enzyme treatment allows two conclusions. First, the loss of surface-associated proteins from HRBC by proteolytic digestion exposed glycosubstances that were reactive with the isolated agglutinin, and possibly other plasma components. The newly exposed glycosubstances probably include glycolipids, since the majority of blood group constituents associated with erythrocyte membranes are glycoconjugates

of the phospholipid ceramide (Wells, 1978).

Neuraminidase treatment also resulted in increased type A cell susceptibility to agglutination with snail plasma, suggesting that exposure of otherwise hidden agglutinin-reactive ligands had resulted from the action of this enzyme. This result is expected, since glycoproteins that possess terminal sialic acid residues generally have galactose or Gal-NAc as the penultimate sugar (Beyer et al, 1979), and the specificity of the isolated agglutinin was found to be in this family of sugars (Chapter 2).

The second conclusion is that retention of agglutinating activity against pronase-treated HRBC after successful adsorption with RaRBC suggests that snail plasma contains at least one other agglutinin whose specificity or affinity differs from the RaRBC agglutinin. This is of special interest since evidence for more than one agglutinin in the plasma of M-line B. glabrata has never been reported. In the light of earlier work reporting more than one agglutinin in the body fluids of numerous groups of invertebrates, including gastropods (Yeaton, 1981), it is not surprising that evidence for multiple lectins in B. glabrata is now available.

Though attempts to show a correlation between the possession of one agglutinin and resistance or susceptibility to Schistosoma mansoni were not successful (Chapter 1), it is still possible that such a relationship exists with an as yet unidentified agglutinin, such as that to fixed sporocysts of S. mansoni (Loker et al, 1984).

Clearly the isolation process yields an increasingly more pure

agglutinin preparation. However the large variability within isolaton steps suggests that either technical problems contribute to occasional failure of protocol steps, or biological variability in separate plasma batches is extreme, possibly being manifested as differential inactivation of the hemagglutinin during the molecule's purification.

It should be noted that small sample size is the reason that the work presented here is not included in the preceding Chapter. The enzyme treatment of erythrocytes was done twice, and the cross-adsorption experiment was performed once, hence the results must be interpreted cautiously.

Table 3.I. Agglutination titers of M-line plasma after enzyme treatment of target erythrocytes.

Cell Type	Treatment		
	None	Pronase	Neuraminidase
Rabbit	128	512	N.D. <sup>a</sup>
Human A	0	256	64
" B	0	128	N.D.
" O	0	128	N.D.

<sup>a</sup> Not done

Table 3.II. Specific Activity of Agglutinin Solution from  
M-line strain of Biomphalaria glabrata

Exp. #	Whole Snail Plasma	DEAE-Sephadex Fraction	Ammonium Sulfate Precipitate
1	18(3.49) <sup>a</sup>	256(0.031)	408(0.007)
2	44(11.6)	3325(0.771)	35310(0.145)
3	12(10.76)	41(0.194)	62(0.162)

<sup>a</sup> Values in parenthesis are actual protein concentrations  
in mg ml<sup>-1</sup>

## REFERENCES

- Bayne, C.J., Buckley, P.M., DeWan, P.C. 1980b. Macrophagelike hemocytes of resistant Biomphalaria glabrata are cytotoxic for sporocysts of Schistosoma mansoni in vitro. J. Parasitol. 66:413-419.
- Beyer, T.A., Rearick, J.I., Paulson, J.C., Prieels, J.-P., Sadler, J.E. and Hill, R.L. 1979. Biosynthesis of mammalian glycoproteins. Glycosylation pathways in the synthesis of the nonreducing terminal sequences. J. Biol. Chem. 254:12531-12541.
- Hartree, E.F. 1972. Determination of protein: A modification of the Lowry method that gives a linear photometric response. Anal. Biochem. 48:422-427.
- Loker, E.S., Yui, M.A. and Bayne, C.J. 1984. Schistosoma mansoni: Agglutination of sporocysts, and formation of gels on miracidia transforming in plasma of Biomphalaria glabrata. Exp. Parasit. 58:56-62.
- Wells, J.V. 1978. Hematologic diseases. In: Basic and Clinical Immunology (H.H. Fudenberg, D.P. Stites, J.L. Caldwell and J.V. Wells, eds.), pp. 452-500. Lange Medical Publications, Los Altos, Calif.
- Yeaton, R.W. 1981. Invertebrate lectins II. Diversity of specificity, biological synthesis and function in recognition. Dev. Comp. Immunol. 5:535-545.

## CHAPTER 4

SCHISTOSOMA MANSONI: LECTIN-DEPENDENT CYTOTOXICITY BY HEMOCYTES  
FROM SUSCEPTIBLE HOST SNAILS BIOMPHALARIA GLABRATA<sup>1</sup>

Carl A. Boswell and Christopher J. Bayne

## ABSTRACT

Normally benign hemocytes from a strain (M-line) of the snail, Biomphalaria glabrata, susceptible to Schistosoma mansoni, became cytotoxic toward the sporocyst stage if the parasite was first treated with the lectin concanavalin A. Concanavalin A binding was inhibitable with -methyl mannoside and killing was dose-dependent. Maximal levels of concanavalin A-induced cytotoxicity were comparable with levels observed when hemocytes from a resistant snail strain (13+16-R1) encountered untreated sporocysts. Induction of the cytotoxic response did not occur if hemocytes alone were pretreated with the lectin. A method incorporating ultraviolet microscopy and the vital fluorescent dye eosin Y was used for discriminating between live and dead sporocysts. This model may prove useful in understanding mechanisms used by invertebrate effector cells in recognition and killing of invading organisms.

<sup>1</sup>In press, Exp. Parasitol., 1985.

## INTRODUCTION

When invertebrate hemocytes react to foreign materials, smaller particles are typically phagocytosed, whereas larger ones are encapsulated (Lackie, 1980; Ratcliffe and Rowley, 1981; Bayne, 1983). It appears that the fate of phagocytosed particles is nearly universal throughout the Animal Kingdom; after formation of a phagosome, this fuses with lysosomes and degradation of the phagolysosome contents occurs. In the case of particles that cannot be degraded, such as colloidal carbon or latex spheres, hemocytes containing these objects may migrate to a site within the animal and become inactive or simply migrate out of the body altogether.

When hemocytes encounter a relatively large object, such as a metazoan parasite, an encapsulation reaction usually occurs in which layer upon layer of hemocytes accumulate around it. Within a susceptible host, if encapsulation occurs at all, hemocytes will not respond further and may disperse after a few days. In a resistant host, the parasite will be killed, often within 24 hr. This sequence of events has been carefully documented in the snail-trematode system used in the present study (Bayne et al, 1980a, b; Lie et al, 1980; Loker and Bayne, 1982).

We are largely ignorant of mechanisms of non-self recognition in invertebrates. Recent studies with molluscs (Renwantz and Stahmer, 1983; van der Knaap et al, 1983; Bayne, 1983) lend support to the notion that lectins may be opsonic, serving as analogs to vertebrate immunoglobulin. It also appears that a complement-like factor found

in the land snail, Helix pomatia, may play an opsonic role (Koch and Nielsen, 1984).

The mechanism(s) of metazoan parasite killing in invertebrate hosts is also poorly understood. In insects, melanization of the encapsulated invader has been implicated as the lethal step in a resistant reaction (Soderhall, 1982). In molluscs, no such single process has been identified. Although changes in the levels of lysozyme activity in granulocytes of clams (Mercenaria mercenaria) and hemolymph of snails (Biomphalaria glabrata) after bacteria-challenge have been reported (Cheng et al, 1975; Cheng et al, 1977), there is no conclusive evidence that lysosomal enzymes play a role in parasite killing. As part of an ongoing project to ascertain the basis for susceptibility and resistance in strains of the schistosomiasis vector, Biomphalaria glabrata, we have used Con A in an in vitro cytotoxicity assay. It has been shown that Con A binds to both snail hemocytes (Yoshino, 1981) and the sporocyst (Yoshino et al, 1977). We reasoned that a molecular bridging between the parasite and normally benign hemocytes, i.e., cells from susceptible hosts, may trigger a cytotoxic response by those hemocytes. In this communication, we show that benign hemocytes kill Con A-treated sporocysts and that this response is dose dependent, with maximum values resembling those recorded for hemocytes from resistant snails.

## MATERIALS AND METHODS

Reagents. All chemicals not otherwise specified were purchased from Sigma Chemical Company, St. Louis, MO, USA. Con A stock ( $500 \text{ ug ml}^{-1}$  in Chernin's balanced salt solution; Chernin 1963) and eosin Y (Cert. # 35, Matheson Coleman and Bell, Norwood, OH, USA) ( $1 \text{ mg ml}^{-1}$  in distilled water) were stored at 4 C. D-galactose and  $\alpha$ -methyl mannoside stocks (100 mM in Chernin's balanced salt solution) and fluorescein isothiocyanate stock ( $1 \text{ mg ml}^{-1}$  in 0.15 M phosphate buffer, pH 7.3) were stored at -15 C until needed. All reagents were diluted with culture medium without fetal calf serum or antibiotics (Medium F base; Stibbs et al, 1979) for use in assays. All glassware used for culturing was siliconized with Sigmacote.

Hemolymph Collection. Snails were maintained in dechlorinated tap water at 26 C and fed lettuce and chalk ad libitum. Individuals susceptible to the PR-1 strain of Schistosoma mansoni were the M-line strain, and resistant ones were the 13-16-R1 strain (Richards, 1975; 1984). To reduce the risk of microbial contamination, snails were soaked in 2% penicillin, streptomycin, and Fungizone (M.A. Bioproducts) in artificial spring water (Ulmer, 1970) for at least 20 min, then dried with cotton swabs and surface-sterilized with 70% ethanol, before being bled. Ten to fifteen millimeter diameter snails were bled by cardiac puncture (Bayne et al, 1980a), and the hemolymph pooled and kept at 5 C until used, never more than 1/2 hr. For experiments utilizing snail plasma,

hemolymph was collected on ice, and the cells removed by centrifugation at 400 xg for 10 min.

In Vitro Assay. Axenically transformed sporocysts (Stibbs et al, 1979) (70-80 in each 6x50mm Sigmacoted test tube) were washed 3x with Medium F base, then soaked in 100 ul of the lectin solution ( $0.2-50 \text{ ug ml}^{-1}$ ) for 1/2 hr. They were then washed 3x with the base and soaked in 100 ul of  $5 \text{ ug ml}^{-1}$  fluorescein isothiocyanate for 20 min. These were washed 2x with Medium F base, 1x with the base supplemented with 10% fetal calf serum and  $100 \text{ ug ml}^{-1}$  gentamycin (M.A. Bioproducts) (complete Medium F), then transferred to similar test tubes in which 150 ul of 1% agarose dissolved in Chernin's balanced salt solution had been previously solidified in the bottom. The supernatant was removed, 200-300 ul of hemolymph or complete Medium F was added and the hemocytes were allowed to settle out for 1 hr. The tubes were then spun at  $17 \times g$  for 12 min. Fluid volume was reduced to 45 ul per tube, and 5 ul of eosin Y ( $50 \text{ ug ml}^{-1}$ ) was added. Sporocysts, along with adhering cells, were immediately transferred to microslides (0.3 mm pathlength, Vitro Dynamics) and placed in a humid chamber at 26 C.

In experiments where hemocytes were treated with Con A, sporocysts were overlayed with a Percoll (Pharmacia Fine Chemicals) cushion ( $1.015 \text{ g ml}^{-1}$  in Medium F base) after transfer to the agarose surface, and 100 ul of medium with or without Con A was layered on top. Snail hemolymph was added carefully so as to mix it with the test reagent while not disturbing the Percoll layer. After 15 min at room temperature, the tubes were centrifuged at 75 xg for 12

min to move the cells through the Percoll. The "microencapsulations" thus formed were washed once with Medium F base then 100 ul of homologous snail plasma was added and the processing for microscopic analysis completed as described above.

Quantification. The viability of sporocysts within these "encounters" was scored within 2 hr of initial hemocyte-sporocyst contact and again after 24 hr. Scoring was done using a Zeiss epifluorescence UV microscope with interchangeable filter sets, one for fluorescein isothiocyanate and one for rhodamine isothiocyanate. Identification, counting, and assessment of sporocyst viability, even when completely encapsulated in a mass of hemocytes, was facilitated by a double fluorochrome stain technique. All sporocysts retain fluorescein isothiocyanate, while only dead sporocysts take up eosin Y. Therefore sporocysts were located by illumination with UV light and use of the fluorescein isothiocyanate filter set. When a sporocyst was located in this way, the filter was switched to the RITC filter set (which causes eosin Y to fluoresce) and all sporocysts that fluoresced red were scored as dead. The percent viability at 2 ( $\%d_2$ ) and 24 ( $\%d_{24}$ ) hr after contact was determined by the formula:

$$\frac{\text{number dead (red)}}{\text{total number (green)}} \times 100$$

A second value, percent killed during the assay, was calculated by the formula:

$$\frac{\%d_{24} - \%d_2}{100 - \%d_2} \times 100$$

This value takes into account the proportion of sporocysts that were not alive at the outset, and provides an estimate of the loss in viability during the experiment. All values reported have been calculated according to this procedure.

Inhibition Experiment. Inhibition of lectin effects was studied by exposing sporocysts to  $10 \text{ ug ml}^{-1}$  Con A in the presence of 20 mM of the inhibiting sugar  $\alpha$ -methyl mannoside, or the non-inhibitor D-galactose.

Controls Experiments. Controls included untreated sporocysts incubated in complete Medium F ("general sporocyst vigor"), sporocysts treated with Con A at relevant concentrations but not exposed to any hemocytes ("lectin control"), untreated sporocysts combined with benign hemocytes ("negative control"), and untreated sporocysts combined with resistant hemocytes ("positive control").

In addition, to determine whether Con A treatment in some way weakened or debilitated sporocysts, we scored survivorship after Con A- and untreated parasites were subjected to osmotic stress (Medium F base:dH<sub>2</sub>O, 1:3) or heat stress (45 C) for 24 hr.

## RESULTS

When sporocysts were soaked in Con A (Con A-sporocysts), they

were killed by benign hemocytes at a level approaching that caused by resistant hemocytes encountering untreated sporocysts, i.e., "positive control" (Fig. 4.I). In comparison, untreated sporocysts which encountered benign cells ("negative control") died at a rate only slightly above that of sporocysts alone ("sporocyst vigor") (Fig. 4.I). Comparisons of controls in the cell-mediated cytotoxicity assay ("lectin control" versus "sporocyst vigor") revealed no effect of the lectin on sporocyst vigor. While preliminary, the physiological stress experiments also failed to discern any difference in the vigor of lectin-treated and untreated sporocysts.

Figure 4.II illustrates the dose-dependency of the lectin-induced response.

Con A appeared to exert its effect in a sugar specific manner, since killing is reduced significantly ( $p < 0.05$ ) when  $\alpha$ -methyl mannoside was included in the lectin solution used for initial sporocyst treatment, but was not reduced nearly as much when D-galactose was used (Table 4.I).

Experiments in which hemocytes but not sporocysts were treated with the lectin (Con A-hemocytes) resulted in low levels of parasite killing (Table 4.II). However, if parasites were also treated with Con A (Con A-sporocysts), killing again reached levels comparable to those obtained with untreated resistant cells and untreated sporocysts ("positive control").

#### DISCUSSION

This report describes the phenomenon of lectin-induced

cytotoxicity in an invertebrate model. Our results imply that benign hemocytes have the same cytotoxic potential as resistant ones. This is consistent with the observation that snails susceptible to one strain of parasite are often resistant to another (Richards, 1975; 1984). That the lectin must be on the target for induction of the killing response is supported by the observation that Con A-hemocytes of susceptible Biomphalaria glabrata did not kill appreciable numbers of untreated Schistosoma mansoni sporocysts, only Con A-sporocysts. However, Con A can form precipitates with B. glabrata plasma components (Stanislawski et al, 1976), thus the plasma could be competitively inhibiting Con A binding to hemocytes. It is not known how long the precipitation reaction takes to develop, but the brief (15 min) incubation period probably allows a substantial proportion of the Con A to freely interact with hemocyte receptors.

Alternatively, the lack of cytotoxic responsiveness by Con A-hemocytes could be due to the clearance of Con A and its receptors from the hemocyte surface (Yoshino, 1982; Yoshino and Davis, 1983) and their consequent unavailability for cross-linking to the parasite. The observation that Con A-sporocysts are still killed by such cells may be the result of either re-expression of Con A receptors on hemocytes shortly after their clearance, or the induction of hemocyte cytotoxicity by some alteration of the parasite's surface not requiring molecular bridging by the lectin. For instance, it has been well established that S. mansoni sporocysts display snail antigens on their surface (Bayne & Stephens, 1983; Yoshino & Bayne, 1983), and therefore the Con A treatment may be covering what might be protective mimicked antigens. Or, the binding of Con A may cause expression of

parasite antigens that the hemocytes recognize as foreign by receptors that are independent from Con A binding sites. Evidence for surface alteration, after Con A exposure, of both adults and the earlier schistosomula stage has been reported (Simpson and McLaren, 1982; van Pijkeren et al, 1983). Finally, it should be noted that allogeneically stimulated mouse cytotoxic T-cells demonstrate the same requirement, i.e. that Con A be on the target for a killing response to be elicited (Rubens and Henney, 1977; Green et al, 1978).

The results of the sugar inhibition experiments suggest that Con A is binding in a sugar-specific manner to the parasite. Work in this lab has shown that using another lectin, wheat germ agglutinin, binding to sporocysts cannot be prevented by addition of the specific "inhibiting" sugar, N-acetyl glucosamine (J.A. Stephens, pers. comm.), suggesting that sporocysts have the potential to bind this lectin nonspecifically.

In another resistant strain (10-R2) of B. glabrata, a plasma factor occurs (Bayne et al, 1980b; Loker and Bayne, 1982) that leads to increased parasite killing by M-line hemocytes. Our finding that the tetravalent lectin, Con A, can induce cytotoxicity in a normally benign hemocyte-target encounter supports the notion that susceptible host snails lack a plasma factor which occurs in resistant hosts that may bridge the host hemocytes and the parasite. Such bridges, analogous to antibody-dependent opsonization, may trigger the cytotoxic response by the hemocyte. Further support for this interpretation requires that the bridging event be confirmed. Also, the possibility must be evaluated that lectin, by being immobilized on

the surface of a particle, activates hemocytes, as reported for cytotoxic T-cells (Ballas et al, 1981).

Up to this time, much of the work on mechanisms and molecules involved in molluscan responses to foreign material has been based on experiments using biologically irrelevant targets (vertebrate erythrocytes, yeast, pollen, or irrelevant microbes). Our findings for Schistosoma mansoni suggest a novel approach to understanding recognition and cytotoxicity responses in Biomphalaria glabrata. Mechanisms elucidated in this gastropod-parasite system may prove to be common to invertebrates in general and may provide useful insights by which direct comparisons can be made with vertebrate recognition and cytotoxic mechanisms.

#### ACKNOWLEDGEMENTS

We gratefully acknowledge the technical assistance of Mary Yui in developing the fluorescence viability assay. We also thank Mary, Craig Flory, and Dr. Robert Hard for critical comments on this manuscript. This work was supported by the U.S. National Institutes of Health through Grant AI-16137 to C.J. Bayne.

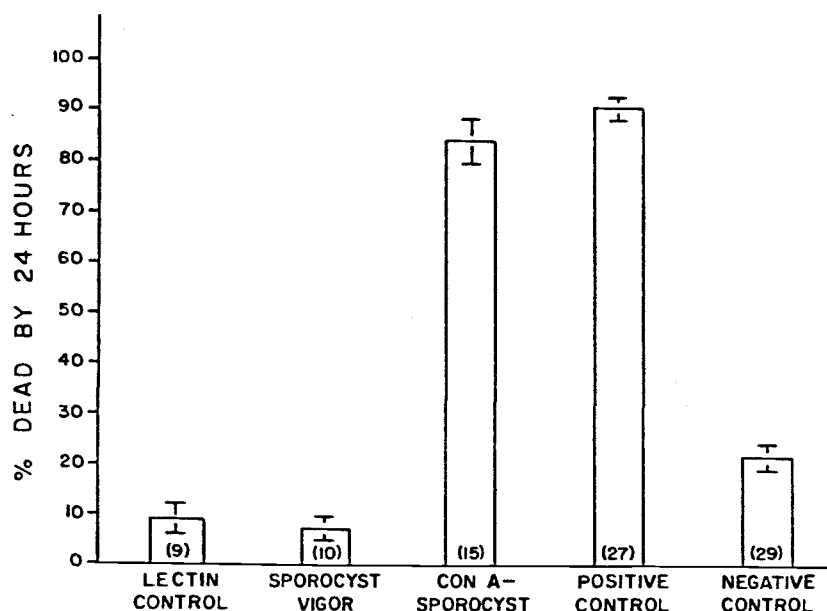


Fig. 4.I. Schistosoma mansoni sporocyst death following specified treatments in vitro. "Lectin Control" represents sporocysts treated with  $10 \text{ ug ml}^{-1}$  Con A then incubated in cell-free Biomphalaria glabrata plasma. "Sporocyst Vigor" represents sporocysts not treated with Con A and incubated in culture medium. "Con A-Sporocysts" represents the level of sporocyst death associated with pretreatment with  $10 \text{ ug ml}^{-1}$  Con A then exposure to benign (M-line) snail hemocytes. "Positive Control" is untreated sporocysts exposed to resistant (13-16-R1) hemocytes. "Negative Control" is sporocyst death observed when untreated sporocysts are incubated with benign hemocytes. The vertical bars are + one standard error of the mean. Numbers in parentheses are the number of experiments from which the means were calculated.

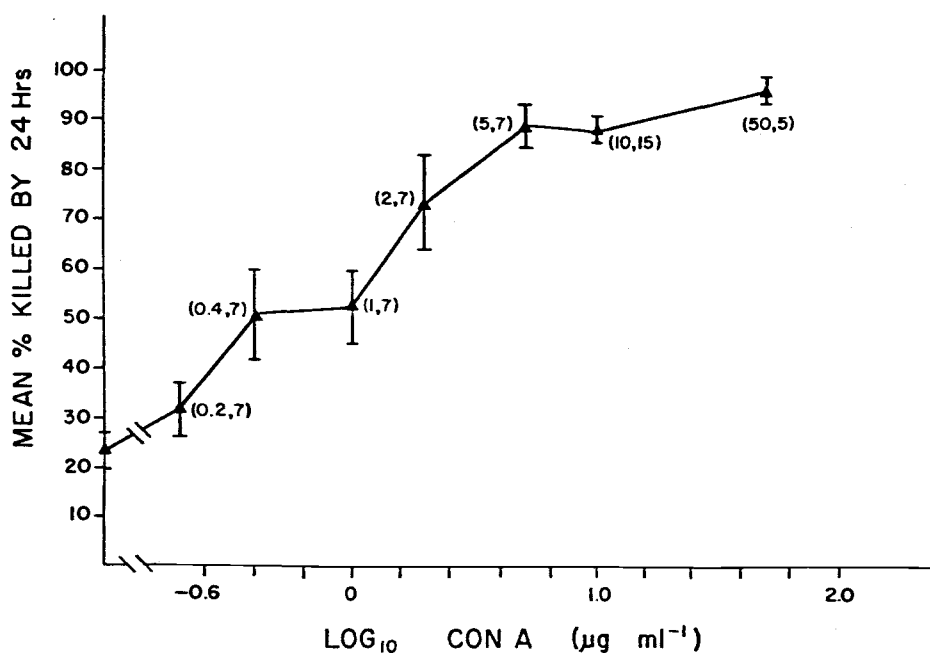


Fig. 4.II. Dose-dependent killing response by benign hemocytes from Biomphalaria glabrata of Schistosoma mansoni sporocysts pretreated with specified levels of Con A. Vertical bars are + one standard error of the mean. Numbers in parentheses are the dose of Con A, in  $\mu\text{g ml}^{-1}$ , followed by the number of trials at that dose.

Table 4.I. Cytotoxicity by Biomphalaria glabrata M-line Hemocytes  
After Co-incubation with Schistosoma mansoni PR-1  
Sporocysts pretreated with Con A<sup>a</sup> and Specified Sugar

Sugar (20 mM)	No. of replicates	Percent sporocysts killed ( $\bar{x} \pm SE$ )
$\alpha$ -methyl mannoside	3	21.5 $\pm$ 13.1 <sup>b</sup>
D-galactose	3	64.6 $\pm$ 18.1
No sugar	3	74.6 $\pm$ 7.3

<sup>a</sup> Sporocysts treated with 10 ug ml<sup>-1</sup> Con A.  
<sup>b</sup> p < 0.05, F=4.316, df=2,9.

Table 4.II. Killing of Schistosoma mansoni sporocysts after treatment of Biomphalaria glabrata M-line hemocytes with Con A<sup>a</sup>

Treatment	No. of replicates	Percent sporocysts killed ( $\bar{x} \pm \text{S.E.}$ )
Con A-hemocytes	3	10.8 $\pm$ 2.7 <sup>c</sup>
No Con A	3	4.6 $\pm$ 1.7 <sup>c</sup>
Con A-hemocytes + Con A-sporocysts <sup>b</sup>	4	69.8 $\pm$ 20.9

<sup>a</sup> Hemocytes were exposed to 50 ug ml<sup>-1</sup> Con A for 15 min.

<sup>b</sup> Sporocysts were exposed to 10 ug ml<sup>-1</sup> Con A for 1 hr.

<sup>c</sup>  $p < 0.01$ ,  $F=10.707$ ,  $df=2,7$ .

## REFERENCES

- Ballas, Z.K., Green, W.R. and Henney, C.S. 1981. Studies on the mechanism of T-cell-mediated lysis. XIII. Lectin-Dependent T-cell-mediated cytotoxicity is supported by Con A coupled to Sepharose beads. *Cell. Immunol.* 59:411-418.
- Bayne, C.J. *Molluscan Immunobiology*. 1983. In: *The Mollusca* (A.S.M. Saleuddin, and K.M. Wilbur, eds.), vol. 5, pt. 2, pp. 407-486. Academic Press, New York.
- Bayne, C.J., Buckley, P.M. and DeWan, P.C. 1980a. Schistosoma mansoni: Cytotoxicity of hemocytes from susceptible snail hosts for sporocysts in plasma from resistant Biomphalaria glabrata. *Exp. Parasitol.* 50:409-416.
- Bayne, C.J., Buckley, P.M. and DeWan, P.C. 1980b. Macrophagelike hemocytes of resistant Biomphalaria glabrata are cytotoxic for sporocysts of Schistosoma mansoni in vitro. *J. Parasitol.* 66:413-419.
- Bayne, C.J. and Stephens, J.A. 1983. Schistosoma mansoni and Biomphalaria glabrata share epitopes: Antibodies to sporocysts bind host snail hemocytes. *J. Invert. Pathol.* 42:221-223.
- Cheng, T.C., Chorney, M.J. and Yoshino, T.P. 1977. Lysozyme-like activity in the hemolymph of Biomphalaria glabrata challenged with bacteria. *J. Invert. Pathol.* 29:170-174.
- Cheng, T.C., Rodrick, G.E., Foley, D.A. and Koehler, S.A. 1975. Release of lysozyme from hemolymph cells of Mercenaria mercenaria during phagocytosis. *J. Invert. Pathol.* 25:261-265.
- Chernin, E. 1963. Observations on hearts explanted in vitro from the snail Australorbis glabratus. *J. Parasitol.* 49:353-364.
- Green, W.R., Ballas, Z.K. and Henney, C.S. 1978. Studies on the mechanism of lymphocyte-mediated cytotoxicity. *J. Immunol.* 121:1566-1572.
- Koch, C. and Nielsen, H.E. 1984. Activation of vertebrate complement by Helix pomatia haemolymph. *Dev. Comp. Immunol.* 8:15-22.
- Lackie, A.M. 1980. Invertebrate immunity. *Parasitology.* 80:393-412.
- Lie, K.J., Jeong, K.H. and Heyneman, D. 1980. Tissue reactions induced by Schistosoma mansoni in Biomphalaria glabrata. *Ann. Trop. Med. Parasitol.* 74:157-166.

- Loker, E.S. and Bayne, C.J. 1982. In vitro encounters between Schistosoma mansoni primary sporocysts and hemolymph components of susceptible and resistant strains of Biomphalaria glabrata. Am. J. Trop. Med. Hyg. 31:999-1005.
- Ratcliffe, N.A. and Rowley, A.F. (eds.) 1981. "Invertebrate Blood Cells", vol. I & II. Academic Press, New York. 641 pp.
- Renwrautz, L. and Stahmer, A. 1983. Opsonizing properties of an isolated hemolymph agglutinin and demonstration of lectin-like recognition molecules at the surface of hemocytes from Mytilus edulis. J. Comp. Physiol. 149:535-546.
- Richards, C.S. 1975. Genetic factors in susceptibility of Biomphalaria glabrata for different strains of Schistosoma mansoni. Parasitology. 70:231-241.
- Richards, C.S. 1984. Influence of snail age on genetic variation of Biomphalaria glabrata for infection with Schistosoma mansoni. Malacologia. 25:493-503.
- Rubens, R.P. and Henney, C.S. 1977. Studies on the mechanism of lymphocyte-mediated cytotoxicity. VIII. The use of Con A to delineate a distinctive killer T cell subpopulation. J. Immunol. 118:180-186.
- Simpson, A.J.G. and McLaren, D.J. 1982. Schistosoma mansoni: Tegumental damage as a consequence of lectin binding. Exp. Parasitol. 53:105-116.
- Soderhall, K. 1982. Prophenoloxidase activating system and melanization - a recognition mechanism of arthropods? A review. Dev. Comp. Immunol. 6:601-611.
- Stanislawski, E., Renwrautz, L. and Becker, W. 1976. Soluble blood group reactive substances in the hemolymph of Biomphalaria glabrata (Mollusca). J. Invert. Pathol. 28:301-308.
- Stibbs, H.H., Owczarzak, A., Bayne, C.J. and DeWan, P.C. 1979. Schistosome sporocyst-killing amoebae isolated from Biomphalaria glabrata. J. Invert. Pathol. 33:159-170.
- Ulmer, M.J. Notes on the rearing of snails in the laboratory. 1970. In: Experiments and Techniques in Parasitology. pp. 143-144. (A.J. MacInnis, and M. Voge, eds.). Freeman, San Francisco.
- van der Knaap, W.P.W., Boots, A.M.H. and Sminia, T. 1983. Immunorecognition in Lymnaea stagnalis. Dev. Comp. Immunol. 7:645-648.
- van Pijkeren, T.A., Tavares, C.A.P. and Gazzinelli, G. 1982. Schistosoma mansoni: Ability of Con A to protect in vitro mechanically transformed schistosomula against the lethal effect of

immune serum plus complement. *Parasitology*. 84:239-252.

Yoshino, T.P. 1981. Concanavalin A-induced receptor redistribution on Biomphalaria glabrata hemocytes: Characterization of capping and patching responses. *J. Invert. Pathol.* 38:102-112.

Yoshino, T.P. 1982. Lectin-induced modulation of snail hemocyte surface determinants: Clearance of Con A-receptor complexes. *Dev. Comp. Immunol.* 6:451-461.

Yoshino, T.P. and Bayne, C.J. 1983. Mimicry of snail host antigens by miracidia and primary sporocysts of Schistosoma mansoni. *Parasite Immunol.* 5:317-328.

Yoshino, T.P., Cheng, T.C. and Renwranztz, L. 1977. Lectin and human blood group determinants of Schistosoma mansoni: Alteration following in vitro transformation of miracidium to mother sporocyst. *J. Parasitol.* 63:818-824.

Yoshino, T.P. and Davis, C.D. 1983. Surface antigens of Biomphalaria glabrata (Gastropoda) hemocytes: Evidence for linkage-independence of some hemolymph-like surface antigens and Con A receptor-bearing macromolecules. *J. Invert. Pathol.* 42:8-16.

## CHAPTER 5

## LECTIN-DEPENDENT CELL-MEDIATED CYTOTOXICITY IN AN INVERTEBRATE MODEL:

CON A DOES NOT ACT AS A BRIDGE.<sup>1</sup>

Carl A. Boswell and Christopher J. Bayne

## ABSTRACT

The plant lectin, concanavalin A (Con A), has been used in an invertebrate model of lectin-dependent cell-mediated cytotoxicity (LDCC). Macrophage-like cells from the susceptible host snail, Biomphalaria glabrata, become cytotoxic effectors when they encounter sporocysts of the parasitic trematode, Schistosoma mansoni, that have been treated with Con A. Using reagents to block putative specific bridging between the target and effector cells, evidence is presented against the molecular bridging hypothesis as the basis for LDCC. We suggest, instead, that surface modulation of the target may be the stimulus that provokes cytotoxicity. This would explain why Con A is effective only when the target has been exposed to it. This example from an invertebrate system is comparable to observations of work on vertebrate LDCC, in which specifically stimulated T-lymphocytes become non-specifically cytotoxic toward cells that have been treated with Con A.

<sup>1</sup>Submitted for publication, Immunology, 1985.

## INTRODUCTION

Wide use has been made of the plant lectin concanavalin A (Con A) to elucidate the mechanisms by which cytotoxic T-lymphocytes (CTL) respond to target cells. Con A is mitogenic for T-cells, and can induce quiescent T-cells to become cytotoxic (Berke et al, 1981a, b). In addition, allogeneically stimulated CTL react nonspecifically if targets are treated with Con A (Bonavida and Bradley, 1976; Gately and Martz, 1977). Hence CTL lyse syngeneic, Con A-treated targets nonspecifically, while only specific, allogeneic reactivity occurs in the absence of lectin treatment.

In the course of studies aimed at understanding the basis for resistance or susceptibility to the human parasite, Schistosoma mansoni, in the intermediate snail host, Biomphalaria glabrata, we have used Con A in an in vitro cytotoxicity assay developed for this host-parasite system. Previous work has shown that Con A binds to both snail hemocytes (Yoshino, 1981) and the schistosome surface (Yoshino et al, 1977). We have found that treatment of the sporocyst stage of the parasite with Con A induces a cytotoxic reaction by hemocytes (macrophage analogs) from susceptible snails that approximates the level of killing observed when cells from resistant snails encounter untreated sporocysts (Boswell and Bayne, 1985). It has been unclear whether the lectin's effect is due to its "bridging" of the hemocytes and their targets, or to some other perturbation in the system.

Evidence presented in this communication suggests a "bridging"

event by Con A between the target sporocyst and snail hemocytes is not the basis for induction of cytotoxicity. Instead, we propose that Con A binding provokes a modulation reaction by the parasite that leads to alterations in its surface character such that benign hemocytes encountering such sporocysts are induced to become cytotoxic.

## MATERIALS AND METHODS

Reagents. All chemicals were purchased from Sigma Chemical Company, unless otherwise stated. Concanavalin A ( $0.5 \text{ mg ml}^{-1}$  in snail saline, CBSS; Chernin, 1963), anti-Con A (13 mg rabbit antibody  $\text{ml}^{-1}$  in 0.01 M phosphate buffered saline, pH 7.2) and eosin Y ( $1 \text{ mg ml}^{-1}$  in distilled water) were stored at 4 C. Fluorescein isothiocyanate (FITC,  $1 \text{ mg ml}^{-1}$  in 0.15 M phosphate buffer, pH 7.3) stock was stored at -15 C. All reagent dilutions and in vitro washes were performed with the culture medium we have designated Medium F base (MFB), developed by Voge and Seidel (1972) and refined by Stibbs et al. (1979). Medium F supplemented with 10% fetal calf serum and  $100 \text{ ug ml}^{-1}$  gentamycin (M.A. Bioproducts) is termed complete Medium F (CMF). All glassware used for culturing was treated with the hydrophobic coating, Sigmacote.

Animals. Snails were maintained at 26 C in aerated tap water and fed lettuce and chalk ad libitum. The two strains of snails utilized in these experiments were the M-line (susceptible) and the 13-16-R1 (resistant) (Richards, 1975; pers comm.). PR-1 S. mansoni was maintained in outbred Swiss-Webster mice, and M-line snails.

Schistosome eggs were harvested from mouse livers 7-8 weeks post-infection (Stibbs et al, 1979). Miracidia hatched from liver-derived eggs were axenically transformed into sporocysts in CMF.

Hemolymph Collection. Snails were prepared for bleeding by first soaking them in 2% PSF (penicillin, streptomycin, fungizone; M.A. Bioproducts) in artificial spring water (Ulmer, 1970) for at least 20 min, then the shells were dried and surface sterilized with 70% ethanol. Snails were bled by cardiac puncture (Bayne et al, 1980b) and the hemolymph pooled and held at 5 C until used, never more than 30 min. When snail plasma was required, hemolymph was pooled, then centrifuged at 400 xg for 10 min at 4 C to remove the cells.

In Vitro Assay. Details of the in vitro assay have been given in Boswell and Bayne (1985). Briefly, sporocysts (70-80 per 6x50mm test tube) were incubated for 30 min in 10 ug ml<sup>-1</sup> Con A (Con A-sporocysts) or MFB, washed, then stained in FITC (100 ul, 5 ug ml<sup>-1</sup>) for 20 min. These were washed, then transferred to similar test tubes with a 150 ul plug of 1% agarose in CBSS in the bottom. The supernatant was removed and 200-300 ul of hemolymph or complete Medium F was added. Cells and sporocysts were allowed to settle at 1 xg for 50 min, then the preparations were spun at 17 xg for 10 min. Fluid volume was reduced to 45 ul, then 5 ul of eosin Y (50 ug ml<sup>-1</sup>) was added to each tube to serve as an indicator of viability. Sporocysts and adhering hemocytes were immediately transferred to Microslides (0.3 mm pathlength, Vitro Dynamics, Rockaway, NJ) and placed in a humid chamber at 26 C.

With a UV microscope and FITC optics, sporocysts were easily

discerned by their bright green fluorescence, even when completely enclosed within a mass of hemocytes. Once a sporocyst was identified the rhodamine isothiocyanate filter set was used to ascertain the uptake of eosin Y. Since eosin Y is a vital dye, only non-viable sporocysts fluoresced red, and hence were visible under these conditions.

The percent sporocysts dead at 2 (%d<sub>2</sub>) and 24 (%d<sub>24</sub>) hr post-contact was determined. From these percentages a revised value, the proportion killed during the assay, was calculated by the formula:

$$\frac{\%d_{24} - \%d_2}{100 - \%d_2} \times 100$$

This revised value takes into account the proportion of sporocysts that were not alive at the outset (less than 10%), and provides a more valid estimate of the loss in viability during the experiment. All values reported are these corrected proportions.

Inhibition Assays. Two experimental approaches were taken to determine if molecular bridging was the stimulus for lectin-induced cytotoxicity (Boswell and Bayne, 1985). In one approach, Con A-sporocysts were exposed to 13, 1.3, or 0.13 mg ml<sup>-1</sup> of rabbit anti-Con A for 1 hr, washed 3x with MFB, then exposed to M-line hemocytes as described above.

In the second approach to test the bridging hypothesis, sugars were included in the medium when initial contact between target and effector cells was made. Thus 20 mM final concentrations of the Con A-inhibiting sugar,  $\alpha$ -methyl mannoside, or the non-inhibiting sugar

D-galactose were added along with snail hemolymph to Con A-sporocysts. This level of  $\alpha$ -methyl mannoside has been shown to be inhibitory when used at the time of Con A treatment of sporocysts (Boswell and Bayne, 1985).

Rosetting Assay. Because the epitope specificity of the anti-Con A was unknown, it was necessary to determine if anti-Con A blocked the ligand binding site on Con A. To accomplish this a rosetting assay, modified from Yoshino et al (1977) was employed. Glutaraldehyde-fixed sporocysts were soaked in  $2 \text{ mg ml}^{-1}$  Con A, washed, then incubated with a 1% suspension of sheep erythrocytes (SRBC) for 1 hr (positive control). Another group of fixed sporocysts was soaked in  $2 \text{ mg ml}^{-1}$  Con A, washed, soaked in  $13 \text{ mg ml}^{-1}$  anti-Con A for 1 hr, and then mixed with SRBC. A third group of fixed sporocysts was left untreated before mixing with SRBC (negative control). These preparations were then washed gently to remove unattached erythrocytes and examined microscopically for sporocysts with attached sheep cells.

Lentil Lectin Assay. Lentil lectin (Lens culinaris lectin) was used in place of Con A in the in vitro assay at 100, 20, and  $4 \text{ ug ml}^{-1}$  to determine if a lectin of sugar binding specificity similar to that of Con A could elicit a similar response from susceptible hemocytes.

Controls. Controls to monitor the effect of in vitro cultivation, i.e. 24 hr in complete Medium F only, and lectin treatment (exposure to lectin, then culture in snail plasma or complete Medium F) on sporocyst vigor were included in all

experiments. Additional controls for antibody and sugar treatments, in which sporocysts which had never encountered lectin were treated with anti-Con A or sugar, were included where appropriate.

Statistics. An analysis of variance (ANOVA) was performed on all data sets.

## RESULTS

Lectin-induced cytotoxicity, as reported in Chapter 4, is confirmed in these experiments (Tables 5.I, 5.II).

Results from anti-Con A treatment of Con A-sporocysts (Table 5.I) fail to indicate a statistically significant increase in killing efficiency in the presence of increasing levels of anti-Con A ( $F=1.86$ ;  $df=3,18$ ;  $p>0.5$ ).

Upon microscopic inspection of the SRBC-rosetting preparations, Con A-treated sporocysts were observed with sheep cells covering their surfaces. Examination of fixed Con A-anti-Con A-treated sporocysts revealed that all rosetting was abrogated.

The results from experiments in which D-galactose or  $\alpha$ -methyl mannoside were included in the LDCC assay give no indication that the presence of an inhibiting sugar ( $\alpha$ -methyl mannoside) reduced cytotoxicity ( $F=0.324$ ;  $df=2,8$ ;  $p>0.5$ ) (Table 5.II).

There was no significant inducing effect when sporocysts were treated with lentil lectin before exposure to susceptible hemocytes, even at ten times the concentration normally used with Con A ( $F=0.52$ ;  $df=3, 16$ ;  $p>0.5$ ) (Table 5.III).

## DISCUSSION

An hypothesis that Con A acts as a molecular bridge between two particles would predict that if ligand binding sites of the Con A were blocked, binding would not occur. If cross-linking of target and effector cells by Con A was the cause for the intense cytotoxic reaction reported here (Table 5.I, no anti-Con A treatment, Table 5.II, no sugar treatment), then killing should have been completely blocked by anti-Con A (as sheep erythrocyte rosetting was blocked) and by the inhibiting sugar,  $\alpha$ -methyl mannoside. The results of these experiments provide evidence that a bridging phenomenon is not the cause for enhanced killing of Con A-sporocysts by benign hemocytes.

It is not possible from these data to determine if anti-Con A bound to sporocysts is itself capable of triggering a cytotoxic response by hemocytes. However we do not think such a possibility is likely, since treatment of sporocysts with anti-sporocyst IgG blocks cytoadherence by hemocytes (Bayne et al, 1984). In addition, non-blocking levels of anti-snail antiserum cross-reactive with sporocysts does not lead to increased killing by hemocytes (Chapt. 6, this thesis).

One explanation for the cytotoxic response following Con A treatment is that Con A bound to the surface of a particle (in this case a sporocyst) triggers a cytotoxic response in hemocytes, much like that reported for cytotoxic T-cells (Ballas et al, 1981). This would also presumably require sugar-specific binding between the

immobilized Con A and hemocyte surface components, hence is subject to the same criticisms as the bridging hypothesis. If hemocytes are being activated by a site on the Con A molecule which is not related to its sugar binding specificity, then Con A bound to sporocysts could activate hemocytes, and hemocytes would be unaffected by inhibiting sugars. One would expect the anti-Con A to interfere with such an interaction, yet the results from anti-Con A treatment do not support this idea.

An alternate hypothesis is that the presence of Con A on the surface of sporocysts causes them to rearrange, turnover, or otherwise modify their surface components, thereby exposing determinants that hemocytes recognize as foreign, which then induces a cytotoxic reaction. There is evidence from work on adult schistosomes that exposure to Con A causes blebbing and sloughing of surface membranes (Simpson and MacLaren, 1982). Similar treatment of schistosomulae resulted in no such tegumental alterations. However, protection of schistosomulae from complement destruction by pretreatment with Con A prompted van Pijkeran et al. (1982) to propose a mechanism in which tegument development of the young worm is affected after Con A treatment. These results suggest a capacity for Con A-induced alteration of the parasite's surface.

The results reported here are germane to other lectin-dependent cell-mediated cytotoxicity (LDCC) studies which have suggested that Con A may possess some peculiar properties. Berke and colleagues (1981a, b) present data that support the notion that Con A modifies the surface of target cells, that it does not bridge the effector and

target, and that MHC receptors are active participants in the recognition step of LDCC, i.e., no new mechanism of recognition is required to explain LDCC.

Green (1982), on the other hand, argues forcibly for a bridging phenomenon. The support for his hypothesis derives from LDCC work using lentil lectin, a lectin with sugar specificity similar to Con A. In this case, lentil lectin treatment of effector cells does result in non-specific target cell lysis, and the phenomenon is blocked when lectin treatment is followed by anti-lectin treatment. It is possible that two separate mechanisms are operating; a true bridging phenomenon when lentil LDCC is concerned, and surface modulation when Con A is used. Our results from experiments using lentil lectin in place of Con A suggest that, indeed, the two lectins influence LDCC differently in the snail-trematode model. At this point, we have no data regarding the effect, on LDCC, of lentil lectin treatment of hemocytes before they encounter sporocysts. The valence of each of these lectins may be the key to their effects. Con A is known to have four sugar binding sites, while lentil lectin has two (Lis and Sharon, 1981). Yahara and Edelman (1973) demonstrated differential mobility of Con A receptors on lymphocyte surfaces depending upon whether intact (tetravalent) or succinylated (divalent) Con A is used. Under specific conditions intact Con A causes patching and capping of lymphocyte lectin-receptor complexes whereas succinylated Con A does not. Other work has shown that snail hemocytes rapidly and non-randomly redistribute and clear (tetravalent) Con A-bound receptors from their surface (Yoshino, 1981; 1982).

That fundamentally similar mechanisms of recognition may occur with cytotoxic T lymphocytes and molluscan hemocytes is an intriguing and provocative concept that deserves further study.

Acknowledgements We wish to thank Jeff Longmate and Dr. Al Nicewander for their statistical counseling, and Drs. Eric S. Loker, Frank Seto, and Tim Yoshino for their critical comments. This work was supported by NIH grant A1-16137 to C.J.B.

Table 5.I. Cytotoxicity by M-line hemocytes after Con A<sup>a</sup>, then anti-Con A treatment of sporocysts

Anti-Con <sub>1</sub> A (mg ml <sup>-1</sup> )	No. of replicates <sup>b</sup>	Proportion dead by 24 hrs ( $\bar{x} \pm \text{S.E.}$ )	Rosette
<u>Experimentals</u>			
13	6	63.4 $\pm$ 13.6	-
1.3	6	78.1 $\pm$ 6.1	N.D. <sup>c</sup>
0.13	5	82.8 $\pm$ 6.8	N.D.
No anti-Con A	5	89.9 $\pm$ 4.6	+
<u>Controls</u>			
13 (no Con A exposure)	6	20.3 $\pm$ 5.4	N.D.
No Con A No anti-Con A	6	19.1 $\pm$ 5.4	-
13 (no hemocytes)	6	11.2 $\pm$ 2.9	+

<sup>a</sup> Sporocysts treated with 10 ug ml<sup>-1</sup> Con A

<sup>b</sup> In each replicate 40-70 sporocysts were scored as living or dead

<sup>c</sup> N.D. = not done

Table 5.II. Cytotoxicity when Con A-sporocysts<sup>a</sup> encounter M-line hemocytes plus specified sugar

Sugar (20 mM)	Number of experiments	Percent killed by 24 hr ( $\bar{x} \pm \text{S.E.}$ )
$\alpha$ -methyl mannoside	3	96.5 $\pm$ 3.5
D-galactose	4	96.3 $\pm$ 2.8
No sugar	4	92.4 $\pm$ 5.5

<sup>a</sup> Sporocysts treated with 10  $\mu\text{g ml}^{-1}$  Con A

Table 5.III. Cytotoxic Response by M-line Hemocytes After Sporocyst Treatment with Lentil Lectin

Lentil lectin ( $\mu\text{g ml}^{-1}$ )	No. of replicates	Percent sporocysts killed ( $\bar{x} \pm \text{S.E.}$ )
100	5	44.7 $\pm$ 12.4
20	5	31.9 $\pm$ 7.8
4	5	31.9 $\pm$ 13.0
0	5	27.0 $\pm$ 7.5

## REFERENCES

- Ballas, Z.K., Green, W.R. and Henney, C.S. 1981. Studies on the mechanism of T-cell-mediated lysis. XIII. Lectin dependent T-cell-mediated cytotoxicity is supported by Con A coupled to Sepharose beads. *Cell. Immunol.* 59:411-418.
- Bayne, C.J., Buckley, P.M. and DeWan, P.C. 1980b. Macrophagelike hemocytes of resistant Biomphalaria glabrata are cytotoxic for sporocysts of Schistosoma mansoni in vitro. *J. Parasitol.* 66:413-419.
- Bayne, C.J., Loker, E.S., Yui, M.A. and Stephens, J.A. 1984. Immune-recognition of Schistosoma mansoni primary sporocysts may require specific receptors on Biomphalaria glabrata hemocytes. *Parasite Immunol.* 6:519-528.
- Berke, G., Hu, V., McVey, E. and Clark, W.R. 1981a. T lymphocyte-mediated cytotoxicity. I. A common mechanism for target recognition in specific and lectin-dependent cytotoxicity. *J. Immunol.* 127:776-781.
- Berke, G., McVey, E., Hu, V. and Clark, W.R. 1981b. T lymphocyte-mediated cytotoxicity. II. Role of target cell histocompatibility antigens in recognition and lysis. *J. Immunol.* 127:782-787.
- Bonavida, B. and Bradley, T.P. 1976. Studies on the induction and expression of T-cell-mediated immunity. V: Lectin-induced nonspecific cell-mediated cytotoxicity by alloimmune lymphocytes. *Transplantation.* 21:94-102.
- Boswell, C.A. and Bayne, C.J. 1985. Schistosoma mansoni: Lectin-dependent cytotoxicity by hemocytes from susceptible host snails Biomphalaria glabrata. *Exp. Parasit.* in press.
- Chernin, E. 1963. Observations on hearts explanted in vitro from the snail Australorbis glabratus. *J. Parasitol.* 49:353-364.
- Gately, M.K. and Martz, E. 1977. Comparative studies on the mechanisms of nonspecific, Con A-dependent cytotoxicity and specific T cell-mediated cytotoxicity. *J. Immunol.* 119:1711-1722.
- Green, W.R. 1982. Studies on the mechanism of lectin-dependent T cell-mediated cytotoxicity: Use of Lens culinaris hemagglutinin A to define the role of lectin. In: *Mechanisms of Cell-Mediated Cytotoxicity*, pp81-100. (W.R. Clark and P. Goldstein, eds.) Plenum Press, New York.
- Lis, H. and Sharon, N. 1981. Lectins in higher plants. In: *The Biochemistry of Plants*, vol. 6, p. 393. (A. Marcus, ed.) Academic

Press, New York.

Richards, C.S. 1975. Genetic factors in susceptibility of Biomphalaria glabrata for different strains of Schistosoma mansoni. *Parasitology*. 70:231-241.

Simpson, A.J.G. and McLaren, D.J. 1979. Schistosoma mansoni : Tegumental damage as a consequence of lectin binding. *Exp. Parasitol.* 53:105-116.

Stibbs H.H., Owczarzak A., Bayne C.J. and DeWan P.C. 1979. Schistosome sporocyst-killing amoebae isolated from Biomphalaria glabrata . *J.Invert. Pathol.* 33:159-170.

Ulmer, M.J. 1970. In: *Experiments and Techniques in Parasitology*, pp. 143-144. (A.J. MacInnis and M. Voge, eds.) Freeman, San Francisco.

van Pijkeren, T.A., Tavares, C.A.P. and Gazzinelli, G. 1982. Schistosoma mansoni: Ability of Con A to protect in vitro mechanically transformed schistosomula against the lethal effect of immune serum plus complement. *Parasitology*. 84:239-252.

Voge, M. and Seidel, J.S. 1972. Transformation in vitro of miracidia of Schistosoma mansoni and Schistosoma japonicum into young sporocysts. *J. Parasitol.* 58:699-704.

Yahara, I. and Edelman, G.M. 1973. The effects of concanavalin A on the mobility of lymphocyte surface receptors. *Exp. Cell Res.* 81:143-155.

Yoshino T.P. 1981. Con A-induced receptor redistribution on Biomphalaria glabrata hemocytes: characterization of capping and patching responses. *J. Invert. Pathol.* 38:102-112.

Yoshino T.P. 1982. Lectin-induced modulation of snail hemocytes surface determinants: clearance of Con A-receptor complexes. *Dev. Comp. Immunol.* 6:451-461.

Yoshino T.P., Cheng T.C. and Renwranz L.R. 1977. Lectin and human blood group determinants of Schistosoma mansoni: alteration following in vitro transformation of miracidium to mother sporocyst. *J. Parasitol.* 63:818-824.

## CHAPTER 6

## ABSENCE OF ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY (ADCC)

IN THE SCHISTOSOMA MANSONI - BIOMPHALARIA GLABRATA MODEL

Prior to the initiation of the cell-mediated cytotoxicity (CMC) assays involving the use of concanavalin A (Chapt. 4, 5), similar experiments were carried out using polyclonal rabbit antiserum raised against plasma from the M-line (susceptible) strain of snail (=anti-M; Bayne, 1980). The rationale for this came directly from the hypothesis that PR-1 S. mansoni sporocysts escape destruction in M-line snail hosts due to the presence on the parasite of surface determinants that mimic M-line antigens, thus disguising the sporocysts so that they avoid appearing foreign. As shown previously (Yoshino and Bayne, 1983), anti-M antibodies bind to axenically transformed sporocysts. If the putative mimicked antigens could be specifically masked with some foreign protein, such as IgG, it was predicted that any protective function of these antigens would be nullified, and normally benign hemocytes would become cytotoxic.

Earlier work (Bayne et al., 1984) revealed that high levels of anti-M bound to sporocyst surfaces barely inhibited adherence of hemocytes to sporocysts. At the same time that cytoadherence was being quantified, identical anti-M-treated sporocysts were analyzed quantitatively by these investigators for the amount of antibody on their surfaces using quantitative immunofluorescent antibody technique (QIFAT). In this way it was shown that concentrations of anti-M could

be used which would block most of the surface antigens recognized by a distinct (anti-sporocyst) antiserum while still permitting a high level of adherence by M-line hemocytes.

#### MATERIALS AND METHODS

The experimental protocol was as described in Chapters 4 and 5, except that sporocysts were soaked in protein-A purified IgG from anti-M (Bayne et al, 1984) for 1 hr, rather than Con A for 1/2 hr. The IgG was used at 145 and 36  $\mu\text{g ml}^{-1}$  in MFB.

An analysis of variance was performed on the resultant data set.

#### RESULTS

The results are shown in Table 6.I. An analysis of variance indicated there was no significant difference in the mean level of killing between parasites treated with anti-M antibody and untreated ones.

#### DISCUSSION

The mimicked antigen hypothesis predicts that removal or masking of mimicked host antigens would negate their protective function, and sporocysts so treated should be attacked by snail hemocytes. Since no enhanced sporocyst killing was observed upon such masking treatment, these results suggest that either the parasite does not rely solely upon mimicked antigens for its survival in susceptible hosts, or the antibodies in this antiserum fail to recognize the relevant epitopes. Furthermore, the mere presence of foreign proteins on the parasite is

not enough to trigger CMC. The observation that anti-10-R2 (resistant) immunoglobulin also binds to sporocysts (Yoshino and Bayne, 1983) indicates that cross-reactive surface determinants do not play a protective role in this particular interaction. In other words, the fact that 10-R2 hemocytes always kill sporocysts, despite the expression of 10-R2 - like epitopes on their surfaces, implies that mimicked antigens are not sufficient for protection.

It is difficult to reconcile the results from these cytotoxicity experiments with those obtained with Con A. Since both reagents (antibody and Con A) are multivalent, both theoretically are capable of cross-linking surface determinants, either on a single sporocyst or between sporocysts and hemocytes, yet they have nearly opposite effects. It is not known if the specific IgGs that bind to the sporocysts also bind to hemocytes, although it has been shown that polyclonal anti-M IgG binds to hemocytes (Yoshino, pers comm). F(ab) regions are assumed to be available for cross-linking, since sporocysts are agglutinated by anti-M to a titer of 40 (Bayne et al, 1984). It is possible that anti-M binds to sporocyst surface antigens that either are not mobile in the plane of the tegument membrane, or do not trigger a surface modulation, as postulated for Con A. An observation that may be germane to this discussion is that specific antibody can be detected on sporocysts 48 hrs after their removal from the antiserum (Bayne et al, submitted for pub.).

If sporocysts were first treated with anti-M, then with Con A, and then assayed for the presence of Con A immediately and 24 hrs later, it should be possible to determine if 1), anti-M bound to or

blocked Con A receptors on the sporocyst, and 2), whether the pretreatment with IgG influenced the putative Con A-induced surface modulation by the parasite. If Con A binding was blocked by pre-treatment with anti-M, escape of such sporocysts from CMC would support the notion that a masking by foreign protein is not enough, and surface rearrangements might be required to induce cytotoxicity by benign hemocytes.

If the presence of IgG influenced not the binding, but the mobility of Con A-bound sporocyst receptors, it would give strong support to the hypothesis that surface modulation of the sporocyst is the stimulus that triggers M-line hemocytes to become cytotoxic toward PR-1 sporocysts.

Table 6.I. Sporocyst killing after treatment with anti-M-line antiserum then exposure to M-line hemocytes.

Anti-M (ug ml <sup>-1</sup> )	Number of experiments	Percent dead after 24 hr ( $\bar{x} \pm \text{S.E.}$ )
145	6	23.5 $\pm$ 4.9
36	6	20.7 $\pm$ 6.8
0	6	27.2 $\pm$ 8.4

## REFERENCES

- Bayne, C.J. 1980. Humoral factors in molluscan parasite immunity. In: Aspects of Developmental and Comparative Immunology, vol. 1, pp. 113-124. (J.B. Solomon, ed.) Pergamon Press, Oxford.
- Bayne, C.J.; Loker, E.S.; Yui, M.A. and Stephens, J.A. 1984. Immune-recognition of Schistosoma mansoni primary sporocysts may require specific receptors on Biomphalaria glabrata hemocytes. *Parasite Immunol.* 6:519-528.
- Yoshino, T.P. and Bayne, C.J. 1983. Mimicry of snail host antigens by miracidia and primary sporocysts of Schistosoma mansoni. *Parasite Immunol.* 5:317-328.

## CHAPTER 7

### CONCLUSION

Efforts to understand how parasites survive in potentially hostile environments, and how hosts prevent lethal infections from developing must include studies at the cellular and molecular levels. In addition, knowledge gained by such studies about systems in invertebrates is important in clarifying the evolutionary origins of the vertebrate immune system. In this thesis, an attempt has been made to identify factors that play an integral role in the processes that result in success or failure of an individual host-parasite encounter.

Because lectins bind avidly and specifically to carbohydrate structures, there is the potential that some of them may bind to and thereby "tag" a foreign body, and thus aid in identifying it as foreign. Chapters 2 and 3 detail the process whereby snail plasma was surveyed for the presence of lectins by monitoring its reactivity to an assortment of erythrocytes, with each erythrocyte displaying its own complement of surface markers. Once a cell type was found that was agglutinated strongly and consistently by the snail plasma, it then became possible to isolate the factor, analyze its physicochemical properties, and assess its possible function in the host-parasite system.

The overall goal for such a study was to identify a soluble plasma component that had some deterministic function in the outcome

following an encounter between Schistosoma mansonii and Biomphalaria glabrata. A functional role for the isolated agglutinin was not discovered, yet it is premature to conclude that agglutinins or opsonins have no part in the interaction between sporocysts and snail host hemocytes. Until all plasma components, including the apparent additional agglutinins described in Chapter 3, are tested and ruled out, receptor-specific substances must be considered possible as soluble mediators of host immune function. Also, vertebrate erythrocytes may not be the optimum indicator to use in searching for such putative substances, since opsonic factors can exist in hemolymph with no hemagglutinating activity (Anderson and Good, 1976; Sminia et al, 1979; Goldenburg and Greenberg, 1983).

Just as we do not know if susceptible B. glabrata possess a soluble molecule that has some function in mediating the compatibility of the relationship, so we are also ignorant of the processes whereby the parasite is killed by a host's hemocytes. It is assumed that there must be some recognition step that initiates a cytotoxic response, and it is further assumed that contact between the effector cells and target is required for recognition to occur. Indeed, previous work with this system (Bayne et al, 1980b; Loker and Bayne, 1982) suggests that contact is required for the killing step; EM studies show a very intimate association between hemocyte pseudopodia and the parasite's surface microvilli. However, these investigators used innately resistant cells as the archetypical cytotoxic model, and it has yet to be shown that when benign cells become cytotoxic, they behave in a similar manner.

From the work described in Chapters 4 and 5 of this thesis, we now have a model with which recognition and cytotoxic mechanisms can be systematically investigated. This (LDCC) model can be readily compared to the natural condition, in which an individual snail may be refractory or susceptible depending upon each miracidium that it encounters in a heterogeneous population of parasites (Basch, 1976). Specifically, the cytotoxic response against Con A-sporocysts in this model depicts a refractory response, while the "non-response" observed when M-line hemocytes encounter untreated sporocysts typifies the susceptible condition. This is in contrast to 10-R2 (and 13-16-R1) snails which are refractory to all S. mansoni strains tested. These two strains have been considered to exhibit the definitive resistant response, yet it is premature to conclude that recognition and killing mechanisms operating in these strains, which are universally resistant to Schistosoma mansoni (Richards 1975, 1984), are necessarily the same as those present in strains with a selective (or induced) resistance.

It is possible that the "recogniton" phenomenon associated with Con A as described in this thesis may be a mechanism used by hemocytes in their normal function as immune effector cells, rather than an artifact stemming solely from the use of Con A. An analogous situation is that proposed by Berke et al (1981a, b) in which specifically activated mouse cytotoxic T-lymphocytes (CTL) are nonspecifically cytotoxic toward target cells that have been treated with Con A. They propose that though killing is non-specific during LDCC, the CTL are using the MHC:foreign antigen combination during the

recognition and triggering process just as they do during specific cytotoxicity. In other words, these workers suggest that CTL do not use a different mechanism for recognition of foreignness during LDCC, they are simply stimulated by an unorthodox rearrangement of the target surface markers that they normally "use".

Such may also be the case in the LDCC described for the snail-schistosome model described here. It is established that M-line snails are susceptible to and fail to kill the PR-1 strain of S. mansoni while the PR-2 strain is killed (Richards, 1975; 1984). Though other explanations are possible, it may be that whatever M-line hemocytes use to identify the PR-2 strain and attack it may also be operating in Con A-dependent cytotoxicity of PR-1 sporocysts.

Evolutionary principles suggest that in the perpetual struggle between parasite and host, there is a universal progression toward an equilibrium in which neither side is dominant. The establishment of such an equilibrium might be due to development of tolerance to a foreign invader's presence by the host, tolerance to a host's reaction to invasion by the parasite, to a decrease in virulence (invasiveness, pathogenicity), to an increased ability in a population of hosts to resist the invader, or any combination of these. By studying the mechanisms, both molecular and cellular, operating in the host and parasite, we can gain a clearer understanding of what determines the success or failure of this most intimate of biological relationships. Furthermore, generalities prompted by such understanding may have applicability to vertebrate immunorecognition processes.

## REFERENCES

- Anderson, R.S. and Good, R.A. 1976. Opsonic involvement in phagocytosis by mollusk hemocytes. *J. Invert. Pathol.* 27:57-64.
- Basch, P.F. 1976. Intermediate host specificity in Schistosoma mansoni. *Exp. Parasitol.* 39:150-169.
- Bayne, C.J., Buckley, P.M. and DeWan, P.C. 1980b. Macrophagelike hemocytes of resistant Biomphalaria glabrata are cytotoxic for sporocysts of Schistosoma mansoni in vitro. *J. Parasitol.* 66:413-419.
- Berke, G., Hu, V., McVey, E. and Clark, W.R. 1981a. T lymphocyte-mediated cytolysis. I. A common mechanism for target recognition in specific and lectin-dependent cytolysis. *J. Immunol.* 127:776-781.
- Berke, G., McVey, E., Hu, V. and Clark, W.R. 1981b. T lymphocyte-mediated cytolysis. II. Role of target cell histocompatibility antigens in recognition and lysis. *J. Immunol.* 127:782-787.
- Goldenburg, P.Z. and Greenberg, A.H. 1983. Functional heterogeneity of carbohydrate-binding hemolymph proteins: Evidence of a nonagglutinating opsonin in Homarus americanus. *J. Invert. Pathol.* 42:33-41.
- Loker, E.S. and Bayne, C.J. 1982. In vitro encounters between Schistosoma mansoni primary sporocysts and hemolymph components of susceptible and resistant strains of Biomphalaria glabrata. *Am. J. Trop. Med. Hyg.* 31:999-1005.
- Richards, C.S. 1975. Genetic factors in susceptibility of Biomphalaria glabrata for different strains of Schistosoma mansoni. *Parasitology.* 70:231-241.
- Richards, C.S. 1984. Influence of snail age on genetic variation in susceptibility of Biomphalaria glabrata for different strains of Schistosoma mansoni. *Malacologia.* 25:493-503.
- Sminia, T., van der Knaap, W.P.W. and Edelenbosch, P. 1979. The role of serum factors in phagocytosis of foreign particles by blood cells of the freshwater snail Lymnaea stagnalis. *Dev. Comp. Immunol.* 3:37-44.

## BIBLIOGRAPHY

- Abdul-Salam, J.A. and Michelson, E.H. 1980. Phagocytosis by amoebocytes of Biomphalaria glabrata: Absence of opsonic factor. *Malacological Rev.* 13:81-83.
- Anderson, R.S. and Good, R.A. 1976. Opsonic involvement in phagocytosis by mollusk hemocytes. *J. Invert. Pathol.* 27:57-64.
- Armstrong, I.L. and Tate, W.P. 1980. A simple device for the dialysis of small volumes. *Anal. Biochem.* 106:469-470.
- Ballas, Z.K., Green, W.R. and Henney, C.S. 1981. Studies on the mechanism of T-cell-mediated lysis. XIII. Lectin-Dependent T-cell-mediated cytotoxicity is supported by Con A coupled to Sepharose beads. *Cell. Immunol.* 59:411-418.
- Basch, P.F. 1976. Intermediate host specificity in Schistosoma mansoni. *Exp. Parasitol.* 39:150-169.
- Bayne, C.J. 1980. Humoral factors in molluscan parasite immunity. In: *Aspects of Developmental and Comparative Immunology*, vol. 1 (J.B. Solomon, ed.) Pergamon Press, Oxford. pp 113-124.
- Bayne, C.J. 1983. Molluscan Immunobiology. In: *The Mollusca* (A.S.M. Saleuddin, and K.M. Wilbur, eds.), Vol. 5, Pt. 2, pp. 407-486. Academic Press, New York.
- Bayne, C.J., Buckley, P.M. and DeWan, P.C. 1980a. Schistosoma mansoni: Cytotoxicity of hemocytes from susceptible snail hosts for sporocysts in plasma from resistant Biomphalaria glabrata. *Exp. Parasit.* 50:409-416.
- Bayne, C.J., Buckley, P.M. and DeWan, P.C. 1980b. Macrophagelike hemocytes of resistant Biomphalaria glabrata are cytotoxic for sporocysts of Schistosoma mansoni *in vitro*. *J. Parasitol.* 66:413-419.
- Bayne, C.J., Loker, E.S., Yui, M.A. and Stephens, J.A. 1984. Immune-recognition of Schistosoma mansoni primary sporocysts may require specific receptors on Biomphalaria glabrata hemocytes. *Parasite Immunol.* 6:519-528.
- Bayne, C.J. and Stephens, J.A. 1983. Schistosoma mansoni and Biomphalaria glabrata share epitopes: Antibodies to sporocysts bind host snail hemocytes. *J. Invert. Pathol.* 42:221-223.
- Berke, G., Hu, V., McVey, E. and Clark, W.R. 1981a. T lymphocyte-mediated cytotoxicity. I. A common mechanism for target recognition in specific and lectin-dependent cytotoxicity. *J. Immunol.* 127:776-781.

- Berke, G., McVey, E., Hu, V. and Clark, W.R. 1981b. T lymphocyte-mediated cytotoxicity. II. Role of target cell histocompatibility antigens in recognition and lysis. *J. Immunol.* 127:782-787.
- Beyer, T.A., Rearick, J.I., Paulson, J.C., Prieels, J.-P., Sadler, J.E. and Hill, R.L. 1979. Biosynthesis of mammalian glycoproteins. Glycosylation pathways in the synthesis of the nonreducing terminal sequences. *J. Biol. Chem.* 254:12531-12541.
- Bonavida, B. and Bradley, T.P. 1976. Studies on the induction and expression of T-cell-mediated immunity. V: Lectin-induced nonspecific cell-mediated cytotoxicity by alloimmune lymphocytes. *Transplantation.* 21:94-102.
- Boswell, C.A. and Bayne, C.J. 1985. Schistosoma mansoni: lectin-dependent cytotoxicity by hemocytes from susceptible host snails Biomphalaria glabrata. *Exp. Parasit.* in press.
- Cheng, T.C., Chorney, M.J. and Yoshino, T.P. 1977. Lysozyme-like activity in the hemolymph of Biomphalaria glabrata challenged with bacteria. *J. Invert. Pathol.* 29:170-174.
- Cheng, T.C., Rodrick, G.E., Foley, D.A. and Koehler, S.A. 1975. Release of lysozyme from hemolymph cells of Mercenaria mercenaria during phagocytosis. *J. Invert. Pathol.* 25:261-265.
- Chernin, E. 1963. Observations on hearts explanted in vitro from the snail Australorbis glabratus. *J. Parasitol.* 49:353-364.
- Cooper, E.L. and Lemmi, C.A.E. 1981. Invertebrate humoral immunity. *Dev. Comp. Immunol.* 5:3-21.
- Dean, D.A. 1983. A review. Schistosoma and related genera: Acquired resistance in mice. *Exp. Parasitol.* 55:1-104.
- Francis, R.T. and Becker, R.R. 1984. Specific indication of hemoproteins in polyacrylamide gels using a double-staining technique. *Anal. Biochem.* 136:509-514.
- Fuke, M.T. and Sugai, T. 1972. Studies on the naturally occurring hemagglutinin in the coelomic fluid of an ascidian. *Biol. Bull.* 143:140-149.
- Fuller, G.M., Raseo, M.A., McCombs, M.L., Barnett, D.R. and Bowlan, B.H. 1973. Subunit composition of haptoglobin 2-2 polymers. *Biochemistry* 12:253-258.
- Gately, M.K. and Martz, E. 1977. Comparative studies on the mechanisms of nonspecific, Con A-dependent cytotoxicity and specific T cell-mediated cytotoxicity. *J. Immunol.* 119:1711-1722.

- Gilbertson, D.E. and Etges, F.J. 1967. Haemagglutinins in the haemolymph of planorbid snails. *Ann. Trop. Med. Parasitol.* 61:144-147.
- Goldenburg, P.Z. and Greenberg, A.H. 1983. Functional heterogeneity of carbohydrate-binding hemolymph proteins: Evidence of a nonagglutinating opsonin in Homarus americanus. *J. Invert. Pathol.* 42:33-41.
- Granath, W.O. and Yoshino, T.P. 1984. Schistosoma mansoni: Passive transfer of resistance by serum in the vector snail, Biomphalaria glabrata. *Exp. Parasit.* 58:188-193.
- Green, W.R. 1982. Studies on the mechanism of lectin-dependent T cell-mediated cytotoxicity: use of Lens culinaris hemagglutinin A to define the role of lectin. In: *Mechanisms of Cell-Mediated Cytotoxicity* (W.R. Clark and P. Goldstein, ed.) pp. 81-100. Plenum Press, New York.
- Green, W.R., Ballas, Z.K. and Henney, C.S. 1978. Studies on the mechanism of lymphocyte-mediated cytotoxicity. *J. Immunol.* 121:1566-1572.
- Hale, L.J. *Biological Laboratory Data*. London: Methuen and Co. Ltd., 1965.
- Hartree, E.F. 1972. Determination of protein: A modification of the Lowry method that gives a linear photometric response. *Anal. Biochem.* 48:422-427.
- Jeong, K.H., Sussman, S., Rosen, S.D., Lie, K.H. and Heyneman, D. 1981. Distribution and variation of hemagglutinating activity in the hemolymph of Biomphalaria glabrata. *J. Invert. Pathol.* 38:256-263.
- Koch, C. and Nielsen, H.E. 1984. Activation of vertebrate complement by Helix pomatia haemolymph. *Dev. Comp. Immunol.* 8:15-22.
- Lackie, A.M. 1980. Invertebrate immunity. *Parasitology.* 80:393-412.
- Laemmli, V.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
- Lie, D.J., Jeong, K.H. and Heyneman, D. 1980. Tissue reactions induced by Schistosoma mansoni in Biomphalaria glabrata. *Ann. Trop. Med. Parasit.* 74:157-166.
- Lis, H. and Sharon, N. 1981. Lectins in higher plants. In: *The Biochemistry of Plants*, vol. 6 (A. Marcus, ed.) p. 393. Academic Press, New York.

- Loker, E.S. and Bayne, C.J. 1982. In vitro encounters between Schistosoma mansoni primary sporocysts and hemolymph components of susceptible and resistant strains of Biomphalaria glabrata. Am. J. Trop. Med. Hyg. 31:999-1005.
- Loker, E.S., Yui, M.A. and Bayne, C.J. 1984. Schistosoma mansoni: Agglutination of sporocysts, and formation of gels on miracidia transformed in plasma of Biomphalaria glabrata. Exp. Parasit. 58:56-62.
- McLaren, D.J. and Terry, R.J. 1982. The protective role of acquired host antigens during schistosome maturation. Parasite Immunol. 4:129-148.
- Merril, C.R., Goldman, P., Sedman, S.A. and Ebert, M.H. 1981. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Science 211:1437-1438.
- Metchnikoff, E. 1905. Immunity in Infective Diseases. Reprinted by Johnson Reprint Corp., New York, 1968.
- Michelson, E.H. and Dubois, L. 1977. Agglutinins and lysins in the molluscan family Planorbidae: A survey of hemolymph, egg-masses, and albumen-gland extracts. Biol. Bull. 153:219-227.
- Noble, E.R. and Noble, G.A. 1982. Parasitology. The Biology of Animal Parasites. Lea and Febiger, Philadelphia. 522pp.
- Pauley, G.B., Krassner, S.M. and Chapman, F.A. 1971. Bacterial clearance in the California sea hare, Aplysia californica. J. Invert. Pathol. 18:227-239.
- Ratcliffe, N.A., and Rowley, A.F. (eds.) 1981. Invertebrate Blood Cells, vol. I & II. Academic Press, New York. 641 pp.
- Renwrantz, L. and Mohr, W. 1978. Opsonizing effect of serum and albumen gland extracts on the elimination of human erythrocytes from the circulation of Helix pomatia. J. Invert. Pathol. 31:164-170.
- Renwrantz, L., Schancke, W., Harm, H., Erl, H., Liebsch, H. and Gercken, J. 1981. Discriminative ability and function of the immunobiological recognition system of the snail, Helix pomatia. J. Comp. Physiol. 141:477-488.
- Renwrantz, L. and Stahmer, A. 1983. Opsonizing properties of an isolated hemolymph agglutinin and demonstration of lectin-like recognition molecules at the surface of hemocytes from Mytilus edulis. J. Comp. Physiol. 149:535-546.
- Richards, C.S. 1975. Genetic factors in susceptibility of Biomphalaria glabrata for different strains of Schistosoma

mansoni. Parasitology. 70:231-241.

Richards, C.S. 1984. Influence of snail age on genetic variation of Biomphalaria glabrata for infection with Schistosoma mansoni. Malacologia. 25:493-503.

Rubens, R.P. and Henney, C.S. 1977. Studies on the mechanism of lymphocyte-mediated cytotoxicity. VIII. The use of Con A to delineate a distinctive killer T cell subpopulation. J. Immunol. 118:180-186.

Sharon, N. 1984. Surface carbohydrates and surface lectins are recognition determinants in phagocytosis. Immunol. Today. 5:143-147.

Simpson, A.J.G. and McLaren, D.J. 1979. Schistosoma mansoni : Tegumental damage as a consequence of lectin binding. Exp. Parasitol. 53:105-116.

Sminia, T., van der Knaap, W.P.W. and Edelenbosch, P. 1979. The role of serum factors in phagocytosis of foreign particles by blood cells of the freshwater snail Lymnaea stagnalis. Dev. Comp. Immunol. 3:37-44.

Soderhall, K. 1982. Prophenoloxidase activating system and melanization - a recognition mechanism of arthropods? A review. Dev. Comp. Immunol. 6:601-611.

Stanislawski, E., Renwanz, L. and Becker, W. 1976. Soluble blood group reactive substances in the hemolymph of Biomphalaria glabrata (Mollusca). J. Invert. Pathol. 28:301-308.

Stein, P.C. and Basch, P.F. 1979. Purification and binding properties of hemagglutinin from Biomphalaria glabrata. J. Invert. Pathol. 33:10-18.

Stibbs H.H., Owczarzak A., Bayne C.J., and DeWan P.C. 1979. Schistosome sporocyst-killing amoebae isolated from Biomphalaria glabrata. J. Invert. Pathol. 33:159-170.

Ulmer, M.J. 1970. Notes on the rearing of snails in the laboratory. In: Experiments and Techniques in Parasitology (A.J. MacInnis and M. Voge, eds.), pp. 143-144. Freeman, San Francisco.

van der Knaap, W.P.W., Boots, A.M.H. and Sminia, T. 1983. Immunorecognition in Lymnaea stagnalis. Dev. Comp. Immunol. 7:645-648.

van der Knaap, W.P.W., Doderer, A., Boerrigter-Barendsen, L.H. and Sminia, T. 1982. Some properties of an agglutinin in the haemolymph of the pond snail Lymnaea stagnalis. Biol. Bull. 162:404-412.

van Pijkeren, T.A., Tavares, C.A.P. and Gazzinelli, G. 1982. Schistosoma mansoni: Ability of Con A to protect in vitro

mechanically transformed schistosomula against the lethal effect of immune serum plus complement. *Parasitology*. 84:239-252.

Vasta, G.R. and Marchalonis, J.J. 1985. Invertebrate agglutinins and the evolution of humoral recognition factors. In: *Cell Receptors and Cell Communication in Invertebrates* (B.A. Cinader, ed.) Marcel Dekker, New York. In press.

Vasta, G.R., Sullivan, J.T., Cheng, T.C., Marchalonis, J.J. and Warr, G.W. 1982. A cell membrane-associated lectin of the oyster hemocyte. *J. Invert. Pathol.* 40, 367-377.

Voge, M. and Seidel, J.S. 1972. Transformation in vitro of miracidia of Schistosoma mansoni and Schistosoma japonicum into young sporocysts. *J. Parasitol.* 58:699-704.

Warr, G.W. 1981. Immunity in Invertebrates. *J. Invert. Pathol.* 38:311-314.

Wells, J.V. Hematologic diseases. In: *Basic and Clinical Immunology* (H.H. Fudenberg, D.P. Stites, J.L. Caldwell and J.V. Wells, eds.) Lange Medical Publications, Los Altos, Calif. USA. pp. 452-500, 1978.

Wheeler, W.E., Lubby, A.L. and Scholl, M.L.L. 1950. The action of enzymes in hemagglutinating systems. II. Agglutinating properties of trypsin-modified red blood cells with anti-Rh sera. *J. Immunol.* 65:39-46.

Williams, A.F. and Gagnon, J. 1982. Neuronal cell Thy-1 glycoprotein: Homology with immunoglobulin. *Science*. 216:696-703.

Yahara, I. and Edelman, G.M. 1973. The effects of concanavalin A on the mobility of lymphocyte surface receptors. *Exp. Cell Res.* 81:143-155.

Yeaton, R.W. 1981. Invertebrate lectins II. Diversity of specificity, biological synthesis and function in recognition. *Dev. Comp. Immunol.* 5:535-545.

Yoshino T.P. 1981. Con A-induced receptor redistribution on Biomphalaria glabrata hemocytes: characterization of capping and patching responses. *J. Invert. Pathol.* 38:102-112.

Yoshino T.P. 1982. Lectin-induced modulation of snail hemocytes surface determinants: clearance of Con A-receptor complexes. *Dev. Comp. Immunol.* 6:451-461.

Yoshino, T.P. and Bayne, C.J. 1983. Mimicry of snail host antigens by miracidia and primary sporocysts of Schistosoma mansoni. *Parasite Immunol.* 5:317-328.

Yoshino T.P., Cheng T.C. and Renwrandtz L.R. 1977. Lectin and human

blood group determinants of Schistosoma mansoni: alteration following in vitro transformation of miracidium to mother sporocyst. J. Parasitol. 63:818-824.

Yoshino, T.P., and Davis, C.D. 1983. Surface antigens of Biomphalaria glabrata (Gastropoda) hemocytes: Evidence for linkage-independence of some hemolymph-like surface antigens and Con A receptor-bearing macromolecules. J. Invert. Pathol. 42:8-16.