AN ABSTRACT OF THE THESIS OF

Maureen Kupke Larson for the degree of Master of Science in Zoology presented on March 4, 1993.

Title: The Axial Organ: Its Potential Role In Echinoid Immunity

Abstract approved Redacted for privacy
Christopher J. Bayne

The echinoderm axial organ is located centrally, at the meeting point of the animals' "circulatory" systems. This, and its unusual histology, have prompted the implication of a number of different functions for the organ, including circulation, excretion and host defense. None of these have been definitively proven. Claims that starfish axial organ cells respond to antigen in an adaptive manner, resembling the vertebrate antibody response, also remain unconfirmed. The purpose of the work on *Strongylocentrotus purpuratus* presented in this thesis was to independently test the hypothesis that the cells of the axial organ respond to antigenic challenge. To determine if the cells are modified quantitatively following antigen encounter, the cell types of the organ were first classified according to morphological characteristics. Cell subpopulations were then monitored quantitatively after *in vivo* exposure to several types of antigens. Axial organ cells were also analyzed for qualitative changes; cells from antigen exposed urchins were assayed for immunocytoadherence to antigen-coated sheep erythrocytes. The coelomic fluids from the same animals were tested for

antigen-specific binding molecules using ELISA assays. Immunocytoadherence and ELISA assays were validated with cells and sera from trout similarly exposed to antigen and control treatments.

Eight subpopulations of axial organ cells were identified. Four of the cells types resemble cells found in the coelomic fluid. A possible precursor cell to the red spherule cell was found to be a member of the axial organ cell population. Changes in the axial organ cell subpopulations after antigen exposure were not significant, regardless of the type of antigen, exposure method or sample time. Cells of the axial organs from TNP-exposed urchins did not bind TNP-SRBC's, and the hemagglutination observed was neither TNP-inhibitable, nor dependent on previous expoure to the TNP antigen. Coelomic fluids from several urchins bound TNP to a greater extent than BSA, but the frequency of binding among urchins was not dependent on their prior treatment. Titrational analysis of one set of urchin coelomic fluids against the hapten, TNP, the carrier, LPS, or an unrelated protein, BSA, revealed identical binding curves with each molecule. This, in addition to a higher titer of binding molecules in the control animals compared to antigen-injected animals, led to the conclusion that the response was nonspecific.

The work presented in this thesis does not support the hypotheses that the urchin axial organ cells respond to antigenic challenge either by a modification in cell types present in the organ, or by the production of cell surface or secreted antigen-specific binding molecules.

THE AXIAL ORGAN:
ITS POTENTIAL ROLE IN ECHINOID IMMUNITY

by

Maureen Kupke Larson

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed March 4,1993

Commencement June 1993

APPROVED:

Redacted for privacy

Professor of Zoology in charge of major

Redacted for privacy

Chair of department of Zoology

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Dean of Graduate School

Date thesis is presented March 4, 1993

Typed by Maureen K. Larson

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ACKNOWLEDGEMENTS

I am sincerely grateful for the patience and support of many people. I have learned a tremendous amount in the past three years, and this would not have been possible without the guidance of others.

My husband, Stuart, has been unselfish in his love and understanding. I appreciate his constant support and kindness. There were times, with this project packed full of negative results, when I felt like giving up, but his listening ear and sense of humor seemed to put my life as a scientist in perspective.

I am especially thankful for my major professor, Chris Bayne, who allowed me to pursue the project of my own choosing. Chris was involved and interested in my project and my progress. Even during his sabbatical he gave unselfishly of his time. He has taught me many skills and in addition, instilled in me confidence and independence.

I appreciate the time given by the members of my graduate committee; Steve Kaattari, Chris Mathews, and Lloyd Swanson. My work with the trout would not have been successful without the guidance of Steve Kaattari and the generosity of both him and his lab.

Barbara Taylor, with her expertise in Nomarski, and her marvelous microscope and camera set-up, was especially helpful with my slides and photographs. I am grateful for her guidance and friendship.

The Bayne Lab has been a fantastic support system. I have never met a more diverse, yet cooperative and supportive group of people. Sarah Fryer has all the qualities of an excellent mentor; expertise, patience, and common sense.

I am extremely grateful for her willingness to help "students". Randy Bender provided useful discussions of background information, and a critical and helpful view of experimental designs. I am also appreciative of his assistance with urchin collection. Jane Menino was always willing to assist in anything from rabbit injections to recovering loose trout. I am grateful for her unselfishness. Jyun Hung Chen provided cultural insight as well as lab assistance. I am sorry that he had to return to Taiwan so soon. Irja Galvan has a very giving heart and I am thankful for her support, and the sharing of her expertise in working with trout. Nora Demers did a great service for me by keeping an eye on my urchins at the coast, and generously donated some of her antiserum for my trout ELISA's.

Finally, I am grateful to my family; my sisters, Marion and Reni, and especially my parents, Konrad and Renate Kupke. I was fortunate to be raised in a home where learning is fostered, and hard work and perseverence are rewarded. I am grateful for the lessons my parents have taught me.

This work was supported by Lab Animal Resources and the Zoology Research Fund.

THE AXIAL ORGAN:

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CHAPTER 1

GENERAL INTRODUCTION

Confronted with a hostile environment rich in opportunistic and infectious agents, metazoans defend their integrity by a variety of immune strategies. Pathogens can include viruses, bacteria, fungi and parasites, so it is imperative that the host response be timely and appropriate. In advanced metazoans, such as the vertebrates, immune function can be described as either innate or adaptive. The interaction between these two types of immunity provides an extremely effective internal defense system.

Physical barriers such as epithelia and mucus serve as a first line of protection. Where an intact skin surface is lacking, secretions containing lytic enzymes and enzyme inhibitors break down bacterial cell walls, and prevent bacterial enzyme activity. If entry does occur, the invading agent encounters a variety of innate cells and molecules that comprise the second line of defense. For example, transferrin, an iron binding protein in the blood, reduces the availability of free iron that is needed by bacteria. Lectins cross-link pathogens

and trigger further protective responses. Host phagocytes such as the monocytes, macrophages and neutrophils engulf foreign material and destroy it. Granulocytes release histamines and other molecules which promote inflammation. As a result, blood supply to the infected area increases and immune cell migration into the surrounding tissue occurs. Natural killer cells recognize virally infected cells and destroy them. Various acute phase proteins such as interferons, C-reactive proteins, and several of the complement components are rapidly released by host cells during an infection. In general these molecules function to recruit host immune cells, localize infection and inactivate the pathogen.

The third level of defense is specific and can be anamnestic. Adaptive responses to antigens are typical of the vertebrates and depend on the presence of lymphocytes. Lymphocytes either react directly with the antigen or they secrete soluble macromolecules, antibodies, which have the ability to specifically bind to the antigen. The lymphoid cells can be divided into two subpopulations, the B and T cells. Antibodies are produced solely by the B lymphocytes.

The structure of an antibody molecule enables it to react specifically with an antigen while simultaneously reacting nonspecifically with other immune cells. The antigen binding portion, Fab, is highly variable so that as a population antibodies can recognize a wide assortment of epitopes. The antibody molecules produced by a single B cell are identical. The region of the molecule that does not interact with the antigen is called the constant region, Fc. There are Fc receptors on a variety of cells including phagocytic ones. The Fc region

can also initiate the classical complement cascade leading to opsonization or lysis of the pathogen.

Antibody molecules are glycoproteins and each consists of two identical light chains and two identical heavy chains linked by disulfide bonds. The type of heavy chain determines the class of antibody isotype produced, while the light chains are of two varieties and combine randomly with the heavy chains. Highly variable regions called complementary determining regions (CDR) located on both the light and heavy chains contact the antigen. In mammalian species, gene rearrangements at gene segments of the CDR's theoretically provide 3.5 x 10^9 antibody possibilities. In addition, somatic recombination after initial antibody response leads to more variation during class switching events. The ability to interact specifically with the enormous variety of antigens would not be possible without these gene rearrangements and mutations.

The second feature of the adaptive immune response of vertebrates is memory. Vertebrates have the ability to respond to a repeat encounter with a pathogen in a powerful and accelerated fashion. Secondary responses to antigens differ from the primary in several parameters. The lag time of the secondary response is decreased substantially, titer is higher, the proportion of isotypes changes, and the affinity of the antibodies for the antigen increases. The cellular basis for memory is clonal selection and expansion. After contact with the antigen, lymphocytes bearing specific receptors are induced to multiply and the number of antibody-producing cells increases significantly.

The vertebrate immune system is an effective alliance of innate and adaptive components. Inborn, nonspecific responses, considered "primitive" in the

hierarchy of immune function, are found in invertebrates as well as vertebrates. As a group, invertebrates face many of the same types of pathogens as vertebrates. Can the origins of complex adaptive responses characteristic of the vertebrates be found in any of the invertebrate phyla? My research focuses on one group of invertebrates, the Echinodermata, in an attempt to unravel the origins of adaptive immune responses.

Echinoderms share the deuterostome lineage with vertebrates and hold an evolutionarily advanced position among invertebrates. Saluted as a "noble group especially designed to puzzle the zoologist" (Hyman, L., 1955), the echinoderms possess an amalgamation of traits found in no other phylum. Their adult symmetry is pentamerous although they originate as bilateral larvae. Echinoderm mobility is achieved by hydraulic pressure within thousands of tiny tube feet. Other striking features include a calcareous dermal endoskeleton, pedicellariae, and many other extraordinary adaptations. However this phylum lacks what might be considered standard features for animals of their evolutionary level, such as a brain and distinct directional flow of blood.

Echinoderms are interesting models for immunological research. Their complex defense system includes molecules that function as vertebrate immune molecules do. Most echinoderms are large and several species can be found intertidally, which makes them easily obtained. These animals adapt well to captivity and their large body volume facilitates the collection of coelomic fluid. At the core of echinoderm host defense lies an unusual assortment of coelomocytes, echinoderm "blood cells".

CELLULAR HOST DEFENSE

V.J. Smith (1981) summarized six different coelomocyte types found in the echinoderms: phagocytic amoebocytes, red and colorless spherule cells, vibratiles, hemocytes, crystal cells, and progenitor cells.

Phagocytic amoebocytes are the largest, 14-30 μm diameter, and most common of the cell types. Two morphologically distinct stages can be observed. Petaloid forms are capable of strong amoeboid movement and phagocytosis. Filiform amoebocytes appear to be important in clotting and can be recognized by their long, thin pseudopodia. Ultrastructural work on phagocytes of the sea urchin, *Strongylocentrotus droebachiensis* (Echinoidea), reveals a cellular structure consistent with their role as phagocytic cells. Many vesicles, heterogeneous in size, occur in the cytoplasm along with membrane-bound phagosomes. The endoplasmic reticulum and mitochondria are modest in development and number respectively (Vethamany and Fung, 1972).

In *Holothuria polii* (Holothuroidea), a sea cucumber, amoebocytes are the only cells producing hemolysins (Canicatti and Ciulla, 1987). The hemolysins are of two varieties, one being calcium dependent and heat labile while the other is calcium independent and heat stable; the latter can be induced by immune stimulation (Canicatti and Ciulla, 1987). After phagocytosing particulate antigens, such as formalized sheep red blood cells (fsRBC's), the amoebocytes tend to fuse into masses called brown bodies (Canicatti and D'Ancona, 1989b)."Stimulated" phagocytes of the urchin *Strongylocentrotus nudus* produce hydrogen peroxide at higher levels *in vitro* than "unstimulated" controls. The

amount of hydrogen peroxide produced differed with respect to the type of red blood cell used as an antigen, indicating that *S. nudus* phagocytes recognize a difference in the surface properties of human and sheep red blood cells. This implies a specific recognition system (Ito, *et al*, 1992).

Spherule cells are the second most abundant cell type in echinoderm coelomic fluid. Also called morula cells, they may or may not contain pigment and are designated red or white (colorless). The red spherule cells of *S. droebachiensis* are 11-14 μm in diameter. The cytoplasm contains abundant membrane-bound spherules of assorted sizes. These spherules are composed of granular material differing in electron density. The cell nucleus is eccentric, with condensed chromatin. Due to the size of the spherules, cytoplasm is sparse (Vethamany and Fung, 1972). The spherules contain a napthoquinone based pigment called echinochrome A. There is evidence that echinochrome A purified from the coelomocytes of *Echinus esculentus* (Echinodea) destroys marine bacteria (Service and Wardlaw, 1984), and that the red spherule cells of another urchin, *Paracentrotus lividus*, inhibited growth of gram negative bacteria (Gerardi, *et al.*, 1991). Holothurians do not possess this cell type.

Colorless spherule cells in *S. droebachiensis* closely resemble their red counterparts. The nucleus is small and eccentrically located. There is no nucleolus and there are few mitochondria. In the sea cucumber *Cucumaria miniata* (Holothuroidea), the colorless spherule cells are the least numerous cell type in the coelomic fluid but are abundant in the connective tissue throughout the body. The spherules of a 20 μm diameter cell average about 3 μm in diameter. Individual spherules consist of an outer "shell" of sulfated acid

mucopolysaccharides and a core of protein most likely complexed to carbohydrates (Hetzel, 1965). Degranulation of the cells occurs frequently in the connective tissue and involves breakdown of the spherule outer "shell" as well as the cell membrane. The scant cytoplasm contains a well developed Golgi, centrioles, free ribosomes, mitochondria and rough endoplasmic reticulum. Lysosomes are scarce (Fontaine and Lambert, 1977). Based on cytochemistry, Canicatti distinguished three types of colorless spherule cells (Types I, II, and III) which differ in spherule content. Type I is basophilic and contains the hydrolytic enzyme arylsulfatase. Type III cells are positive to Schmorl's reaction, suggesting melanin content (Canicatti, *et al.,* 1989a).

Brown bodies are structures related to host defense, in which coelomocytes aggregate around foreign material. In *Holothuria polii*, brown bodies occur naturally in the coelomic cavity. Their presence is not linked to disease or damage. The aggregating cells are phagocytes and spherule cells. Histologically, the brown bodies consist of one to several nodules of flattened amoebocytes surrounding residual foreign material. The nodules are immersed in an extracellular, granulated matrix with spherule cell types I, II, and III present. The granular material stains positive for Schmorl's reaction (Canicatti, *et al.*, 1989b).

Vibratile cells are an unusual cell type, ocurring mainly in echinoids. Although small, 5-10 μm in diameter, they are flagellated and highly motile. Vibratiles are extremely sensitive to fixation, but have been described as spherical, with an irregular nucleus containing condensed chromatin. The cytoplasm contains membrane-bound vesicles enclosing fine irregular filaments of mucus. The long

flagellum is of the 9+2 variety and orginates near the nucleus. A Golgi is present but mitochondria are uncommon. The presence of polyanions and polysulfates in both the cytoplasm and granules is evidenced by positive alcian blue staining (Vethamany and Fung, 1972). Large concentrations of mucopolysaccharides released by these cells initiate the clotting process (Bertheussen and Seljelid, 1978).

Hemocytes have been found only in holothurians. These cells contain hemoglobins which have lower molecular weights (Farmanfarmaian, 1966) and different absorption spectra (Crescitelli, 1945) than the analogous vertebrate molecule. Crystal cells are also found only in holothurians. They enclose one to three crystals of unknown origin and composition.

Nonmotile, small leukocytes are the second most numerous cell type in the coelomic fluid of *Cucumaria miniata*. Small and conservative in shape, these cells have a high nuclear to cytoplasmic ratio. Ribosomes are plentiful, and in some cells a well developed rough endoplasmic reticulum is present. This cell is believed to be a progenitor cell since many intergradations between them and amoebocytes, and them and spherules have been seen (Fontaine and Lambert, 1977). These cells probably exist in all echinoderms although descriptions have come from only holothurians and asteroids.

Coelomocyte origins remain puzzling. *Strongylocentrotus purpuratus* (Echinodea) phagocytes, and red and colorless spherule cells incorporated tritiated thymidine *in vitro* when cultured in the presence of the radiolabeled molecule for 20 minutes. Incorporation also occurred with the same cell types *in vivo* after injection of tritiated thymidine and sampling 1 hour later. This

process is not seen with vibratile cells unless injected animals are serially sampled. In the latter case, labeled vibratiles appear in circulation sometime between 1 and 29 days post injection. Thus, some proliferating cell type differentiates into vibratiles. One possible source is the parietal peritoneum since some cells from this area are filled with granules which stain violet with toluidine blue indicating a mucopolysaccharide content similar to that of mature vibratiles. Another discovery was the correlation between high thymidine uptake in phagocytes and correspondingly high values for both the red and colorless spherules. This may suggest that the DNA synthesis of these cell types is under the control of one or more common factors present in the coelomic fluid (Holland, *et al* 1965).

Studies of body wall transplantation reactions in echinoderms reveal specific anamnestic responses for allogeneic grafts (Hildemann and Dix, 1972; Karp and Hildemann, 1976; Coffaro and Hinegardner, 1977; Coffaro, 1980). Specific responses to other foreign materials have also been observed. *Strongylocentrotus purpuratus* injected with radiolabeled bovine serum albumin (BSA) and human serum albumin (HSA) or radiolabeled molecules (greater than 25 kD) from *S. purpuratus* coelomic fluid cleared more than 85% of the BSA and HSA within 22 hours while less than 50% of the *S. purpuratus* molecules were cleared. In addition, competition experiments showed that radiolabeled BSA clearance was inhibited by BSA addition but not HSA addition (Hilgard and Phillips, 1968). *S. purpuratus* injected with three different types of bacteria were compared in their ability to clear these pathogens from circulation. Primary clearance rates were found to differ for each type of bacteria, suggesting an

ability to distinguish between them. Secondary clearance rates were not significantly different from primary rates. Coelomocyte number was altered after the primary injection, with declining percentages for phagocytes and red spherule cells and an increase in vibratile cells (Yui and Bayne,1983).

NON-CELLULAR HOST DEFENSE

In close association with coelomocytes, components of non-cellular defense mechanisms have been discovered which foreshadow vertebrate components. *Holothuria polii* coelomic fluid possesses a rich selection of lysosomal enzymes, including acid and alkaline phosphatases, β glucuronidase, amino peptidase, acid and alkaline proteases, lipase, α amylase, peroxidase (Canicatti, *et al.,* 1991) and arylsulfatase (Canicatti, 1988). The hemolytic activity of *H.polii* can be inhibited by high concentrations of zinc. This effect also occurs in the mammalian complement system, although, unlike mammmalian complement where a polymerization of C9 occurs in the presence of zinc, no corresponding variations in the molecular weights of sea cucumber coelomic fluid components occurs (Canicatti and Grasso, 1988). Lysozyme has also been detected in the coelomocyte lysate of *H. polii*. Similar to egg white lysozyme, the lytic activity is stable upon heating, and is sensitive to both pH and ionic strength. The presence of lysozyme activity in the sea cucumber is in agreement with findings in other invertebrates (Canicatti and Roch, 1989).

Three species of sea urchin (Echinoidea), *Anthocidaris crassipina, Pseudocentrotus depressus*, and *Hemicentrotus pulcherrimus*, possess, in their

coelomic fluid, agglutinins for red blood cells from a variety of mammalian species. Rabbit erythrocytes showed the highest degree of susceptibility. The activity is calcium dependent; with the addition of EDTA agglutination is abolished. Activity is not temperature dependent (McKay, *et al.*, 1969; Ryoyama,1974). Lectins, glycoproteins which exhibit specific and reversible sugar binding activity, are responsible for cross-linking and opsonizing bacteria, viruses, fungi, and other parasites. A lectin from *A. crassipina* has been sequenced and found to be homologous to the rat lectin, mannose binding protein (Giga, *et al.*, 1987).

Sea Star Factor (Prendergast and Liu, 1976) purified from coelomocyte lysate exhibits many of the properties of mammalian lymphokines. These include delayed inflammatory skin reactivity in mice, monocyte chemotaxis, migration inhibitory properties and macrophage activation (Prendergast, 1976). When experimentally released into the coelomic wall of the sea star, *Asterias forbesi (Asteroidea)*, Sea Star Factor causes the adherence and spreading of coelomocytes (Prendergast, *et al.*, 1983).

Sea urchin coelomic fluid also contains complement-like activity (Bertheussen, 1983; Leonard, *et al.*, 1990; Ito, *et al.*, 1992). Addition of cell-free coelomic fluid to various mammalian erythrocytes results in opsonization for both murine macrophages and *Strongylocentrotus droebachiensis* phagocytes (Bertheussen, 1983). Similar results have been reported using the phagocytes of *Strongylocentrotus nudus* (Ito, *et al.*, 1992). The activity is heat labile and is inhibited by complement inhibitors such as zymosan, cobra venom factor, ammonium ions and others, suggesting a resemblence to the alternate pathway

(Bertheussen, 1983). The idea of a complement-based system in echinoderms was supported earlier by the discovery of C3b receptors on the phagocytes of *S. droebachiensis*. A strong opsonic effect was found by coating sheep red blood cells with human C3b. This effect was markedly increased when C3b was converted to C3bi (Bertheussen, 1982). Work on the hemolytic system in *Asterias forbesi* indicates a multicomponent system similar to the alternate complement pathway. Dilution of lytic coelomic fluid with fluid from the coelom of a nonlytic animal actually increases hemolysis. In addition, heating causes a shift in titer, but not a complete loss of hemolytic function. These two observations support the idea of interaction between at least two participants in the lytic activity. Divalent cations, excluding calcium or magnesium, are essential, and lysis is sensitive to PMSF treatment, indicating a serine protease component in the system (Leonard, *et al.*, 1990). The evidence indicates a complement-like system.

Concentrated, purified proteins obtained from either coelomic fluid or lysed coelomocytes have been found to activate murine thymocytes and stimulate fibroblast proliferation and protein synthesis. Thymocyte proliferation is stimulated directly by starfish lymphocyte activation factor and to a greater extent in the presence of submitogenic concentrations of concanavalin A. Incubation with antibody to human IL-1 significantly inhibits this protein's thymocyte proliferation ability and the protein has been characterized as a primitive IL-1-like molecule (Beck, 1986).

Components of echinoderm defenses described thus far have been of an innate nature. In addition to these studies on the immunobiological properties

of coelomic fluid and coelomocytes, research has suggested that the axial organ may also play a role in host immune responses. Of interest is the implication that the cells within the axial organ may function in an adaptive manner.

THE AXIAL ORGAN

The axial organ, an enigmatic structure, has been associated with several functions, none of which has been substantially demonstrated. The organ is located below the madreporic plate, lies along the stone canal and is the central meeting point for the perivisceral coelom, the hemal system, the perihemal system, and the water vascular system (Millott and Vevers, 1964). Absent in holothurians, the axial organ varies considerably in size and development within members of the phylum. Structural studies have been published by Boolootian and Campbell (1964), Millott and Vevers (1968), Bachmann and Goldschmid (1978), Anteunis (1985), and most recently by Welsch and Rehkamper (1987).

The axial organ is an assembly of free cells, connective tissue and cell debris floating in a fluid matrix. Penetrated by canaliculi, the organ has a spongy, glandular appearance. The free cells are identical to those found in the coelom and are able to wander between compartments and can directly penetrate the somatocoelic epithelium (Bachmann and Goldschmid, 1978).

Tiedeman (1816) described the axial organ as a heart-formed, contractile channel which is connected to the ring lacunae. The pumping action was also noted by Boolootian and Campbell (1964) who called the axial organ a primitive heart. Coelomocyte aggregation in the lacunae and canaliculi of the axial organ

following injury or injection of foreign cells led to the conclusion that the axial organ is involved in host defense (Millott, 1969). Ferritin injected into the perivisceral coelom was rapidly ingested by coelomocytes and later accumulated in the axial organ. The description of used cell aggregation led to the belief that the axial organ is a center for storage and degradation of cells and waste (Jangoux and Schaltin, 1977).

Radioactive tracer fed to *Echinaster graminicolus* (Asteroidea), a starfish, accumulated in the axial organ six to twelve hours after feeding (Ferguson, 1984). Ferguson suggested that the organ concentrates nutrients that reach it in a fluid state. Recent work implies an ultrafiltrational role for the structure. The idea is based in part on the ultrastructural characterization of specialized coelomic epithelial cells which are likened to podocytes of the vertebrate kidney (Welsch, *et al.*, 1987).

Axial organ cell populations from the starfish *Asterias rubens* (Asteroidea), injected with either bovine serum albumin or whale myoglobin, were separated by agglutination using soy bean agglutinin (SBA), and compared in their ability to bind fluorescent conjugates of the injected proteins. Fluorescence was classified as membrane bound or internal. It was reported that the SBA agglutinable population had significantly more membrane fluorescent cells than the non-agglutinable one. Membrane fluorescence was not observed if the FITC-labeled protein differed from the injected one, and no significant membrane fluorescence was observed in cell populations obtained from untreated animals (Leclerc, Panijel, *et al.* 1980).

The injection of *Asterias rubens* with trinitrophenylated polyacrylamide beads

(TNP-PAA beads) or fluorescein isothiocyanate-labeled polyacrylamide beads (FITC-PAA beads) *in vivo*, followed seven days later by the removal and culture of axial organ cells in the presence of the same antigen, resulted in the production of "antibody-like" molecules (Brillouet, *et al.*, 1984). It was reported that, in the presence of mammalian complement and 2-mercaptoethanol, this molecule caused lysis of sheep red blood cells labelled with the same hapten as previously encountered by the animal. Both nylon wool adherent and nonadherent cell components had to be present for lysis to occur, but either cells or culture supernatants could be utilized, indicating a released soluble factor. Addition of increasing amounts of monovalent dinitrophenyl (DNP-OH) inhibited the lysis (Brillouet, *et al.*, 1984). The factor, precipitated with 50-70% ammonium sulfate and gel filtered, was eluted in a volume corresponding to 132,000 daltons (Delmotte, *et al.*, 1986).

The work of Leclerc, Brillouet, and colleagues suggests not only a function for the axial organ but also the presence in an invertebrate of a specific adaptive response. The importance of these possibilities led me to independently test the hypothesis that the axial organ functions immunologically. Quantitative and qualitative descriptions were done of the axial organ cells in *Strongylocentrotus purpuratus*, and changes in the cellular subpopulations in response to antigenic challenge were monitored. I also examined the hypothesis that axial organ cells produce molecules specific for an injected antigen. In the latter study I utilized trout to validate the assays, as these fish are known to respond adaptively to challenges such as those used in this study.

CHAPTER 2

AXIAL ORGAN CELLS OF *STRONGYLOCENTROTUS PURPURATUS* DESCRIPTION AND QUANTIFICATION

INTRODUCTION

The axial organ is a slender, tapered structure which lies parallel to the stone canal (Figure 1). Glandular in appearance, the axial organ is supported by connective tissue and filled with free cells in a fluid matrix. It is part of the hemal system and connects the oral and aboral hemal rings. Named for its central positon along the radial axis of the animal, the axial organ can communicate with all of the fluid-filled systems. This position makes the axial organ an interesting candidate for internal surveillance and host defense.

LOCATION OF THE AXIAL ORGAN

The body cavity of an echinoderm can be divided into compartments. The perivisceral coelom is the cavity which houses digestive and reproductive organs. The tubular coelomic system consists of fluid-directing channels which

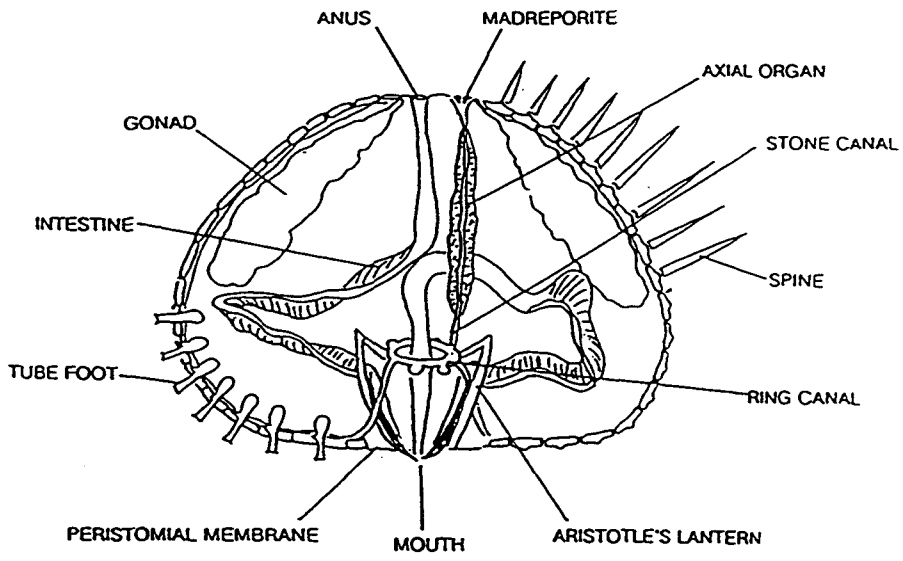

FIGURE 1
Vertical section through the central portion of the test of a sea urchin. All systems shown radiate pentamerously from the center of the animal. This simplified diagram depicts the oral-aboral orientation of the axial organ along the stone canal. (Modified from McRae, Transactions of the Royal Society of New Zealand, 1986)

are functionally separate but physically interconnected. This system includes the water vascular, hemal, and perihemal systems. The water vascular system provides the hydrostatic pressure needed for movement. It includes a calcified "stone" canal that extends inward from a porous ossicle called the madreporite. The stone canal connects to the ring canal, and from there the system radiates throughout the animal and provides fluid pressure for thousands of tube feet.

The hemal system is channeled within the connective tissue of the coelom. In starfish the lacunae generally contain a viscous fluid and crowded amoeboid cells. The system includes up to four ring elements. These rings (oral, aboral, gastric and pyloric) are connected by a pulsating vessel which runs parallel to and is interconnected with the axial organ. Connected to the oral and aboral hemal rings are radial hemal vessels that extend into the tube feet and gonads. Contractions of muscle cells surrounding some of the vessels circulate fluid in a back and forth motion. The perihemal system surrounds and mirrors the hemal system in its gross anatomy. Due to its central position within the animal, the axial organ links the entire circulatory network.

ANATOMY OF THE AXIAL ORGAN

In cross section the axial organ consists of a highly chambered tissue surrounding an eccentrically located axial sinus. Running along the oral-aboral axis of the sinus is the pulsating vessel, so named for its myogenic contractions

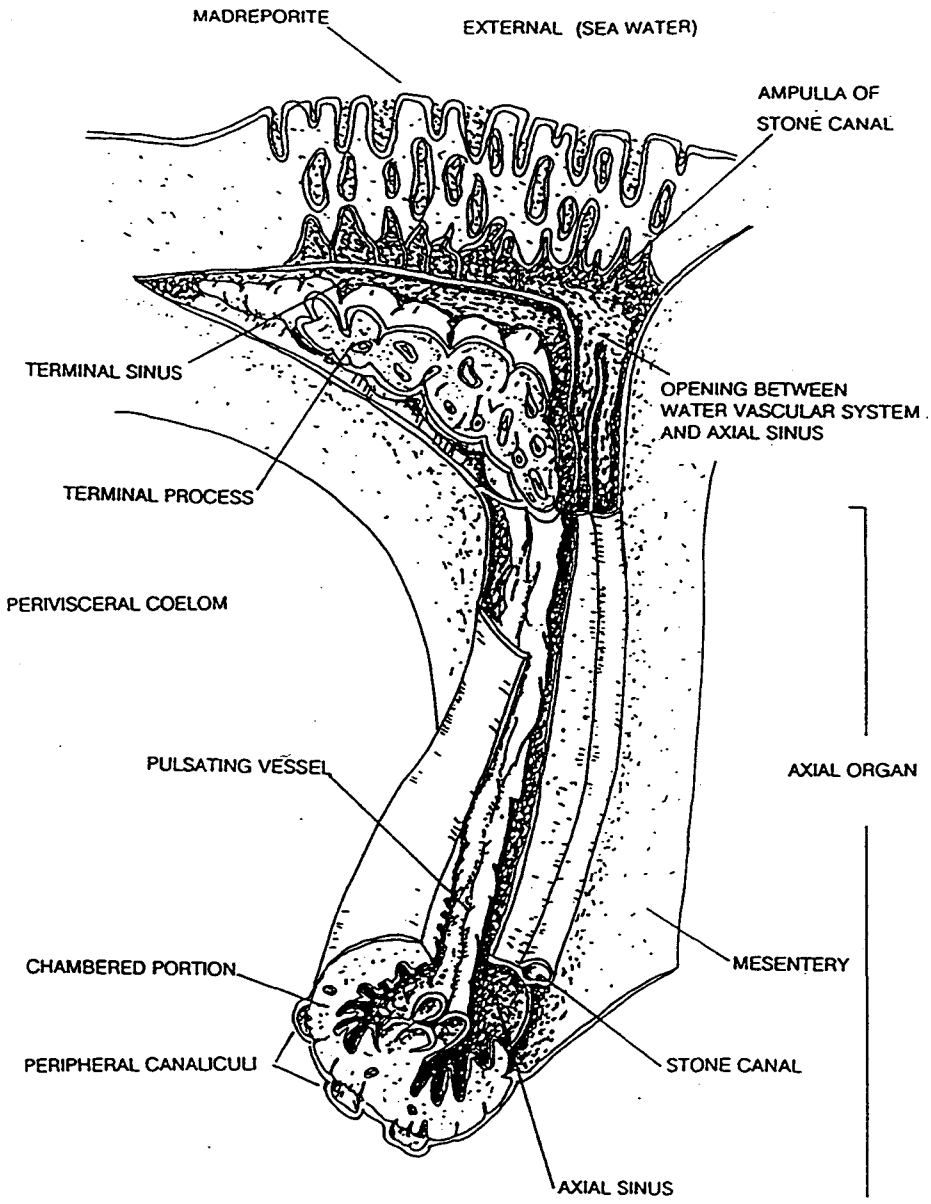

FIGURE 2
Semischematic longitudinal representation of the axial complex of *Sphaerechinus*. The chambered portion of the axial organ surrounds and connects with the pulsating vessel. An opening between the stone canal and the axial sinus allows axial organ communication with the water vascular system. Peripheral canaliculi link the axial organ to the fluid filled perivisceral coelom. (Modified from Bachmann and Goldschmid, 1978)

(Figure 2). The chambered portion of the axial organ contains compartments some of which lack continuous connective tissue walls. Coelomocytes wander freely between these compartments. The peripheral canaliculi communicate with the surrounding perivisceral coelom and, although lined with flattened epithelial cells, they often lack free coelomocytes (Millott and Vevers, 1968). Lacunae form a branched network covering the surface of the chambered portion, and connect directly to the rest of the hemal system. In the lacunae several coelomocytes can be found. Accumulation of granular material is greatest in the periphery of this region (Bachmann and Goldschmid, 1978).

At the aboral end of the axial organ, the chambered portion terminates. The pulsating vessel continues aborally to the terminal process. Below the madreporite is an opening in the septum between the stone canal and the axial sinus. At this point the axial organ is connected to the water vascular system.

AXIAL ORGAN RESPONSE TO INJURY

Injuries to the test of the urchin *Arbacia punctulata* (Echinoidea) resulting in loss of coelomic fluid affect the axial organ in a distinctive manner. Cells, cell debris, and secretions leave the lacunae and move into the pulsating vessel. The result is a swollen appearance to the organ (Millott, 1969). When a combination of ciliates (*Uronema)* and bacteria are injected into the coelom, the axial organ swells to the point of becoming spherical with cavities expanding into thin-walled bladders. Within both the bladders and the central cavity, streaming of amoebocytes is evident. In the perivisceral coelom conspicuous red "clouds" of

cells can be seen swirling near the axial organ. A superficial examination of other body systems does not reveal obvious changes (Millott, 1966).

There is also evidence for quantitative changes in axial organ cell subpopulations following injection of foreign proteins. In *Asterias*, animals injected with bovine serum albumin or whale myoglobin exhibited an increase in the soy bean lectin agglutinable subpopulation by nearly 20% (Leclerc, *et al.*, 1980.).

AXIAL ORGAN CELLS

Echinoidea

Several cell types have been identified on the basis of ultrastructural criteria within intact axial organs from *Sphaerechinus granularis* (Echinoidea). Monociliated epithelial cells possessing cytoplasmic extensions interdigitate with neighboring cells and with the basal lamina. The cilium arises from a deep invagination surrounded by a collar of lamellae. These cells resemble the choanocyte-like cells found in various coelomic cavities of echinoderms (Norrevang and Wingstrand, 1970). Pinocytic vesicles in the collar region as well as microvilli and protrusions of the cell surface suggest highly active transport functions (Bachmann and Goldschmid, 1978).

Four types of free cells have been described in this organ: morulas, phagocytes, leukocyte-like cells, and fibroblasts. The leukocyte-like cells and fibroblasts are round with few, short pseudopodia. The fibroblasts are

distinguished by their close association with collagen fibers (Bachmann and Goldschmid, 1978).

TABLE 1
CHARACTERISTICS OF AXIAL ORGAN CELLS
FROM *ASTERIAS RUBENS*

Cell Characteristic	Adherent	Nonadherent
Lectin Binding [1]	SBA, WGA	Con A
Mitogens	LPS[2],Nocardia[3]	Con A[2], Limulin[2], PWM[2]
Phagocytosis of latex beads or *C. albicans* [4]	No	Yes
Causes angiogenesis in vertebrates [1]	No	Yes

A summary of the work done by M. Leclerc and colleagues on the subpopulations of cells in the axial organ of the starfish *Asterias rubens* is presented in Table 1. Cells were teased from the organs and were initially separated by their adherence to nylon wool.
References: 1; (Leclerc, Brillouet, *et al.*, 1980), 2; (Brillouet, *et al.*, 1981), 3; (Leclerc, *et al.*, 1988), 4; (Bajelan, *et al.*, 1990)

Asteroidea

Unlike those studied in echinoids, the axial organ cells from *Asterias rubens* were removed from the surrounding connective tissue. The cells were then separated on the basis of surface receptors, and viewed as distinct subpopulations (Leclerc, Brillouet *et al.*, 1980; Brillouet, *et al.*, 1981; Leclerc, *et al.*, 1988). The authors' work emphasized the similarities between vertebrate lymphocytes and some of the axial organ cells from *Asterias rubens*. Recent descriptions have included terms such as B- and T-like cells.

Axial organ cells from *Asterias rubens* have been separated on the basis of adherence to nylon wool. Characteristics of the subpopulations are listed in Table 1. Ultrastructural analysis of the nonadherent population reveals two types of cells. The small lymphocyte-like cells, 4 μm in diameter, have a high nuclear to cytoplasmic ratio. The nucleolus is round and compact. The external side of the nuclear membrane bears ribosomes, which in the cytoplasm are monomeric. The Golgi is poorly developed and lysosomes are small and few in number. Mitochondria are also sparse, and the endoplasmic reticulum is moderately extensive. These cells account for about 20% of the whole axial organ population of *Asterias rubens* (Anteunis, *et al.*, 1985). Based on the work summarized in Table 1, Leclerc identified this cell type as having T-like qualities.

The second type of nonadherent cell is 7-8 μm in diameter. The nucleus is eccentric with either condensed or diffuse chromatin. The ribosomes are numerous and the Golgi is well developed. Mitochondria are large with swollen, clear matrices. Many vesicles are present. The extent of the endoplasmic reticulum varies. Some of these cells stain positively for endogenous peroxidase and have been compared to vertebrate mononuclear phagocytes. This axial organ cell subpopulation accounts for 40% of the whole (Anteunis, *et al.*, 1985).

The nylon wool adherent population also contains two subpopulations. One consists of 5-6 μm diameter cells which account for 5-10% of the whole axial organ population. These have a high nuclear to cytoplasmic ratio, although not as high as the small nonadherent cells. The endoplasmic reticulum is variable and large multivesicular bodies are present. The characteristics listed in Table 1 led Leclerc to suggest a B-like function for these cells. This implied functional

similarity to vertebrate B cells was supported with experiments in which axial organ cells separated from the total population by soy bean agglutinin (SBA) bound previously encountered antigen at a higher frequency than cells that were not SBA agglutinable. This suggests the production of a specific membrane receptor, a characteristic of B lymphocytes (Leclerc, *et al*, 1980). The 7-8 μm cells in the adherent population resemble those larger cells in the nonadherent population (Anteunis, *et al*., 1980).

Axial organ cells from *Asterias rubens* mixed with axial organ cells from another starfish, *Marthasterias glacialis*, exhibited a cytotoxic reaction, but this did not occur when allogeneic cells were targeted (Luquet, *et al*., 1984). Similar results were obtained when mouse tumor cells were targeted, but results may have been compromised by osmotic differences between the two species. *In vivo* inoculation of heat killed *Staphylococcus aureus* induced an increase in spontaneous cytotoxicity by axial organ cells. These types of responses are reminiscent of those that characterize Natural Killer cells in mammals (Leclerc and Luquet, 1983).

HYPOTHESIS

On the basis of previous work with *Asterias rubens* and *Arbacia punctulata*, I hypothesized that an antigenic challenge would alter the relative percentages of axial organ cell subpopulations in the urchin, *Strongylocentrotus purpuratus*. To test this hypothesis, I first characterized the axial organ cells. Classification was based on morphological properties. The subpopulations were then

monitored following antigenic challenge. In an effort to identify potentially effective antigen characteristics, and to optimize exposure methods and schedules, experiments included several different designs. The results reported in this chapter include data from fifty-six animals.

MATERIALS AND METHODS

ANIMALS:

Sea urchins (*Strongylocentrotus purpuratus)* averaging 136 ± 4 grams were collected year-round at Boiler Bay, Oregon. The animals were housed for two weeks in an open sea water aquarium at the Hatfield Marine Science Center, Newport Oregon. Healthy individuals were transferred to Oregon State University and maintained in 15°C circulating sea water. Urchins were fed *Laminaria sp.* supplemented with green leaf lettuce *ad libitum*.

AXIAL ORGAN REMOVAL:

Axial organs were removed aseptically through incisions in the aboral portion of the sea urchin tests. The axial organs were rinsed extensively in sterile chilled media. Cell suspensions of individual axial organs were made by massaging the organ through 75 μm porous nylon netting (Tetko, # 3-75/45) with a sterile syringe bulb. All glassware was silicon coated (Sigmacote, Sigma) and sterilized. Since the cell population was heterogeneous in both size and fragility, the cells were not centrifuged after separation from the organ. Cells were enumerated with a hemacytometer and viability determined with propidium iodide.

ESTIMATION OF CELLULAR VIABILTY:

Ten microliters of a 500 μg/ml stock propidium iodide solution was added to a 200 μl aliquot of the cell suspension. Cell viability was scored using the RITC filter set of a Zeiss epifluorescence microscope.

$$\text{VIABILITY} = \frac{\text{Total cell number - (propidium iodide positive nuclei)}}{\text{Total cell number}}$$

ANTIGEN PREPARATION:

Several antigens were assayed for their effects on axial organ populations.

Bovine Serum Albumin (BSA, Fraction V, Sigma A-3912) was prepared in sterile Instant Ocean at 50 mg/ml. Animals received injections of 200 μl. Control animals received similar volumes of Instant Ocean.

Keyhole Limpet Hemocyanin (KLH, Sigma H-2133) was prepared at a concentration of 2 mg/ml in sterile Instant Ocean. Before injection, equal volumes of KLH solution and Freund's Incomplete Adjuvant, FICA, were emulsified and animals received 100 μl per injection. Control animals received 100 μl Freund's Incomplete Adjuvant emulsified in sterile Instant Ocean, or were not injected.

A particulate form of KLH used for immersion exposures was prepared by

mixing 10 mg KLH with 15 ml of a sterile bentonite suspension in Instant Ocean. The bentonite suspension consisted of 1 gram of bentonite (Sigma B3378) added to 15 ml Instant Ocean. The suspension was autoclaved for 15 minutes and cooled to room temperature before KLH addition. The 15 ml of suspension was added to 1 liter of sea water. Each urchin was immersed in 150 ml of this suspension for 30 minutes followed by two 10-minute rinses with the same volume of sea water. Control animals received immersions in either bentonite suspensions without protein, or plain sea water.

The particulate form of KLH for injection was made by adding 1 mg KLH to a sterile 100 μg/ml suspension of bentonite in 1 ml Instant Ocean. Urchins received 100 μl injections. Control animals received either bentonite suspensions without protein or were not injected.

INJECTION SCHEDULES:

Four separate experiments are presented in this chapter. The intent was to optimize the effect of antigen exposure by varying antigens, methods of exposure and sampling times.

Experiment 1: Animals were injected once with either BSA or Instant Ocean. Urchins were injected through the peristomial membrane using a 26 gauge, 1/2 inch needle. Sampling took place at days 5, 7, 9, and 11 post injection. A total of 16 animals were harvested.

Experiment 2: Animals were injected with KLH in FICA three times with 8 days between the first and second exposure and 11 days between the second and third. Sampling took place 28 days, 35 days, and 57 days after the last exposure. Thirteen animals were sampled.

Experiment 3: Animals exposed to KLH on bentonite were divided into three groups. Group 1 received three immersions 1 week apart. This was followed by an injection at week four and harvest at week seven. Group 2 received 2 immersions one week apart. This group was injected at week three and harvested at week 10. Group 3 received the same two immersions as group 2, was injected at week 11 and harvested at week 16. Fifteen animals were used.

Experiment 4: Animals received six injections of KLH in FICA with two week intervals between each. All of the animals were harvested seven weeks after the final exposure. Twelve animals were used.

CELL COUNTS:

Cells were separated from the axial organ and cellular viability was determined by propidium iodide exclusion. One hundred cells were counted and categorized under phase microscopy. Cell numbers were expressed as percent of the whole population.

LECTIN STAINING:

Axial organ cells were characterized by their affinity for various lectins. Fluorescein isothiocyanate labeled lectins were added to cell suspensions for a final concentration of 100 μg/ml in Instant Ocean. Cells were incubated for 15 minutes on ice followed by two 15 minutes rinses with Instant Ocean. Cells were viewed under fluorescence microscopy and positive cells were identified.

Specificity of lectin binding was assayed by preincubating the lectins with appropriate monosaccharides for 20 minutes at room temperature before adding to the axial organ cells. Three different concentrations of sugars were used; 110 mM, 55 mM, and 27.5 mM.

YEAST PHAGOCYTOSIS

The phagocytic capacity of axial organ cells was determined by yeast phagocytosis. Tetramethylrhodamine-5-isothiocyanate (TRITC) labeled yeast (labeled by Linda Bleeker) were added to 100 μl of an axial organ cell suspension made in Instant Ocean buffered with 2 mM Hepes (pH 7.9). The yeast were added at a ratio of 10 yeast/1 axial organ cell. Phagocytosis was allowed to proceed for 1 hour at 17°C and the liquid was removed. 100 μl of trypan blue made in Instant Ocean was added and allowed to stain the cells for 3 minutes. The cells were rinsed 3 times with Instant Ocean and were viewed under fluorescence. The internalized yeast fluoresced brightly, while the

unphagocytosed yeast fluorescence was quenched by the trypan blue stain. Phagocytic cells were categorized according to morphological features.

RESULTS

AXIAL ORGAN CELLS FROM *STRONGYLOCENTROTUS PURPURATUS*

Cells from the axial organ of *S. purpuratus* can be seen in Figure 3. This photo shows the variety of cell types and the accumulation of granules, which is characteristic of the organ. The cells were categorized on the basis of morphological characteristics. Eight subpopulations were identified, four of which are unique to the organ and four of which resemble cells found in the coelomic fluid. Cells of type 1 are bipolar (Figure 4) with cell bodies measuring 6-10 μm and two thin processes each extending up to 20 μm from opposite ends of the cells. These constitute the most numerous of the cell types, accounting for a third of the cells of the axial organ. The membranes bind wheat germ agglutinin (WGA), peanut agglutinin (PNA), and *Tetragonobulus* (Tet) lectins indicative of β-D-GlcNAc(1-4)GlcNAc, β-D-Gal(1-3)GalNAc, and α-L-fucose carbohydrate groups respectively. Staining with the fluorescein labeled lectins can be seen in Figure 5. The binding to PNA could be inhibited by 28 mM D-galactose. The binding to WGA could not be inhibited by the highest concentration of N-acetylglucosamine assayed, 110 mM. Sugar inhibition was not tested for Tet.

Cell Type 2 is glass adherent and is the second most abundant cell type in the urchin axial organ. The diameter is 15-20 μm with pseudopodia extending from the entire surface (Figure 6). The cell readily phagocytoses yeast.

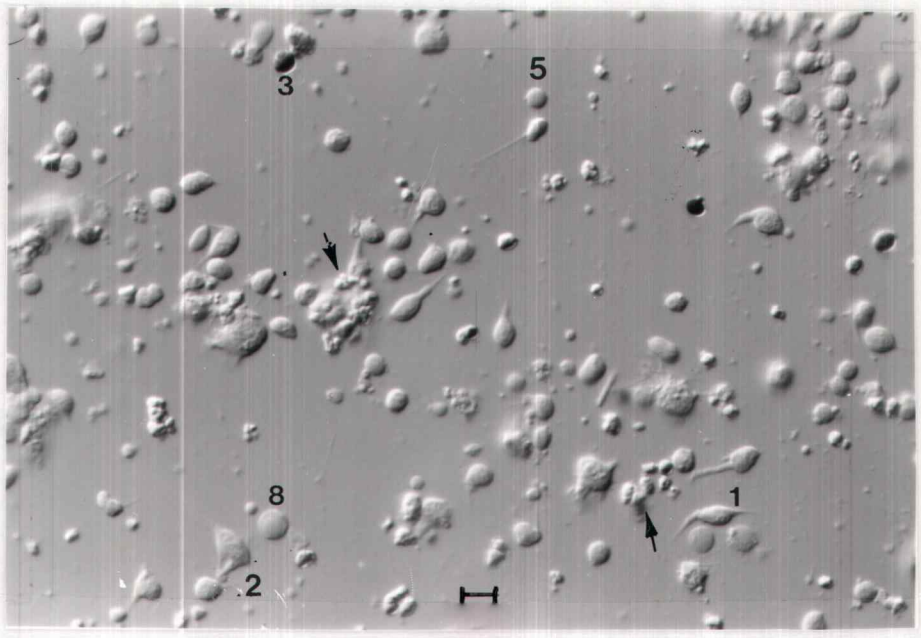

FIGURE 3
Photograph represents a sample of *Strongylocentrotus purpuratus* whole axial organ cell population. Notice clumps of granules (arrowheads) and identified cell types. Bipolar Cell (1), Glass Adherent Cell (2), Red cell (3), small, "lymphocyte-like" cell (5), large nonspread cell (8). Cells viewed under Nomarski, x400. Scale bar represents 10 μm.

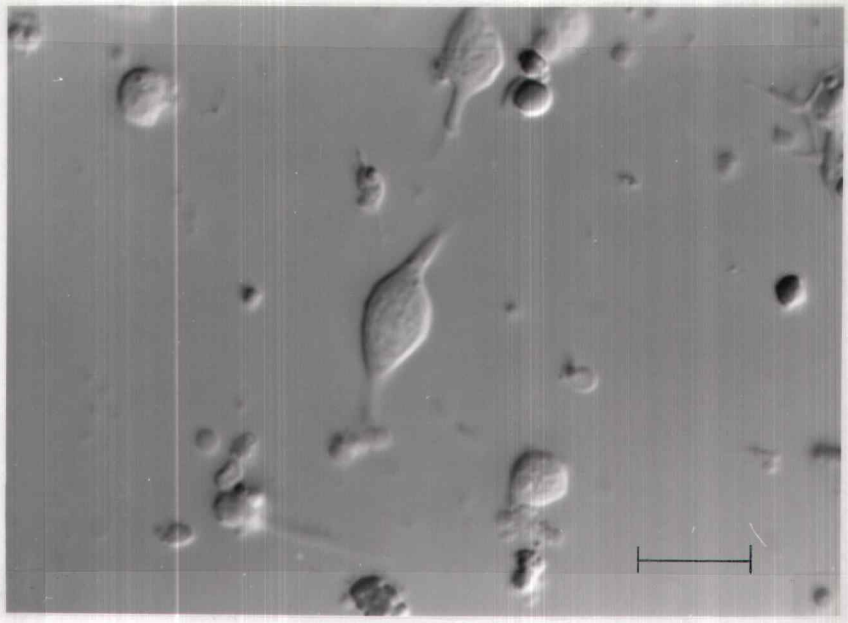

FIGURE 4
Cell Type 1 from the urchin axial organ. These bipolar cells account for a third of the total population. Nomarski x1000. Scale bar represents 10 μm.

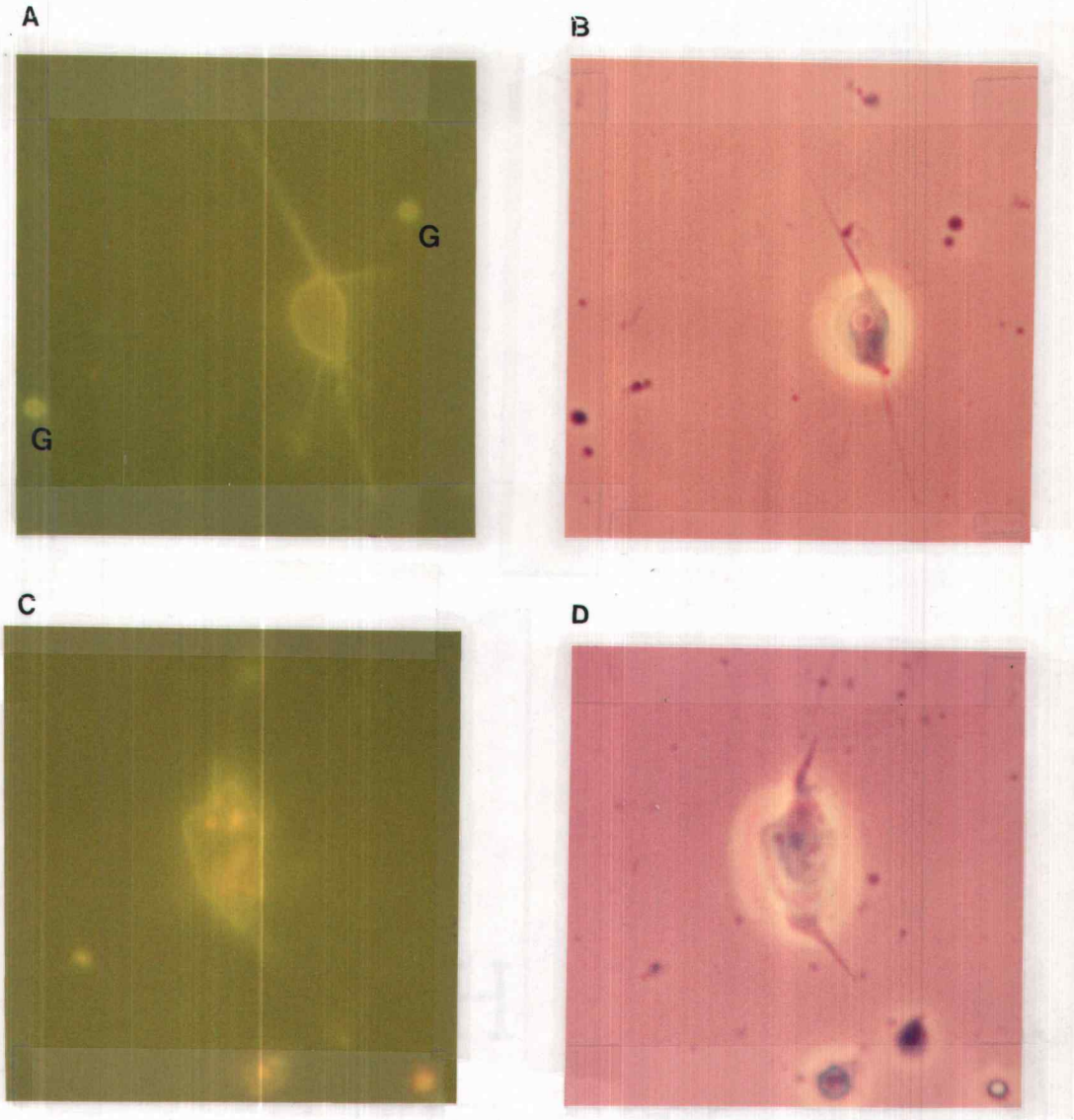

FIGURE 5
Cell Type 1 was characterized by membrane carbohydrate content. FITC-labeled lectins were added to live cell preparations and positive cells were classified. A) Bipolar cell (Type 1) stained with FITC-PNA. B) Same cell under phase optics. C) Bipolar cell fluorescently labeled with FITC-Tet. D) same cell under phase. Notice autofluorescent granules (G). x1000

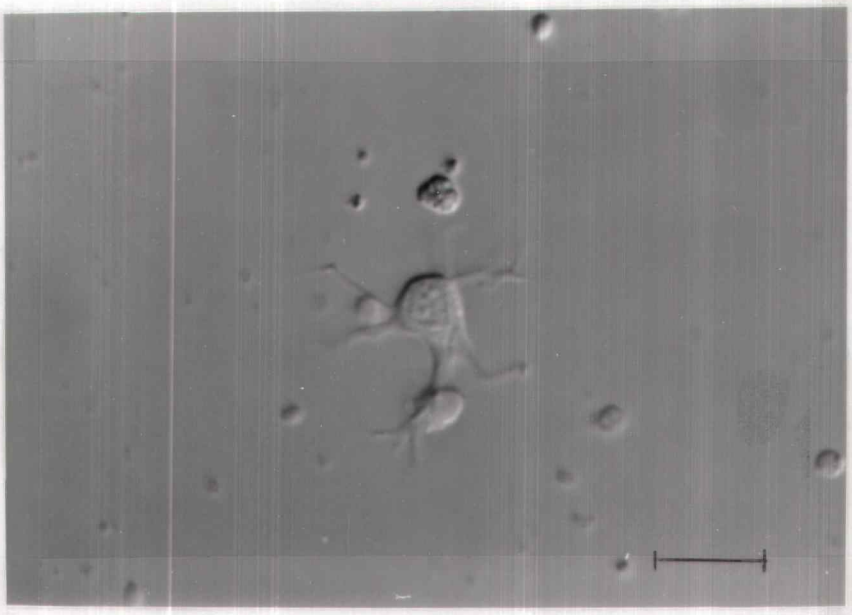

FIGURE 6
Glass adherent Cell Type 2 from axial organ of *S. purpuratus* displays spread pseudopodia on a glass coverslip. These cells constitute a third of the total axial organ cell population. Nomarski x1000. Scale bar represents 10 μm.

Cells of type 3 are red to brown and in most instances granulated. They vary in size, color, and extent of granulation. This variation can be seen in Figure 7. In its largest form, 15 μm in diameter, the cell is highly granulated. Its red raspberry-like appearance and amoeboid movement are identical to characteristics of the red spherule cell found in coelomic fluid of the urchin. Both the red spherule cell and the large form of cell type 3 have surface carbohydrates which bind potato-lectin (Figure 8) and WGA. Neither binding can be inhibited by 110 mM N-acetylglucosamine. The smaller cells do not bind these lectins. They exhibit several degrees of granulation and range in color from red to orange to brown. These smaller forms are absent in the coelomic fluid.

The fourth cell type is flagellated and highly motile. There are few in the axial organ. Cell type 4 is spherical, granulated, and appears to be the same as the vibratile cell found in the coelomic fluid (Figure 9). This cell type aggregates with many other cell types. It is often seen associated with cells of the same type, and occasionally balls of flagellated cells can be seen swirling in live cell preparations. The sticky nature of these cells suggests mucopolysaccharide content, which would confirm their identity as vibratile cells.

Cell type 5 is the third most common cell type in the organ (Figure 10). It was not observed in coelomic fluid. Small in size (<6 μm in diameter) with a high nuclear to cytoplasmic ratio, these cells have a lymphocyte-like appearance.

The sixth cell type is a highly granulated cell, 10 μm in diameter, which occurs at a low frequency in the urchin axial organ (Figure 11). The size, color,

granulation, and amoeboid movement suggest that this cell type is the white spherule cell present in urchin coelomic fluid. Released granules from this cell may be responsible for the characteristic granular nature of the axial organ.

Cell Type 7 is glass adherent and phagocytic (Figure 12). It differs from Type 2 in size, measuring up to 60 μm across when spread. This cell is seldomly seen in the axial organ (1.67 ± .09%) and appears to be the phagocytic coelomocyte.

The last axial organ cell subpopulation is comprised of large cells similar in appearance to cell type 5, but greater in size (Figure 13). These cells were initially (in Experiment 1) categorized with cell type 5, but their size and appearance are similar to a glass adherent cell (Type 2) without pseudopodia. At 12-15 μm in diameter, cell Type 8 has been given a separate category which accounts for about 4% of the total population.

A summary of the cell type characteristics is given in Table 2.

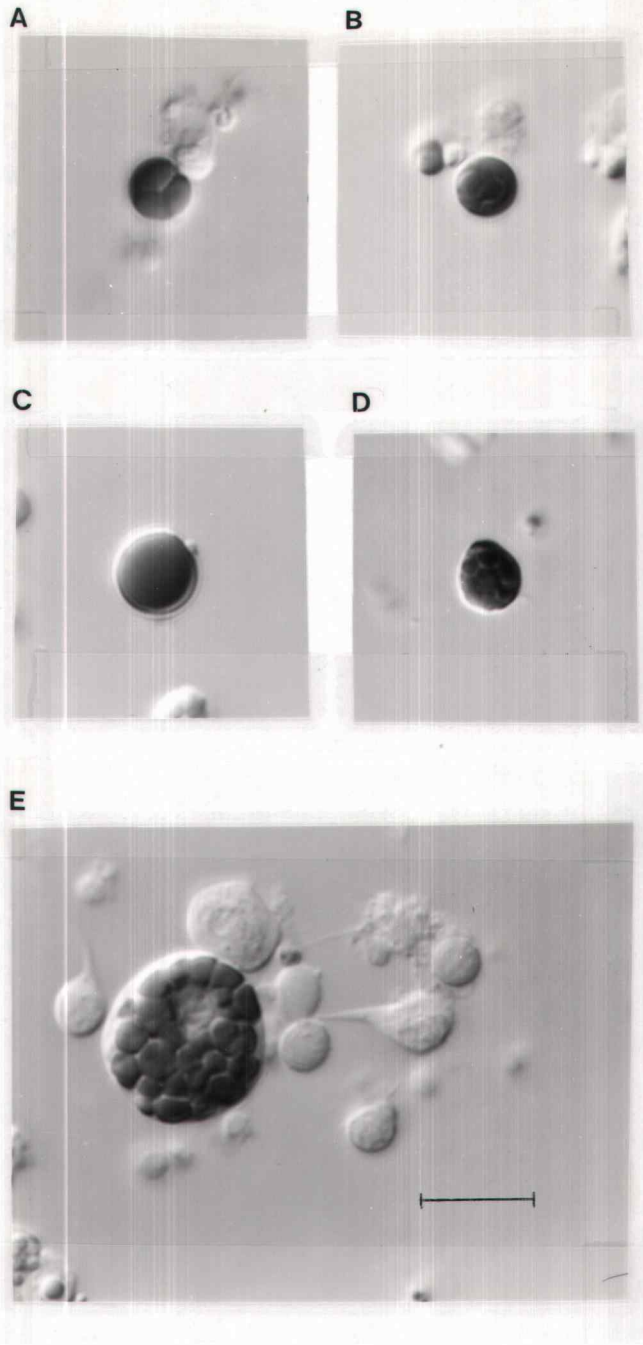

FIGURE 7
Various forms of Cell Type 3 from the axial organ. Cells vary in color and granulation. Non-granulated cells are usually dark brown in color (C) while the large, heavily granulated ones (E) are red and look identical to the red spherule cells found in urchin coelomic fluid. Moderate granulation and intermediate color is characteristic of most type 3 cells (A,B,D). These may be developing red spherule cells. Nomarski, x1000. Scale bar represents 10 μm.

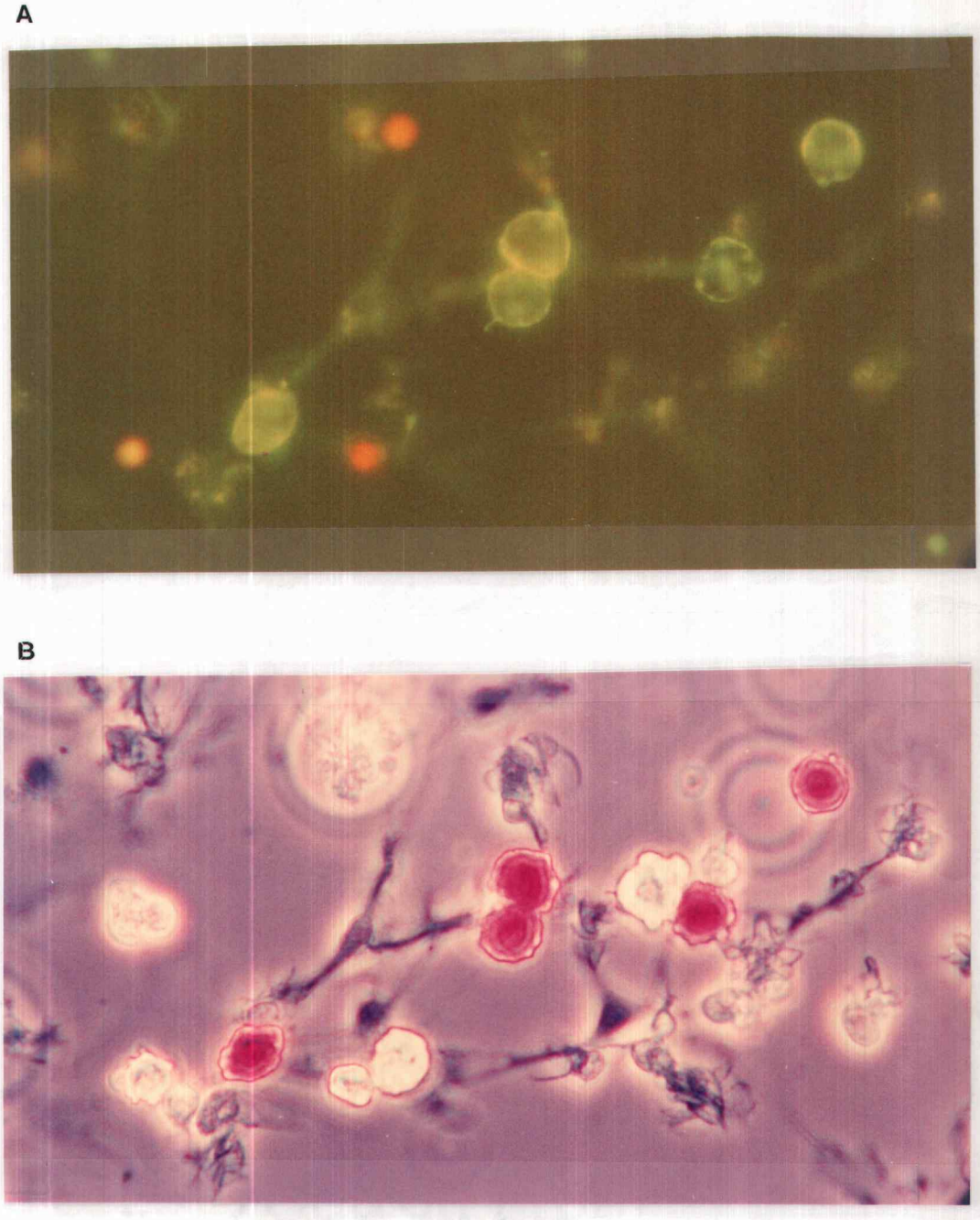

FIGURE 8
A) Red spherule cells from coelomic fluid labeled with FITC-Potato lectin. B) Same cells under phase optics. x400.

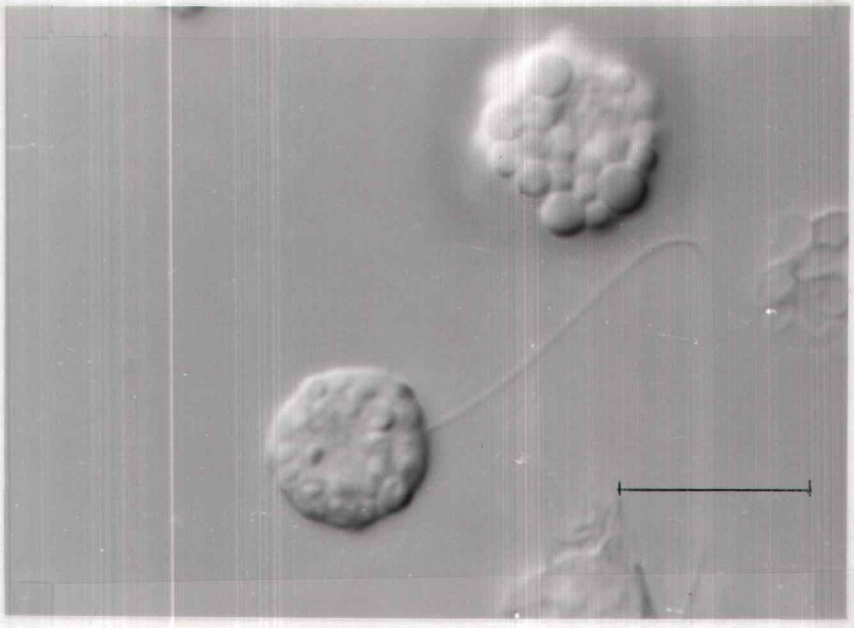

FIGURE 9
Cell Type 4 is granulated and highly motile. The outstanding feature of this group is the flagellum. This cell is seldomly seen in the axial organ, and is identical to the vibratile cell found in the coelomic fluid of the urchin. Nomarski, x1000. Scale bar represents 10 μm.

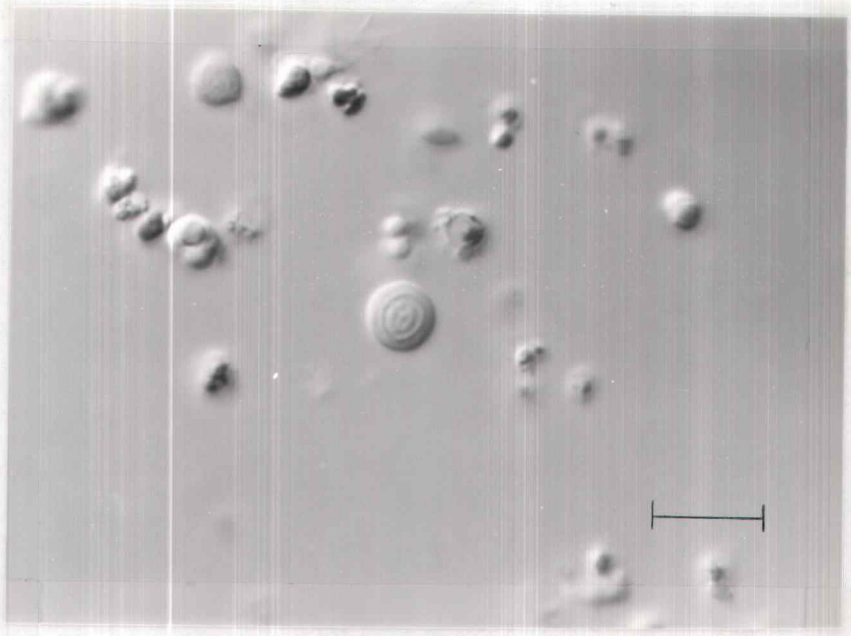

FIGURE 10
These small cells have a high nuclear to cytoplasmic ratio and are the third most numerous cell in the urchin axial organ. Classified as Type 5, they are not found in the coelomic fluid. Nomarski, x1000. Scale bar represents 10 μm.

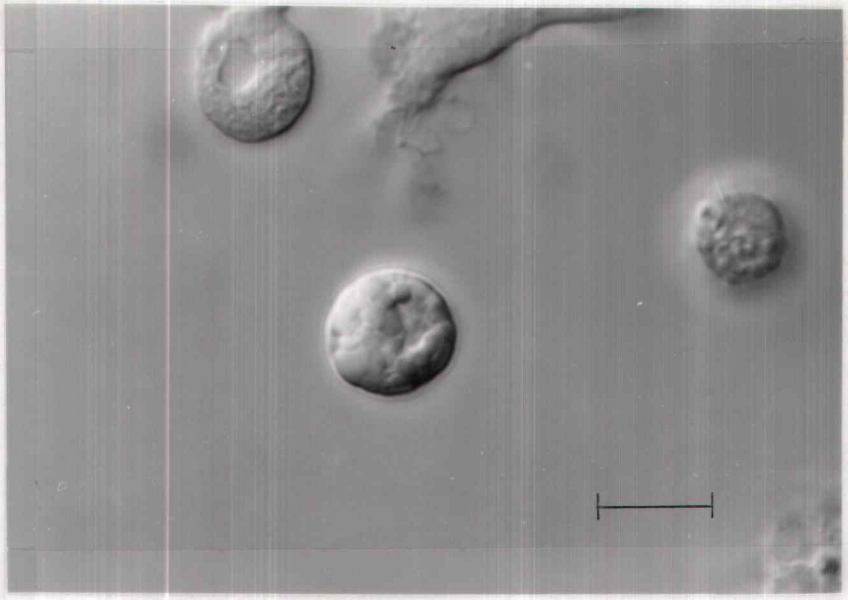

FIGURE 11
Cell Type 6 is highly granulated and similar in appearance to the white spherule cell found in the coelomic fluid. It occurs infrequently in the axial organ. Nomarski, x1000. Scale bar represents 10 μm.

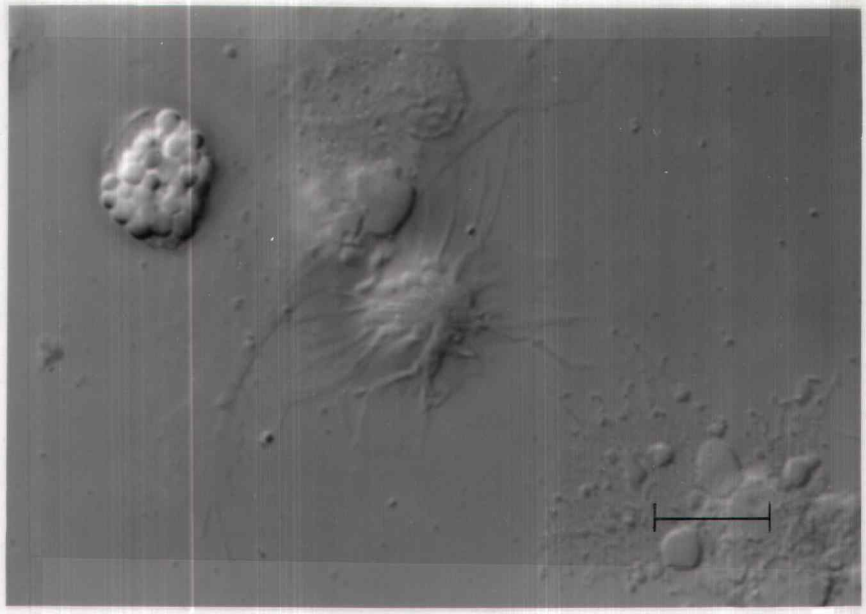

FIGURE 12
This photograph shows a large phagocytic cell characterized as Cell Type 7. These cells are identical to the phagocytes found in the coelomic fluid, and are rare inhabitants of the axial organ. Cells were allowed to spread on the glass coverslip for 45 minutes. Nomarski, x1000. Scale bar represents 10 μm.

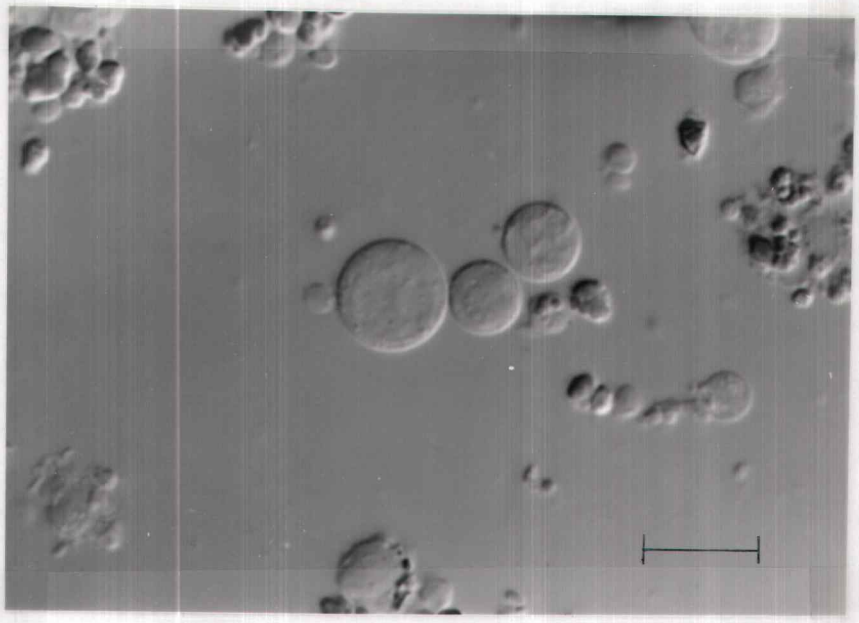

FIGURE 13
Cell type 8 can be seen in this photograph with two "lymphocyte-like" cells (Type 5). The large size of this cell makes it similar to Type 2, although it does not display the pseudopodia characteristic of that type. Nomarski, x1000. Scale bar represents 10 μm.

TABLE 2
CHARACTERISTICS OF CELL SUBPOPULATIONS
ISOLATED FROM *STRONGYLOCENTROTUS PURPURATUS* AXIAL ORGANS

TYPE	SIZE (μm)	MORPHOLOGY	MOBILITY
1	20	bipolar	none observed
2	15-20	pseudopodia	amoeboid
3 a	15	highly granulated, spherical	amoeboid
b	7-10	little to no granulation, spherical	none observed
4	6-8	granulated, spherical	flagellated
5	5	spherical	none observed
6	10	highly granulated, spherical	amoeboid
7	30-60	pseudopodia	amoeboid
8	12-15	spherical	none observed

Axial organ cells from *S. purpuratus* were observed under phase microscopy and classified according to morphological characteristics.

Size is defined as the diameter for spherical cells, and the sum of cell-body-diameter and length of pseudopodia for spread cells.

TABLE 2 (continued)
CHARACTERISTICS OF CELL SUBPOPULATIONS
ISOLATED FROM *STRONGYLOCENTROTUS PURPURATUS* AXIAL ORGANS

TYPE	N/C	LECTIN BINDING	FEATURES	PERCENT OF TOTAL
1	1/2	WGA, PNA, Tet	not glass adherent	33 ± 2.6 %
2	1/4-1/3	WGA	glass adherent, phagocytic	32 ± 2.8
3 a	1/5	Potato, WGA	similar to Red Spherule*	8.6 ± 3.5 (3a and 3b)
b	nucleus not visible	none	orange → brown	included above
4	2/3	none	similar to Vibratile*	1.5 ± .79
5	4/5	WGA	lymphocyte like	15.0 ± 1.4
6	1/5-1/4	WGA	similar to White Spherule*	2.6 ± 1.0
7	1/6	all lectins internalized	similar to Phagocyte*	1.67 ± .09
8	nucleus not visible	not tested	may be nonspread type 2	3.8 ± 1.1

Nuclear to cytoplasmic ratios (N/C) were determined from cytospun cell preparations stained with Leishmann Giemsa. Cell type 8 was not observed in the cytospun preparations. The pigmentation of Cell Type 3b prevented observations of the nucleus.
Cell percentages are expressed as the average of twelve non-injected control animals.

*Refers to coelomocyte subpopulations (see Smith, 1981)

QUANTITATIVE CHANGES IN THE CELL SUBPOPULATIONS

Analyses of the axial organ cell subpopulations of control animals from all experiments did not reveal seasonal variations in relative abundance. Nor were any correlations found between urchin size (grams) or length of captivity and percentages of cell types. As a consequence, analysis of cell types was performed independent of those variables.

Four experimental designs were used to test the effects of antigenic challenge on subpopulations of axial organ cells. The experiments tested a variety of antigen types, doses, methods of administration, and sample periods. In the body of the thesis, data from only one cell type are reported; the remainder of the graphs can be viewed in Appendix B.

Experiment 1

The antigen, bovine serum albumin (BSA), was injected at a dose of 1 milligram per animal. Axial organs were examined at 5, 7, 9, and 11 days after one injection. The sampling times were based on work done by Leclerc and colleagues (Brillouet, *et al.*, 1984). Figure 14 presents the data for the glass adherent cell Type 2. Differences between animals injected with BSA and those injected with Instant Ocean were not significant when sample days were analyzed separately. In Figure 15, the data from all sample days have been combined. The purpose of this analysis was to increase the sample sizes within experimental and control groups. The combined data were analyzed for differences in the means and were examined for general trends in variance and

distribution. The difference between the mean values for BSA versus Instant Ocean injected animals was significant at the $p = .009$ level. This was the only cell type which was altered in Experiment 1.

PERCENT OF AXIAL ORGAN CELLS REPRESENTED BY CELL TYPE 2
IN BUFFER-INJECTED AND BSA-INJECTED URCHINS,
GRAPHED AGAINST SAMPLE DAY.

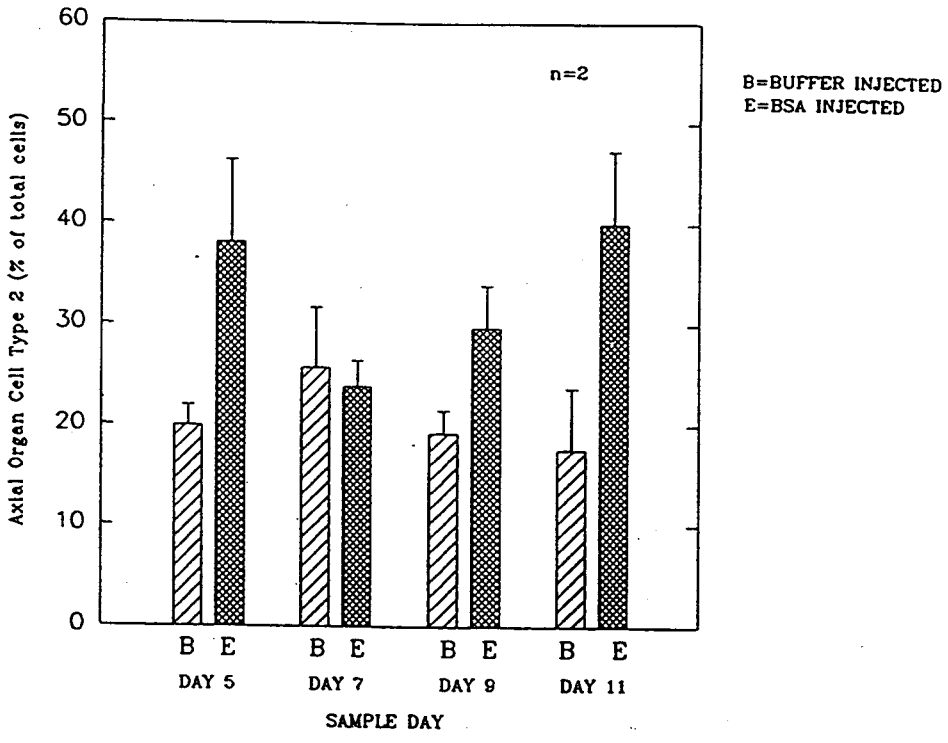

FIGURE 14
This graph displays data from two urchins at each of four sampling days. The urchins were injected once with BSA in Instant Ocean (E) or Instant Ocean alone (B). The axial organs were removed on the days indicated and cells were classified and counted. This graph depicts the data for Cell Type 2. Cell counts are expressed as percent of total cell population. The differences in the means between BSA injected and buffer injected animals was not significant on any sample day. (Student's t test.)

PERCENT OF AXIAL ORGAN CELLS REPRESENTED BY CELL TYPE 2
IN BUFFER-INJECTED AND BSA-INJECTED URCHINS.

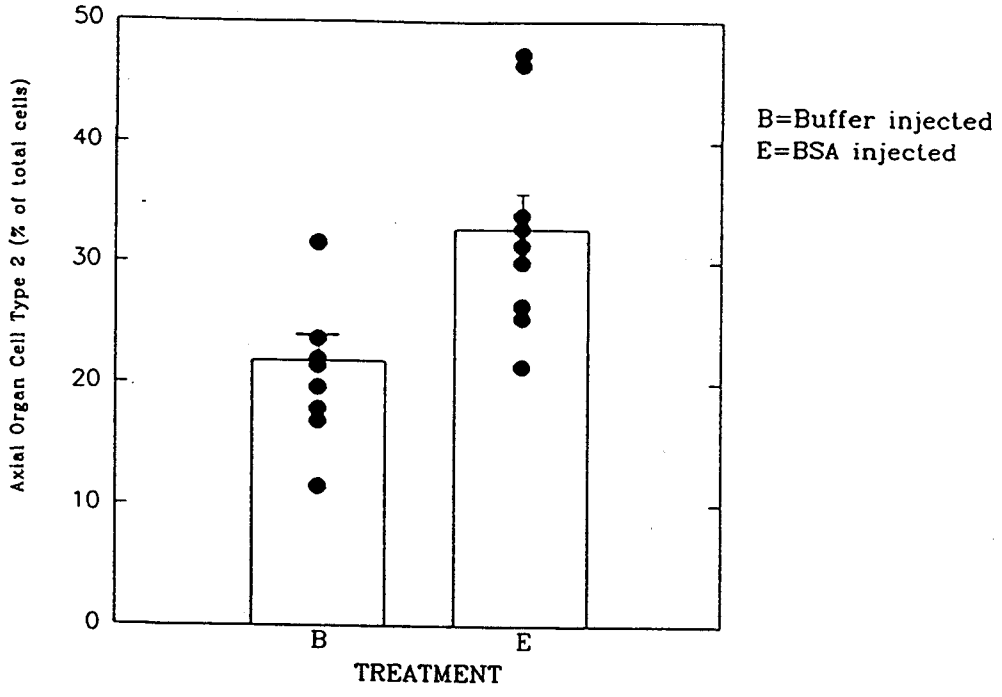

FIGURE 15
The data from the four individual sample days (displayed in Figure 14) have been combined in this graph. Axial organ Cell Type 2 percents are compared between BSA (E) and Instant Ocean (B) injected animals. The difference in the means is significant at p=.009 (Student's t test)

The three other experiments were analyzed in a similar manner. Experiment 2 results for axial organ cell type 2 can be seen in Figure 16. Keyhole limpet hemocyanin (KLH) was injected in Freund's Incomplete Adjuvant (FICA). This experiment included an FICA/Instant Ocean control group and a non-injected group. Three injections (described specifically in Materials and Methods) were followed by samplings between 28-57 days after the final injection. When sample days were analyzed separately, differences in the mean percent of cell type 2 were not significant. Figure 16 depicts the combined data from all sample days. Increasing numbers of cell type 2 can be seen in the KLH injected animals, but differences in the means of the treatment groups were not significant. Cell types 1 and 3-8 also were not altered significantly with respect to control groups.

Experiment 3 was designed to more closely approach natural conditions of antigen exposure. Urchins were immersed in a sea water suspension of KLH adsorbed onto bentonite, and subsequently injected with the same antigen. This type of immunological challenge has been used in fish (Egidius, 1979) and oysters (Hardy, *et al.*, 1977) and can enhance immune responses. Control urchins were either not exposed, or exposed to bentonite in Instant Ocean. Figure 17 depicts the results for cell type 2. No differences among the means of experimental and control animals can be seen for cell type 2 and none were found for any of the other cell types in the axial organ.

Animals in experiment 4 received six injections of KLH in FICA at two week intervals and were sampled 7 weeks after the final injection. Figure 18 shows results for cell type 2. The non-injected control animals display an unusually small variation in percent of the type 2 cell subpopulation, such that it appears

as if both the buffer (FICA/buffer) and protein (KLH in FICA) injections lead to higher variation. But the variation within experimental animals did not differ from that found in experiments 1-3. No differences can be seen in the means among treatment groups for any cell type.

PERCENT OF AXIAL ORGAN CELLS REPRESENTED BY CELL TYPE 2
IN NON-INJECTED, FICA-INJECTED, AND KLH-INJECTED URCHINS.

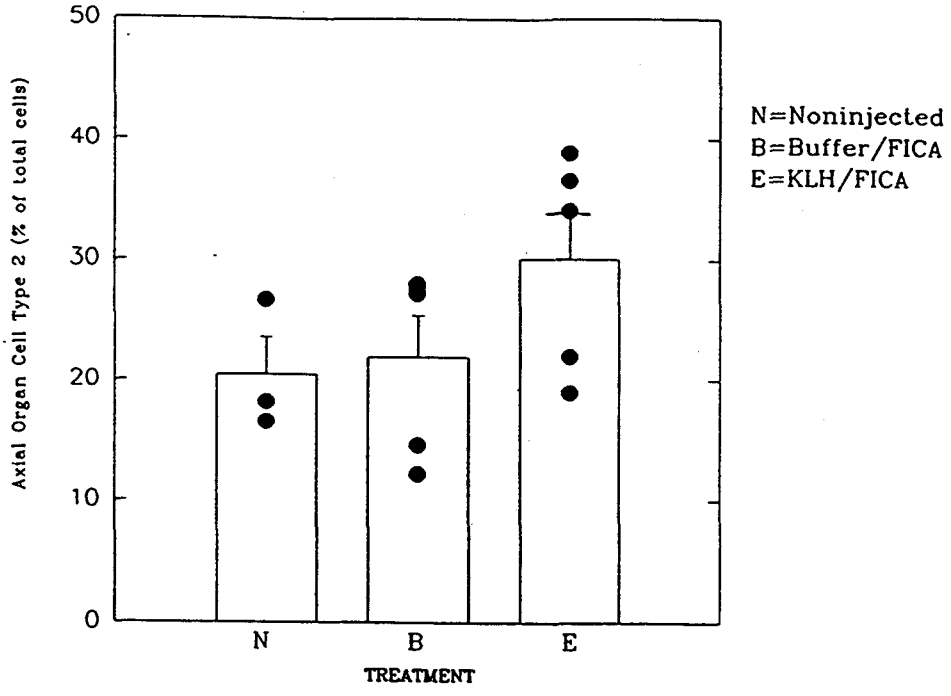

FIGURE 16
Animals in this experiment received three injections of KLH emulsified in FICA (E), or Instant Ocean emulsified with FICA (B). A third group was not injected (N). Axial organs were removed and cell percentages quantified. This graph depicts the data from Cell Type 2. The differences in the means were not significant.

PERCENT OF AXIAL ORGAN CELLS REPRESENTED BY CELL TYPE 2
IN NON-INJECTED, BENTONITE-INJECTED,
AND KLH/BENTONITE-INJECTED URCHINS.

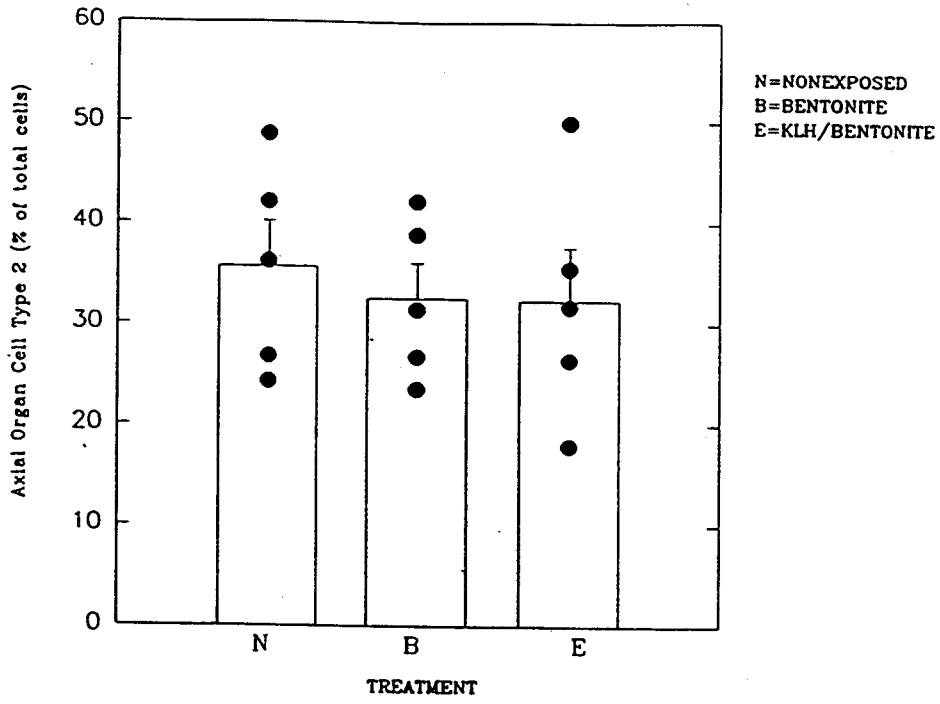

FIGURE 17
Urchins in the experimental treatment group were exposed to KLH adsorbed onto bentonite (E) while control urchins received either plain bentonite (B) or were not exposed. The method of exposure was a combination of immersion and injection.(For exposure details see Materials and Methods.) Data for cell type 2 are plotted and differences in the means were not significant.

PERCENT OF AXIAL ORGAN CELLS REPRESENTED BY CELL TYPE 2 IN NON-INJECTED, FICA-INJECTED, AND KLH-INJECTED URCHINS.

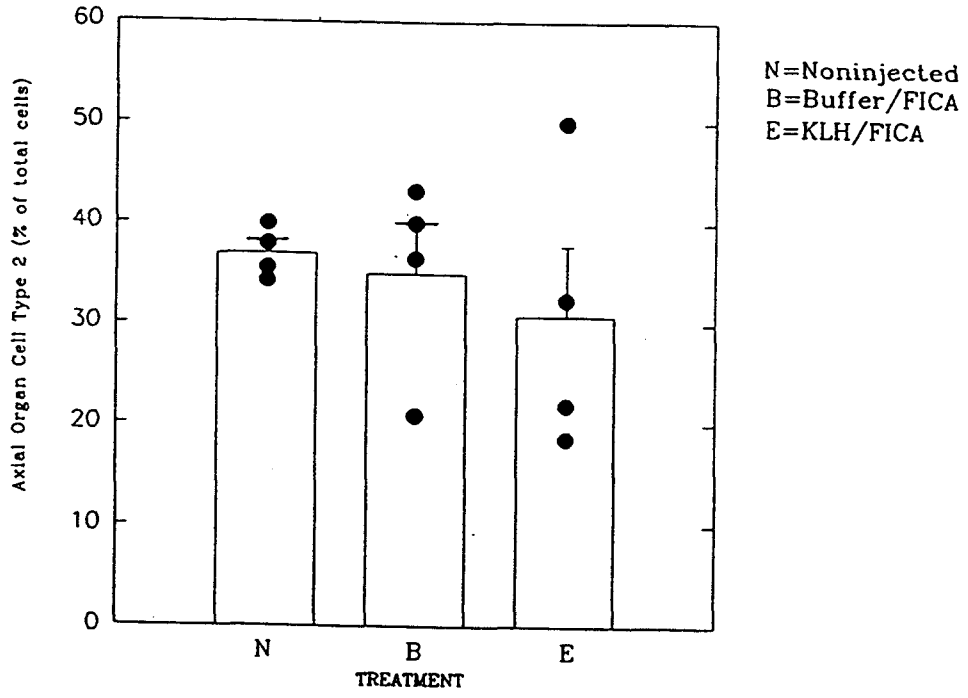

FIGURE 18
Animals in this experiment received extended exposure to KLH resulting from six injections followed by a seven week period before sampling. Animals were injected with KLH in FICA (E), FICA (B), or remained non-injected (N). Differences in the mean percent for cell type 2 were not significant.

DISCUSSION

In an attempt to determine the role of the axial organ in echinoid immunity, cells of the *Strongylocentrotus purpuratus* axial organ were characterized morphologically, and quantitative changes in cell subpopulations were monitored following antigenic challenge.

Eight subpopulations were identified. Four of the cell types are similar to the free cells found in the urchin coelomic fluid: the phagocytic cell, the vibratile cell, and the red and white spherule cells. Coelomocytes have been reported previously in the *Sphaerechinus granularis* (Echinoidea) axial organ (Bachmann and Goldschmid,1978). As a whole, cell types 3, 4, 6, and 7 (cells which resemble coelomocytes) accounted for less than 15% of the entire cell population, and no significant changes in the percent of any coelomocyte types was noted when animals were exposed to antigen.

The four other cell types were not apparent in the coelomic fluid. The bipolar cell (cell type 1) and the glass adherent cell (cell type 2) were the most numerous cell types in the axial organ. The bipolar cell identified in *S. purpuratus* has characteristics similar to the podocytes described in *Encidaris sp.* and *Echinometra sp.* The podocytes are distinguished by several cellular processes from which delicate pedicels derive (Welsch and Rehkamper, 1987). Cell type 1 also resembles the epithelial cells described in intact axial organs of *Sphaerechinus sp.* The epithelial cells interdigitate with neighboring cells through long cytoplasmic extensions (Bachmann and Goldschmid, 1978).

Cell type 2, the glass adherent cell, resembles the phagocytic cells described in the axial organ of *Asterias rubens,* although the starfish cell is smaller than the one in *S. purpuratus* (Anteunis, *et al.*, 1985). Cell type 2 is also similar to the phagocytic cell described in the *Sphaerechinus* axial organ, having large phagocytic pseudopodia and elongated, narrow processes (Bachmann and Goldschmid, 1978).

Cell type 5 is a small cell with a high nuclear to cytoplasmic ratio. It accounts for 15% of the total population. Axial organs from *A. rubens* contain similar cells which were described (Leclerc, Brillouet, *et al.*, 1980; Brillouet, *et al.*, 1981; Leclerc, *et al.*, 1988; Bajelan, *et al.*, 1990) as having B- and T-like qualities based on characteristics presented in Table 1. Holothurian coelomic fluid contains small progenitor cells which may also be similar to the cell described here (Smith, 1981). Without ultrastructural analysis, it is difficult to confirm the similarities between cell type 5 and either of these types from other echinoderm classes.

Cell type 8 resembled cell type 5 in shape, but was more of the size of cell type 2. In some preparations, only a few spread type 2 cells could be located. Instead there was an abundance of type 8 cells. This suggests that perhaps cell type 8 is a nonspread stage of cell type 2. More work is needed to clarify this.

In four experiments several types and doses of antigens, as well as different exposure methods and sample days, were used. Only one cell type showed a significant change during treatment. Cell type 2 increased significantly in BSA-injected animals over buffer-exposed animals. This difference was not evident when sample days were graphed separately. Caution must be used in the

interpretation of these data since experiment 1 did not include a non-injected control group. In experiment 3, a similar trend was evident between buffer and protein injected animals for cell type 1, but the significance of these data was lessened by a non-injected control group whose values fell between the buffer- and protein-injected values. Appendix B contains the cell type data not presented in this chapter.

TABLE 3
TOTAL NUMBERS OF CELLS IN AXIAL ORGAN CELL PREPARATIONS:
RESULTS FROM THREE EXPERIMENTS

EXPERIMENT	NON-INJECTED	BUFFER-INJECTED	PROTEIN-INJECTED
5	21.3 ± 11.9 (n=6)	21.6 ± 7.1 (n=5)	17.6 ± 11.8 (n=6)
6	5.7 ± 3.5 (n=8)	2.9 ± 1.2 (n=8)	4.6 ±3.2 (n=8)
7	5.9 ± 1.9 (n=7)	7.0 ± 2.3 (n=7)	5.9 ± 2.4 (n=7)

Total numbers of axial organ cells from three experiments are presented. Each count is a multiple of 1×10^{6} cells. The experiments contained a nonexposed group, a buffer exposed group and a protein exposed group. Axial organs cell suspensions were prepared and cells were enumerated on a hemacytometer. When treatment groups are compared within an experiment, no differences in the cell number exist. When total cell number is compared among experiments, differences do exist. This suggests a group effect which may be related to time of collection or conditions during captivity. Details of the experimental designs for these experiments are presented in Chapter 3.

Since cell numbers within specific cell types are expressed as percents of the total population, it is important to monitor the total number of axial organ cells among treatment groups. In a second set of experiments (see Chapter 3), the overall number of axial organ cells was monitored following antigenic challenge. Table 4 presents the data from the three experiments. Since the antigens and sample times differed from those in the experiments presented here, it is impossible to draw definitive conclusions. It should be noted, however,

that within experiments the total number of cells is consistent among treatment groups. This was not the case among experiments.

An interesting discovery from the work presented in this chapter is the presence, in the urchin axial organ, of colored cells which differ from red spherule cells found in the coelomic fluid. Classified with the red spherule cells in cell type 3, these smaller cells are less granulated and vary in color from red to orange to brown. It could not be determined morphologically if these cells were developing or degrading red spherule cells, or a different cell type altogether. Future experiments could include granule content analysis of the small colored cells. Cellular components which distinguish coelomocyte subpopulations have been isolated. For instance, red spherule cells contain echinochrome a, which has bacteriostatic effects (Service and Wardlaw, 1984). Melanin and its precursors have been found in the holothurian, *Thyone briareus* (Millott, 1950), and an enzyme system capable of synthesizing melanin has been found in the spherule cells of *Diadema antillarum* (Söderhäll and Smith, 1986). Characterization of granule chemistry could reveal the relationship between coelomocytes and the colored cells found in the axial organ.

The hypothesis that axial organ cell populations would change in response to antigenic challenge was not supported by the data obtained in this set of experiments. There are several reasons which could account for this. First, the identification of cell types was based primarily on morphological characteristics. Each cell type identified could potentially contain more than one subpopulation which may differ in cell membrane receptors, or ultrastructure. For instance, small lymphocyte-like cells with high nuclear to cytoplasmic ratios, separated

from the axial organs of starfish, were separated into subpopulations by Leclerc and colleagues based on differences in their surface carbohydrates. Microscopically, the distinction between soy bean agglutinable cells and nonagglutinable cells was impossible to make. Lectins used for the characterization of cell membranes revealed the existence of two subpopulations. In vertebrates, a similar situation is seen in the T lymphocyte population. Surface receptors such as CD4 and CD8 are markers for distinct subpopulations of helper cells and cytotoxic cells. The axial organ of *S. purpuratus* could be responding to the antigens by the differentiation of cell types within the organ, which would not necessarily be detected by the criteria used here. Further work using lectins, histochemistry, and ultrastructural analysis could reveal more subtle changes in the cells populating this organ.

Although several experimental designs were followed, the doses and types of antigens may have been unsuitable for *S. purpuratus*. It is also possible that the kinetics of any response were such that detectable changes were not seen at the times of sampling.

Larger sample sizes for each experiment may also have increased the significance of the differences among treatments. The variation in percent of cell types between animals appears to be extremely large and this creates a statistical dilemma which may be corrected by larger sample sizes.

Since the cell types of the axial organ of *S. purpuratus* have been morphologically characterized, it would be worthwhile and interesting to broaden the scope of this research to investigate the topographic relationships between the cell types identified here. Sectioned preparations of the axial organ stained

with fluorescently labeled lectins identified in this work as probes for distinct cell types could enhance knowledge of the role each cell plays in the intact organ.

CHAPTER 3

QUALITATIVE CHANGES IN THE AXIAL ORGAN CELLS AND COELOMIC FLUID COMPONENTS OF *STRONGYLOCENTROTUS PURPURATUS* FOLLOWING ENCOUNTER WITH ANTIGEN

INTRODUCTION

Investigations in asteroids (Leclerc, *et al*, 1980) and echinoids (Millott, 1969) suggest axial organ involvement in echinoderm host defense. The organ's central location in the coelomic cavity and its abililty to interact with the fluid filled chambers in the coelom are consistent with suggestions of a role in immunity.

To test the hypothesis that the axial organ functions in echinoid immunity, investigations presented in Chapter 2 focussed on quantitative changes in axial organ cell subpopulations in response to antigenic challenge. No significant or consistent modifications in relative proportions of cell types were detected in the axial organ of *Strongylocentrotus purpuratus* following a variety of antigen

exposures. These results were surprising considering the work done by Millott which described axial organ swelling in response to injury. Perhaps axial organ modifications could not be detected by quantifying cell subpopulations. It is possible that changes in axial organ cells are qualitative in nature.

The work on *Asterias rubens* (Leclerc, *et al*, 1980) suggests that axial organ cells respond to immune challenge in a manner similar to vertebrate lymphocytes. Subpopulations of axial organ cells from starfish injected with either bovine serum albumin or whale myoglobin bound fluorescent conjugates of those proteins at a higher level than alternative proteins. In other work (Brillouet, *et al.*, 1984), complete axial organ cell populations were reported to produce antibody-like molecules in response to antigenic exposure. The cells were exposed both *in vivo* and *in vitro* to trinitrophenylated polyacrylamide beads (TNP-PAA), and the antibody-like molecule was detected in both the cell lysate and culture supernatant, suggesting a secretory nature. Functional homology to vertebrate antibody was shown in a hemolytic assay, in which TNP-sRBC's, incubated with axial organ cells or axial organ cell culture supernatant, were lysed in the presence of mammalian complement components (Brillouet, *et al.*, 1984). The antibody-like molecule was purified and characterized by SDS-PAGE as a tetrameric protein, made up of 30 kDa polypeptide chains lacking disulfide bonds (Delmotte, *et al.*,1986). These authors suggested that echinoderm axial organ immune function is reminiscent of the immune system of the vertebrates.

The idea that a specific recognition system capable of adaptive responses to antigen exists in an invertebrate phylum is intriguing yet controversial.

Ancestors of echinoderms and chordates are very likely related, and a common "ancestral" immune system may have evolved into the echinoderm and vertebrate immune systems which exist today. Thus, the echinoderms are important subjects in any examination of the origins of the vertebrate immune system.

HYPOTHESIS

Based on the work performed by Leclerc and colleagues, I hypothesized that, in response to antigenic challenge, axial organ cells from *Strongylocentrotus purpuratus* would produce molecules capable of specific antigen recognition. I tested this hypothesis by exposing sea urchins to a variety of antigens and assaying both the axial organ cells and the coelomic fluid for the production of specific antigen-binding molecules.

The assays used in this set of experiments were carefully chosen for their sensitivity and appropriateness to marine invertebrates. Phylogenetically distant sources of defense molecules, such as complement, were not used, and care was taken that experiments were performed at temperatures and osmolarities physiological for the animals. Assays were validated with cells and serum from immunized rainbow trout. These fish are known to produce adaptive immune responses to the antigens tested (Arkoosh, 1989). Trout provided suitable models for antigen exposure, assay development, and data interpretation.

Two assays were used to detect responses. In the first, immunocytoadherence, particulate antigen carriers are bound by receptor-

bearing cells, such as lymphocytes. Cells bearing membrane receptors for the antigen form "rosettes", typically a lymphocyte bound by five or more antigen labeled particulates. Antigen specific molecules secreted by plasma cells agglutinate the particulates and form complexes consisting of a lymphocyte surrounded by several layers of cross-linked particulates. Binding specificity can be demonstrated by inhibition with free antigen. This technique is used successfully in vertebrate sytems and can be easily modified for use with invertebrate cells.

The second protocol utilized is the enzyme-linked immunosorbent assay (ELISA). It can be used as an extremely sensitive immunoassay to detect and quantify serum antibody levels in vertebrates. Its sensitivity is advantageous for circumstances which limit available serum quantities and for animals whose antibody titers are minimal. These qualities prompted use of the ELISA in the testing of *S. purpuratus* coelomic fluid for the presence of molecules specific for an injected antigen.

The ELISA requires a multi-well plate whose surface is coated with the antigen of interest. The serum/coelomic fluid is added and allowed to interact with the antigen under physiological conditions. Unbound molecules are removed by extensive washing at each step of the assay. Molecules from the serum/coelomic fluid which have bound the coated antigen are detected by a primary antibody. A secondary antibody can be used to amplify the signal. An enzyme is attached to the antibody and, in a final step, a substrate whose colored product can be detected spectrophotometrically is added. An outline of

the ELISA can be seen in Figure 19. With this basic ELISA design, titers can be determined and molecule specificity demonstrated.

This chapter presents data from rainbow trout, *Oncorhynchus mykiss*, and urchins, *Strongylocentrotus purpuratus*. Splenic lymphocytes from trout immunized with TNP-KLH (trinitrophenylated keyhole limpet hemocyanin) validated the immunocytoadherence assay, and immunized trout sera were used in the development of the ELISA protocol. Axial organ cells and coelomic fluid from urchins exposed to an identical antigen were then assayed. In addition, two other groups of urchins injected with different forms of the TNP antigen were tested.

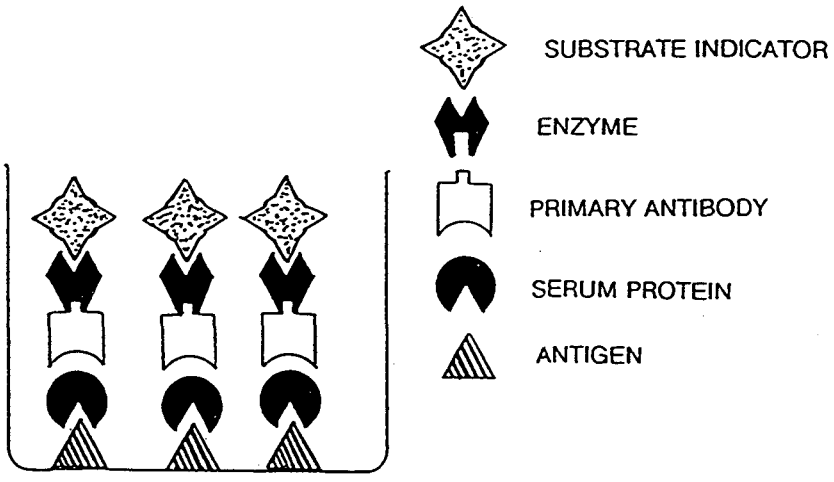

FIGURE 19
The general outline for the detection of serum antibodies using an ELISA. The well of an ELISA plate is initially incubated with antigen diluted in coating buffer. The antigen which has not bound the well's surface is removed with extensive washing. The exposed surfaces of the well are blocked with an unrelated protein. The serum or fluid believed to contain specific molecules against the antigen is added to the well. At every step, unbound molecules are washed away. The serum molecules which have bound the antigen are detected with a primary antibody to which enzyme molecules are attached. Finally, a substrate solution which contains a chromagen is added. The chromagen is measured spectrophotometrically.

MATERIALS AND METHODS

ANIMALS

Sea urchins (*Strongylocentrotus purpuratus)* averaging 136 ± 4 grams were collected year-round at Boiler Bay, Oregon. The animals were housed for two weeks in an open sea water aquarium at the Hatfield Marine Science Center, Newport Oregon. Healthy individuals were transferred to Oregon State University and maintained in 15°C circulating sea water. Urchins were fed *Laminaria sp.* supplemented with green leaf lettuce *ad libitum*.

Thirty rainbow trout (*Oncorhynchus mykiss*) averaging 200 grams each were supplied by Richard Ewing of the Oregon Department of Fish and Wildlife. The trout were maintained in 1.7 m diameter tanks equipped with flow through well water at 12-14 °C and were fed Oregon Moist Pellet.

Six New Zealand White rabbits were supplied by the Laboratory Animal Resource Center at Oregon State University.

ANTIGEN PREPARATION

The method for preparing trinitrophenylated-keyhole limpet hemocyanin (TNP-KLH) was modified from Rittenberg and Amkraut (1966). Briefly, 210 mg of KLH (Sigma, H 2133) was added to 20 ml ddH2O and was gently rocked for 30 minutes at room temperature. After the KLH had dissolved, 5 ml of 5X cacodylate buffer (see Appendix A) was added for a final concentration of 0.28 Molar. The tube was wrapped in foil and a solution containing 43.2 mg trinitrobenzenesulfonic acid (TNBS) in 6 ml 0.28 M cacodylate buffer was added dropwise to the KLH solution. The mixture was rotated for 3 hours at room temperature. Dialysis against three 1 liter volumes of Phosphate Buffered Saline (PBS) (see Appendix A) was done at 4° C over a two day period. The conjugate was filter sterilized and stored at 4° C in sterile siliconized Eppendorf tubes.

A particulate form of TNP-KLH was prepared by adding 1 mg of TNP-KLH to 1 ml of a sterile 100 μg/ml suspension of bentonite in Instant Ocean. The suspension was rotated for 1 hour at room temperature.

Trinitrophenylated-lipopolysaccharide (TNP-LPS) was prepared as described by Jacobs and Morrison (1975). Briefly, 50 mg of *E. coli* lipopolysaccharide (Sigma, L 2880) was dissolved in 2.5 ml of 0.28 M cacodylate buffer and adjusted to pH 11.5. A solution of 30 mg picrylsulfonic acid in 2.5 ml 0.28 M cacodylate buffer was added dropwise to a test tube containing the LPS solution. The tube, wrapped in foil, was rotated for 2 hours at room temperature. The conjugate was then dialyzed against three 1 liter volumes of PBS at 4° C

over a two day period and pasteurized at 70° C for thirty minutes. The TNP-LPS was stored at 4° C in sterile siliconized eppendorf tubes.

Trinitrophenylated bovine serum albumin (TNP-BSA) was prepared by dissolving 30 mg BSA in 5 ml cacodylate buffer. A solution of 125 mg TNBS in 2.5 ml cacodylate buffer, (see Appendix A) was added dropwise to the foil-covered test tube containing the BSA solution. The mixture was rotated for 2 hours at room temperature. The conjugate was separated from the TNP by extensive dialysis against three 1 liter volumes of PBS at 4° C over a two day period. The conjugate was stored at 4° C.

Trinitrophenylated glycine was prepared by dissolving 30 mg glycine in 1 ml of cacodylate buffer. A solution of 100 mg TNBS in 2 ml cacodylate buffer was added dropwise to the foil-covered tube containing the glycine solution. The mixture was rotated for 2 hours at room temperature. Due to the similar molecular weights of the reactants and products, dialysis was not performed and the conjugate in cacodylate buffer was diluted upon addition to the immunocytoadherence assay. The conjugate was stored at 4° C.

Trinitrophenylated sheep red blood cells (TNP-SRBC) were prepared according to the method of Rittenberg and Pratt (1969). Sheep red blood cells were washed three times in 1X Modified Barbital Buffer (MBB) (See Appendix A), with centrifugation of 400xg for 10 minutes between washings. To 500 μl packed sRBC's, 200 μl 5% TNBS in 3.3 ml 0.28 M cacodylate buffer was added. In a foil-covered tube, the mixture was rotated for 20 minutes at room temperature. Centrifugation at 400xg was followed by washing in 5 mM

glycylglycine in 1X MBB. Washings continued in 1X MBB until the supernatant was clear. TNP-SRBC were stored in MBB at 4° C for not longer than a week.

ANESTHETIZING TROUT

Rainbow trout were anesthetized in a 50mg/L ethyl aminobenzoate (benzocaine) solution (Summerfelt and Smith, 1990). Due to low solubility in water, a 10% stock solution of benzocaine was prepared in absolute ethanol. Five milliliters of the stock solution was added to 10 liters of water. Fish placed in the anesthetic were removed as soon as they could be handled easily. Care was taken to avoid exposing the trout to lethal doses of benzocaine.

ANTIGEN EXPOSURE

Urchins were injected through the peristomial membrane using a 26 gauge, ½ inch needle. Three antigens used were: soluble trinitrophenylated keyhole limpet hemocyanin (TNP-KLH), TNP-KLH adsorbed onto bentonite, and trinitrophenylated lipopolysaccharide (TNP-LPS). The soluble antigens, TNP-KLH and TNP-LPS, were emulsified in Freund's Complete Adjuvant, FCA, (Sigma, F 4528). The final concentration of each antigen was 1μg/1μl. Experimental urchins received injections of 100 μl of antigen. Control urchins received either 100 μl of Freund's complete adjuvant emulsified with an equal volume of sterile Instant Ocean, 100 μl sterile bentonite in Instant Ocean, or were non-injected. Urchins were sampled at nine or ten weeks for the presence

of specific antigen-binding molecules in the coelomic fluid as well as on axial organ cells.

Anesthetized trout were injected intraperitoneally with 100 μg TNP-KLH emulsified in an equal volume of Freund's Complete Adjuvant (FCA). The final injected volume was 0.1 ml. Control fish received injections of Phosphate Buffered Saline (PBS) in complete adjuvant. The fish were sacrificed nine to ten weeks after injection at which time serum was collected for antibody detection and spleens were removed for enumeration of specific antigen-binding cells.

COELOMIC FLUID AND SERA COLLECTION

Coelomic fluid was removed from urchins through a puncture in the peristomial membrane. For coelomocyte counts the coelomic fluid was pulled into a syringe containing an equal volume of anticoagulant (Bertheussen, 1978) (see Appendix A). When cell free coelomic fluid was required, no anticoagulant was used and cells were allowed to aggregate. Within 30 minutes of collection, the cellular aggregates were removed by centrifugation at 250 x g for 10 minutes. Cell-free coelomic fluid was stored at -70° C.

Blood was collected from anesthetized trout from the caudal vessels through a lateral penetration from a position just ventral to the lateral line. The blood was allowed to clot for 1 hour at room temperature. The clot was rimmed and allowed to constrict overnight at 4° C. The clot was removed by centrifugation

at 400 x g for 10 minutes, followed by a second spinning of the supernatant to remove residual cells. The sera were stored at -70° C.

AXIAL ORGAN AND SPLEEN REMOVAL

Axial organs were removed aseptically through incisions in the aboral portion of the sea urchin tests. The axial organs were rinsed extensively in sterile chilled RPMI-1640 adjusted to urchin osmolarity (See Appendix A). Cell suspensions of individual axial organs were made by gently pushing the organ through sterile 75 μm porous nylon netting (Tetko, # 3-75/45) with a sterile syringe bulb. Cold, sterile Urchin RPMI-1640 was used to wash the cells through the netting. All glassware was siliconized (Sigmacote, Sigma) and sterilized. Since the cell populations were heterogeneous in both size and fragility, the cells were not centrifuged after separation from the organ. Cells were enumerated by using a hemacytometer, and viability was determined with propidium iodide (See Chapter 2).

Spleens were removed from fish that had been killed by cerebral concussion. The spleens were washed in sterile RPMI-1640 and were massaged through 100 μm netting in a manner similar to that used on the axial organs. Each cell suspension was washed once by centrifugation at 400 x g for 10 minutes at 4° C. After adjusting the volume to 2 ml, the cell suspension was layered onto 10 ml of a Percoll (Sigma, P 1644) (62% in RPMI-1640) cushion and spun for 30 minutes at 400 x g to separate the red blood cells from the

splenic leukocytes. The top of the gradient was removed and washed three more times in fish media. Cells were enumerated using a hemacytometer.

IMMUNOCYTOADHERENCE

Estimates of the number of antibody-producing cells in trout spleens were made according to Ruben, *et al.* (1981). A 50 μl aliquot of splenic leukocytes (4-5 x 10^6 cells/ml) from rainbow trout were exposed to sheep red blood cells which had been previously labeled with the injected antigen, TNP. The volume of red blood cells was 25 μl and the number was adjusted so that the final ratio of red blood cells to splenic leukocytes would be 5:1. The mixture was incubated overnight at 17° C and the antibody-producing cells were distinguished microscopically as fish cells surrounded by at least 5 sRBC's. The rosettes were enumerated by using a hemacytometer, and values are expressed as the number of rosettes per number of leukocytes. To determine specificity, 50 μg of TNP-glycine was added to the leukocytes as an inhibitor for 1.5 hrs at 17° C before the addition of the sRBC's. Another set of control leukocytes were exposed to glycine alone. A third control set included exposure of leukocytes to unlabeled sRBC's. Urchin axial organ cells were analyzed for immunocytoadherence in a similar manner.

DETERMINATION OF ANTIBODY ACTIVITY

Fish serum was assayed for the presence of antibodies against the hapten, TNP, by using an enzyme-linked immunosorbent assay (ELISA). Wells of 96-well plates (Costar, EIA/RIA #3590) were coated with 100 μl of 200 ng/ml TNP-BSA in coating buffer (see Appendix A) for 1 hour at 37° C. The wells were then blocked with 300 μl 1% BSA in Tween Tris Buffered Saline (TTBS) (see Appendix A) for 1 hour at 37° C and washed three times with TTBS followed by three washes with Tris Buffered Saline (TBS). One hundred microliters of a 1:1000 dilution of individual trout serum in blocking buffer (1% BSA in TTBS) was incubated in each well for one hour. Five washings with TTBS and five washes with TBS followed each incubation. All immunological probes were added in 100 μl aliquots. The fish serum was detected in one of two ways. Either a biotinylated monoclonal antibody (developed by Gregory Warr, and provided by Steve Kaattari) against fish immunoglobulin was added at 1:2000 dilution in blocking buffer for 1.5 hrs at room temperature, or the fish serum was detected by the addition of 1:10,000 dilution of rabbit anti-rainbow trout serum (a generous gift from Nora Demers) for 1.5 hrs at room temperature, followed by 1:1000 dilution of biotinlyated goat anti-rabbit antibody for one hour at room temperature. After the addition of one of the two biotinylated probes, a 1:1000 dilution of avidin peroxidase was added for 1 hr at room temperature. The substrate, a solution of 2,2-Azino-bis(3-Ethylbenzthiazoline-6-sulfonic acid) (ABTS) and hydrogen peroxide in a citrate buffer (see Appendix A) was then added, and optical density readings at 420 nm were initiated immediately and

continued at 5 minute intervals for 45 minutes. The increases in optical density over time were graphed and reaction rates for each well were calculated. A single optical density reading for each well was then used to evaluate antibody concentration (end-point analysis). The optical densities chosen were measured while reaction rates remained linear.

Antibody titers in immunized animals and nonimmunized controls were compared by an ELISA using serial dilutions of pooled trout serum. This method was described by Arkoosh and Kaattari (1990). Briefly, it consists of estimating the maximum absorbance of the immunized sera, and determining 1/2 max. The amount of serum needed to produce 1/2 max is determined from the immunized trout, and compared to the amount of serum required from each control animal to achieve the same level of absorbance. The reciprocal of this value gives an standard measure of titer.

Antibody specificity for TNP was demonstrated by the addition of 50 μl 1 mM TNP-lysine (a gift from Steve Kaattari) in TTBS to 50 μl of a 1:500 dilution of sera 30 minutes before addition to the ELISA plate. Control wells received either sera preincubated with lysine alone or only TTBS.

Coelomic fluid was analyzed for the presence of molecules that specifically bound the hapten TNP. An ELISA protocol was modified from that used for the trout. Wells of a 96-well ELISA plate were coated with 20 ng TNP-BSA, BSA, or LPS in 100 μl coating buffer (see Appendix A). The coating antigen was incubated in the wells at 37° C for 1 hour. The contents of the wells were removed and 300 μl of 1% BSA in TTBS (Blocking Buffer) was added as a block. This was incubated for 1 hr at 37°C. The wells were then washed 3 times

with TTBS followed by washes with TBS. All subsequent immunochemical additions were made in volumes of 100 μl and all incubations were followed by 5 washes in TTBS and 5 washes in TBS. Coelomic fluid was added at 1:10 in blocking buffer and incubated overnight at 4°C. Rabbit antisera to sea urchin coelomic fluid antigens was added at 1:5000 in blocking buffer and incubated 1.5 hrs at room temperature. Biotinylated goat-anti-rabbit antibody was added at 1:1000 dilution followed by a 1:1000 dilution of avidin peroxidase. The substrate solution (see Appendix A) was added, and spectrophotometric readings were immediately started at 420 nm and were continued at 5 minute intervals for 1 hour. End-point analysis was used to evaluate the concentration of specific antigen-binding molecules for each well.

PRODUCTION OF RABBIT ANTISERUM TO SEA URCHIN ANTIGENS

Three 12-week-old New Zealand White rabbits were used to produce each kind of antiserum. The first antigen consisted of 450 μg protein from sea urchin coelomic fluid and coelomocyte lysate. The antigen was prepared by drawing coelomic fluid into a syringe containing an equal volume of 0.4% Trition X-100. The cell lysate was then filtered through sterile 100 μm nylon netting and mixed with an equal volume of Freund's Complete Adjuvant. The second antigen was prepared from 0.2% Triton X-100 lysed axial organ cells. The protein content, as measured with BCA reagents (Sigma), was 675 μg per rabbit injection. The antigen was emulsified in an equal volume of Freund's Complete Adjuvant.

Each rabbit was prebled from the marginal ear vein and serum was tested

for baseline antibody levels. A total of 1.5 ml of antigen was injected subcutaneously at multiple sites by using a 25 gauge needle. Three weeks later a second series of antigen injections of the same volume and protein concentration was given subcutaneously emulsified in Freund's Incomplete Adjuvant. Two weeks afterward the rabbits were ear bled and antibody titer was estimated. Final blood collection was done five days later by cardiac puncture. The blood was allowed to clot at room temperature for 1 hour. The clot was rimmed and allowed to contract overnight at 4° C. The clot was removed by centrifugation at 10,000 x g for 10 minutes at 4° C. The sera were aliquoted and stored at - 70° C.

RESULTS

IMMUNOCYTOADHERENCE IN TROUT

Oncorhynchus mykiss immunized with trinitrophenylated keyhole limpet hemocyanin (TNP-KLH) in Freund's Complete Adjuvant (FCA) were sampled nine weeks later. Splenic leukocytes from the trout were incubated overnight at 17°C with TNP-labeled sheep red blood cells (TNP-sRBC's), and antibody-producing- cells (APC's) were characterized and enumerated. Figure 20a shows an APC, defined in this system as a trout leukocyte bound by at least five TNP-sRBC's. Due to its appearance, the formation has been described as a rosette. The number of TNP-specific APC's in the spleens of TNP-KLH-injected, adjuvant-injected, and non-injected trout were compared. An average of 17,000 APC's per 1 x 10^6 leukocytes were estimated in the TNP-KLH-injected trout. Significantly fewer APC's were seen in control animals. Adjuvant-injected animals averaged 1,500 APC's per 1 x 10^6 splenic leukocytes and no rosettes were seen in non-injected animals. Specificity of antibody binding was shown by inhibition with free TNP-glycine. Following overnight incubation at 17°C, agglutinated TNP-sRBC's were also noted in addition to rosettes. The agglutination of TNP-sRBC's was TNP-glycine inhibitable (Figure 20d and 20e.) The number of agglutinated TNP-sRBC's was reduced if the cells were examined after only 3 hours at 17°C. This suggests that the antibody produced by the splenic leukocytes was secreted

and cross-linked the TNP-sRBC's. The results of immunocytoadherence in *Onchorynchus* can be seen in Figure 21.

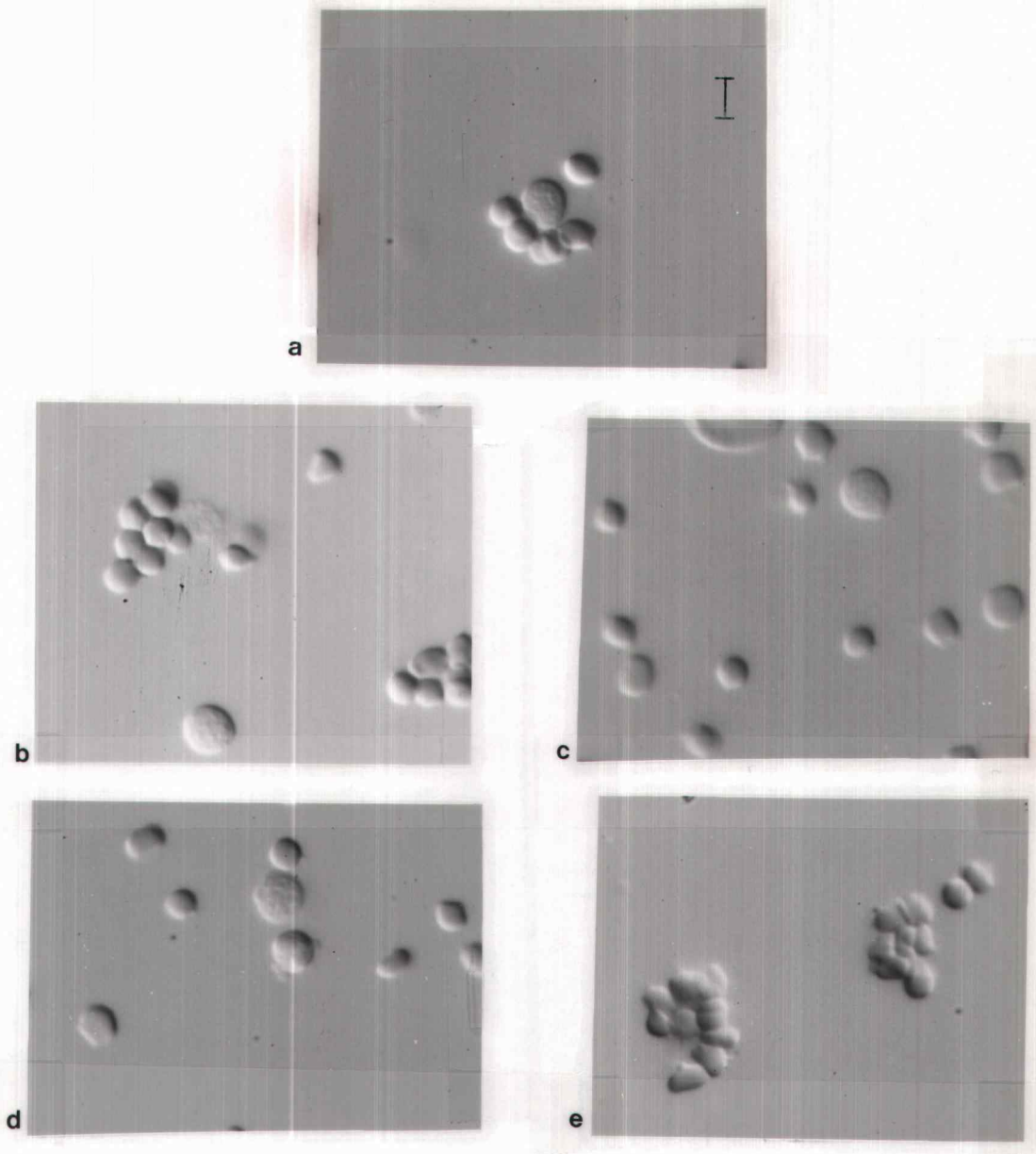

FIGURE 20
Immunocytoadherence with *Oncorhynchus mykiss* splenic leukocytes.
Splenic leukocytes from TNP-KLH-injected trout, and control trout were incubated in the presence of TNP-sRBC's. a) A rosette is defined as a leukocyte bound by at least 5 TNP-sRBC's. b) After overnight incubation at 17°C, TNP-sRBC's incubated with leukocytes from TNP-KLH-injected trout formed aggregates. c) This hemagglutination was not seen when control animal leukocytes were used. d) The agglutination was TNP-glycine inhibitable. e) The agglutination could not be inhibited by glycine. These results indicate that the leukocytes from injected trout secreted TNP-specific antibodies. 1000X Nomarski. Scale bar represents 10 μm.

ANTIBODY-PRODUCING CELLS IN SUSPENSIONS OF SPLENIC LEUKOCYTES FROM RAINBOW TROUT.

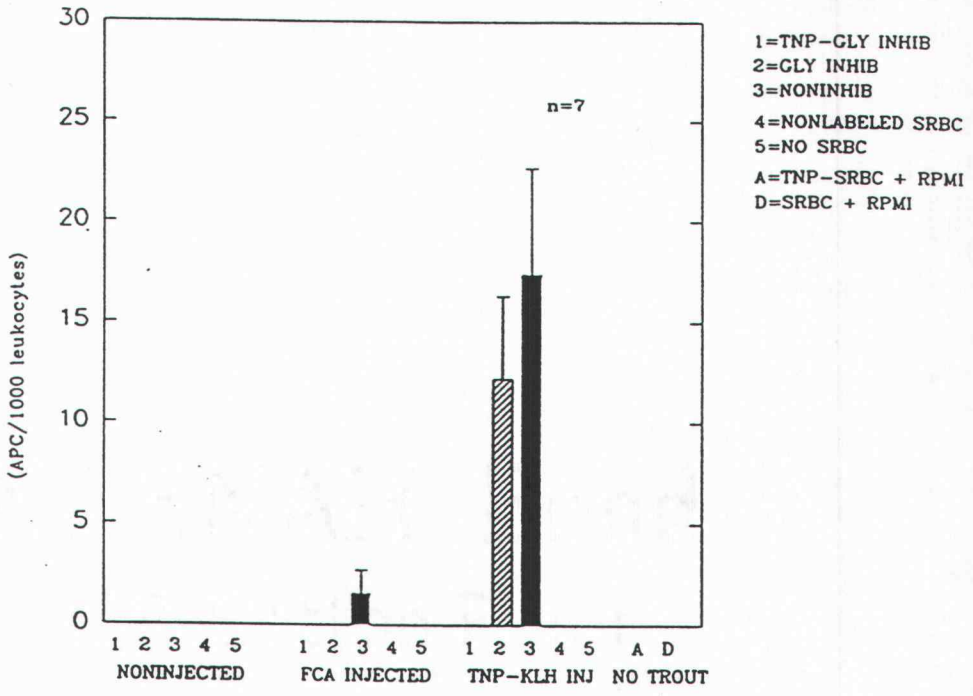

FIGURE 21
This graph presents the immunocytoadherence data from twenty-one trout. Seven animals were in each treatment group. The treatments consisted of TNP-KLH injection (TNP-KLH), adjuvant injection (FCA), and no injection (NON). Splenic leukocytes from these animals were incubated overnight (o.n.) at 17°C under the defined conditions. 1) Preincubation (PI) of leukocytes with TNP-glycine for 1.5 hrs, followed by o.n. with TNP-sRBC's. 2) PI with glycine, followed by o.n. with TNP-sRBC's 3) PI with media, then o.n. with TNP-sRBC's. 4)PI with media, then o.n. with sRBC's. 5) PI with media, no erythrocytes added. Control tubes contained A) TNP-sRBC's and media, D) sRBC's and media. Rosette forming cells in each tube were enumerated and APC's per leukocyte were calculated. The TNP-KLH-injected trout had the largest number of specific APC's.

IMMUNOCYTOADHERENCE IN URCHINS

Axial organ cells, from urchins injected nine weeks earlier with TNP-KLH in FCA, were incubated with TNP-sRBC's in a manner similar to that used for trout. In order to perform the assay in conditions that were physiological for the urchin cells, TNP-sRBC's were slowly adjusted to urchin osmolarity by stepwise washings in increasingly higher salt concentrations, and were subsequently added to the axial organ cell suspensions. After incubation overnight at 17° C, the cells were examined for APC's. No rosette formations were seen in TNP-KLH-injected groups or in FCA-injected or non-injected control groups.

TNP-KLH adsorbed onto bentonite was injected into another set of urchins nine weeks before sampling. The rationale behind the use of this antigen was to provide a particulate form of TNP-KLH which may have been more efficiently processed by the urchin cells. These animals were examined for the presence of APC's in the axial organ after incubation with TNP-sRBC's. No rosettes were seen in any treatment group. Instead, agglutination of TNP-sRBC's was noted (Figure 22). This phenomenom also occurred in trout leukocyte suspensions and suggested secreted antibody production. Thus, aggregates of more than three TNP-sRBC's were enumerated in the axial organ cell suspensions. Figure 23 depicts agglutination data from urchins injected with TNP-KLH adsorbed onto bentonite, bentonite alone, and non-injected controls. Differences in the mean number of TNP-SRBC aggregates per axial organ cell were not significant between treatment groups. The agglutination was not TNP-glycine-inhibitable.

It does appear that the TNP label on the SRBC was important in the aggluination process since nonlabeled sRBC's were not agglutinated as readily.

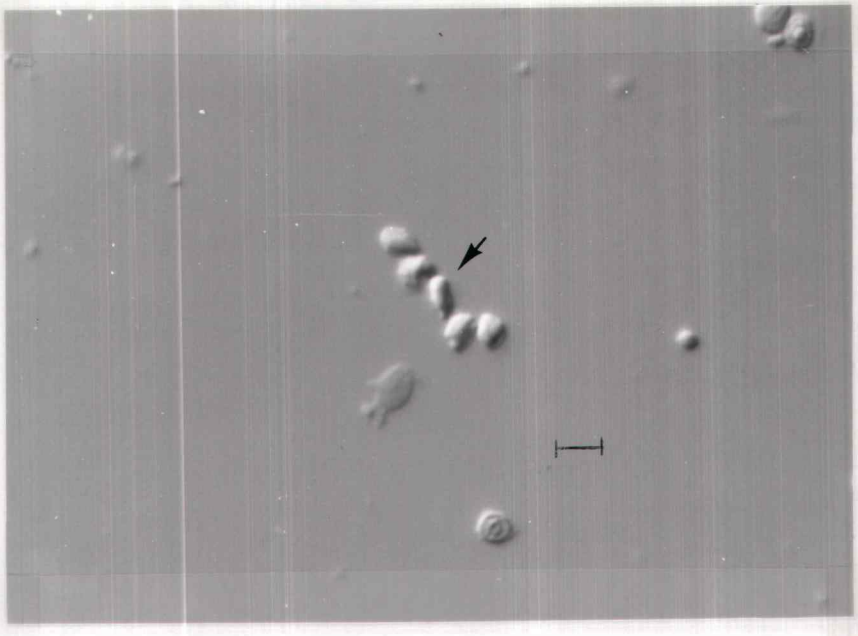

FIGURE 22
Agglutination of TNP-sRBC's occurred after incubation with axial organ cells (arrowhead). This hemagglutination was not TNP-glycine inhibitable and did not depend on previous antigen exposure. Nomarski 1000X. Scale bar represents 10 μm.

AGGLUTINATION OF sRBC's IN SUSPENSIONS WITH AXIAL ORGAN CELLS FROM NON-INJECTED, BENTONITE-INJECTED, AND TNP-KLH/BENTONITE-INJECTED URCHINS.

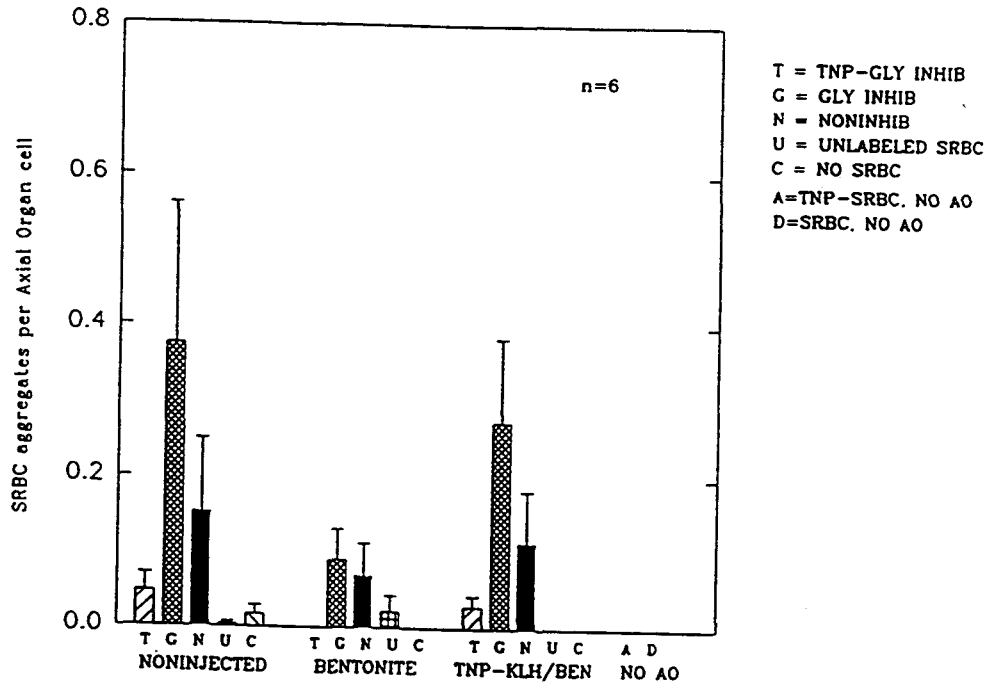

FIGURE 23
Hemagglutination with *S. purpuratus* axial organ cells.
Rosette formations were not detected in axial organ cell suspensions. Agglutination of TNP-sRBC's occurred and was compared among treatment groups. In this experiment the urchins were injected with TNP-KLH adsorbed onto bentonite, bentonite alone, or were non-injected. Axial organ cells from these animals were incubated with sheep red blood cells under the following conditions: T) Preincubation (PI) with TNP-glycine for 1.5 hrs followed by overnight incubation with TNP-sRBC's. G) PI with glycine, followed by o.n. with TNP-sRBC's. N) PI with media followed by o.n. with TNP-sRBC's. U) PI with media, then o.n. with sRBC's. C) PI with media, then o.n. with media. A) TNP-sRBC's incubated with media. D) sRBC's incubated with media. Aggregates composed of more than three cells were enumerated, and numbers were expressed as aggregates per axial organ cell. Differences between the mean number of aggregates were not significant among treatment groups. Agglutination was not specific.

Urchins which received injections of trinitrophenylated lipopolysaccharide (TNP-LPS) in adjuvant (FCA) were scored for immunocytoadherence and this was compared with values for animals injected with adjuvant alone and for non-injected control animals. LPS is known to be a T-independent antigen in vertebrates and does not require the interaction of B and T lymphocyte subpopulations for the generation of an antibody response. Since the presence of interacting subpopulations of axial organ cells has not been established in *S. purpuratus*, an antigen which requires fewer cellular interactions in vertebrates might be an appropriate antigen for a specific immune response in an invertebrate.

Axial organ cells from the TNP-LPS-injected animals did not form rosettes in the presence of TNP-sRBC's, but hemagglutination did occur. There were no significant differences in mean numbers of TNP-SRBC aggregates per axial organ cell among treatment groups. TNP-sRBC's were agglutinated significantly more than nonlabeled sRBC's in all treatment groups and agglutination was not inhibited by free TNP-glycine. Figure 24 depicts the data from this experiment.

AGGLUTINATION OF sRBC's IN SUSPENSIONS WITH AXIAL ORGAN CELLS FROM NON-INJECTED, FCA-INJECTED, AND TNP-LPS-INJECTED URCHINS.

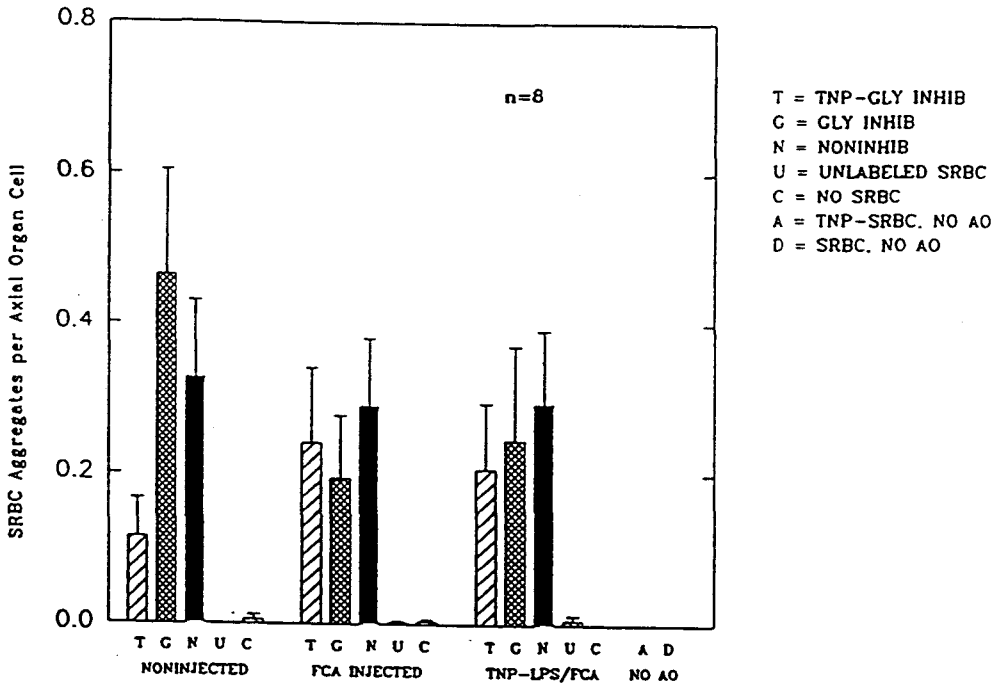

FIGURE 24
Hemagglutination in axial organ cell suspensions from TNP-LPS-injected urchins was compared with hemagglutination in FCA- and non-injected control animals. Axial organ cells were incubated under the same conditions as those given in Figure 24. Differences in the mean number of aggregates per axial organ cell were not significant among treatments, and agglutination was not inhibitable by TNP.

ENZYME-LINKED IMMUNOSORBENT ASSAY USING TROUT SERA

Sera from trout injected with TNP-KLH in FCA were examined for the presence of molecules specifically binding TNP in an ELISA assay. Initially, individual trout sera were examined separately by using two immunological probes. The first probe was a monoclonal antibody to trout immunoglobulin (WARR-mAb). The second, a polyclonal probe, was made in rabbits to general trout serum antigens. The monoclonal confirmed the presence of TNP specific antibodies in the trout sera. The second probe proved that a specific antibody signal could be detected in an ELISA assay using a polyclonal probe. It was important to establish positive results with the use of a polyclonal probe, since the only available primary antibody probes for the urchins were polyclonal sera made in rabbits against general coelomic fluid antigens.

Figures 25a and 25b compare the absorbance patterns for sera from trout injected with TNP-KLH, FCA or non-injected controls. These data represent a set of three individual trout. The rest of the trout data can be viewed in Appendix C. All trout assayed gave similar results. Sera from TNP-KLH-injected trout probed with WARR-mAb has its maximum absorbance in the wells containing TNP (TNP-BSA) (Figure 25a). Since the WARR-mAb detects only trout immunoglobulin, this suggests antibody binding to TNP. This binding is not evident in control animals or in wells containing unrelated proteins. Figure 25b shows the same trout sera probed with the polyclonal sera to rainbow trout. The most striking contrast between Figure 25a and this set of data is the high background levels of binding when the polyclonal is used. This expected result can conceal the specific

antigen-binding signal. The ELISA binding pattern (EBP) for trout sera depicted in Figure 25b suggests specificity in their immune response. The pattern can be seen in TNP-KLH-injected trout where binding to wells containing TNP is higher than to unrelated molecules.

ANTI-TNP ANTIBODIES IN RAINBOW TROUT SERA DETECTED WITH MONOCLONAL SERA AGAINST RAINBOW TROUT IgM.

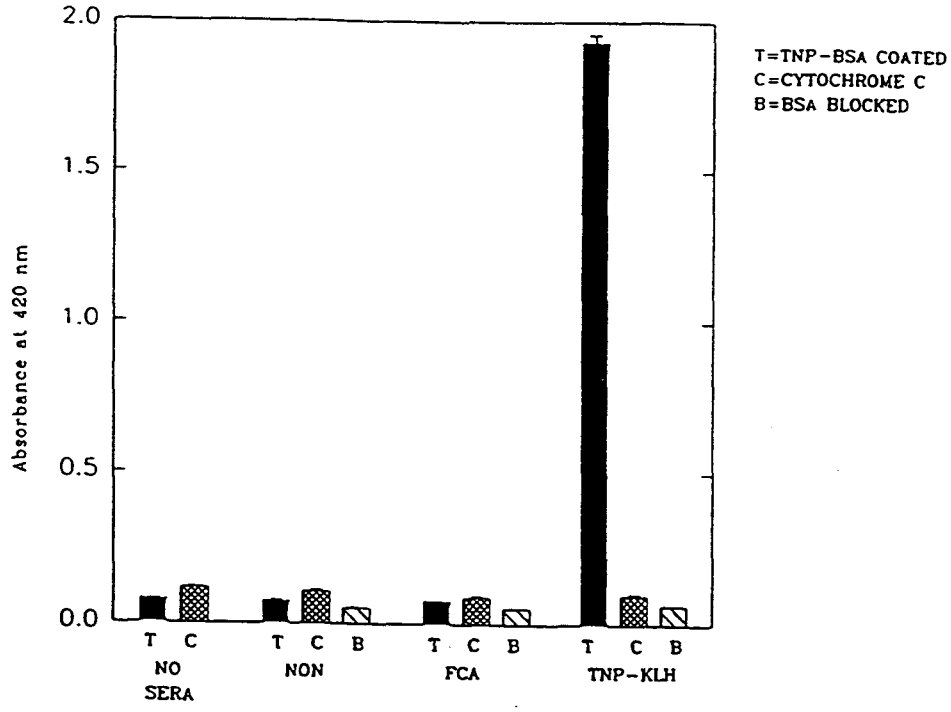

FIGURE 25a
Trout sera binding to TNP-BSA (T), cytochrome c (C), or BSA blocked (B) wells in an ELISA plate were probed with a monoclonal antibody against trout IgM. The data show the presence of specific antibodies to TNP in TNP-KLH-injected trout. This binding does not occur with the sera from adjuvant (FCA) or non-injected animals (NON). Plates were read 15 minutes after substrate addition.

ANTI-TNP ANTIBODIES IN RAINBOW TROUT SERA DETECTED WITH POLYCLONAL SERA AGAINST TROUT SERUM PROTEINS.

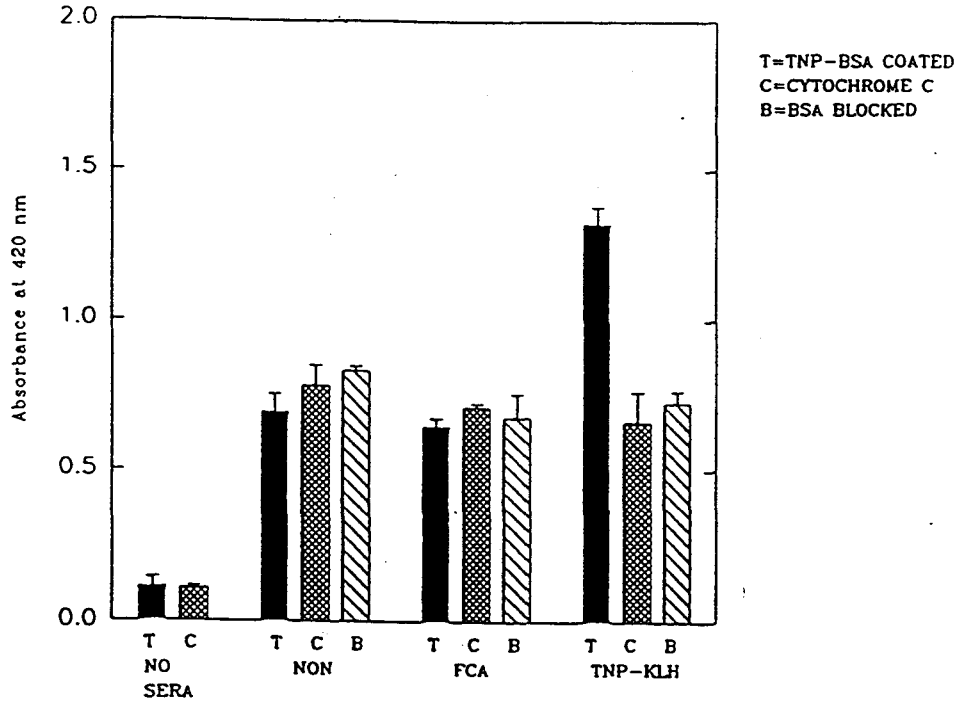

FIGURE 25b
The same trout sera depicted in Figure 26a have been allowed to bind to TNP-BSA (T), cytochrome c (C) or BSA blocked wells (B) and were then detected with polyclonal serum made against general trout serum antigens. The background binding is elevated, but the specific binding signal in the TNP-KLH-injected trout can still be detected. Plates were read 15 minutes after substrate addition.

TABLE 4
TROUT ELISA RESULTS

AG	NON	FCA	TNP	TOT	X^2	p
TNP-KLH	0/8 (2.66)	0/8 (2.66)	8/8 (2.66)	8/24	12.54	.005

Twenty four trout sera were tested individually for the production of TNP-specific binding molecules in an ELISA. The trout belonged to three treatment groups: TNP-KLH-injected (TNP), Adjuvant-injected (FCA), or non-injected (NON). The number of trout exhibiting the ELISA binding pattern (EBP) indicative of the production of a TNP-specific molecule is shown for each treatment group. Below the observed value is an expected value, in parentheses, which was calculated on the premise that the EBP is a random occurrence and does not depend on immunization with TNP. The X^2 value is significant, suggesting that the EBP's in this experiment are dependent on injection with TNP-KLH ($p = .005$).

Table 4 shows trout ELISA data from the polyclonal assays. The data are presented in this format for later comparison with the data from the urchin experiments. Chi-squared estimates for the normal probability of EBP's occurring in any treatment group are provided in parentheses below the observed values. The occurrence of EBP's is significantly different from that which would occur randomly. Thus, the treatment did influence the results seen in these animals.

Further characterization of the trout antibody response to TNP was performed with trout sera pooled from the eight trout in each treatment group. Binding of immune trout serum antibodies to TNP could be inhibited by free TNP-lysine (See Figure 26). This was not observed with the control sera. Titration of pooled trout sera indicated that the antibody levels of TNP-KLH-injected trout were at least 100 times greater than the titers in FCA- and non-injected controls (See Figure 27). The specificity of the antibody response and the elevated titers in immunized animals suggest an adaptive immune response. This would be expected for this group of vertebrates.

INHIBITION OF TROUT-SERUM-BINDING TO TNP-BSA WITH TNP-LYSINE.

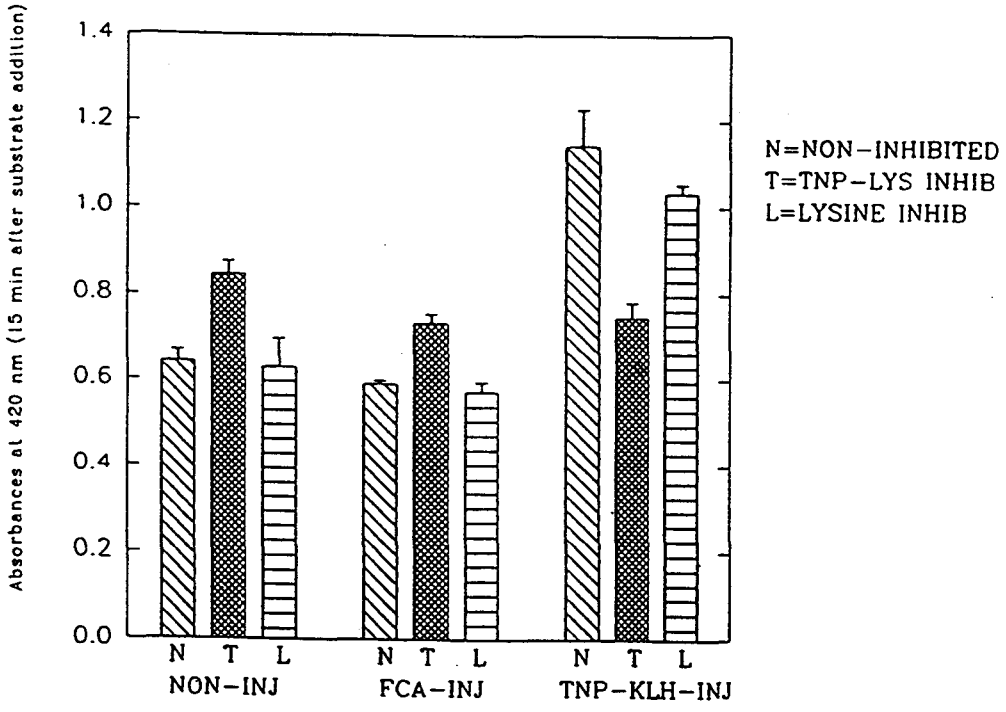

FIGURE 26
The binding of trout serum molecules to TNP-BSA can be inhibited by preincubating the serum with TNP-lysine (T) but not lysine (L) alone. The difference between the noninhibited binding and the TNP-lysine inhibited binding is significant ($p=0.012$). This occurs in the TNP-KLH-injected trout sera but not the adjuvant (FCA) or non-injected (NON) controls.

TITRATIONAL ANALYSIS OF ANTIGEN-BINDING MOLECULES IN POOLED SERA FROM NON-INJECTED, FCA-INJECTED, AND TNP-KLH-INJECTED TROUT.

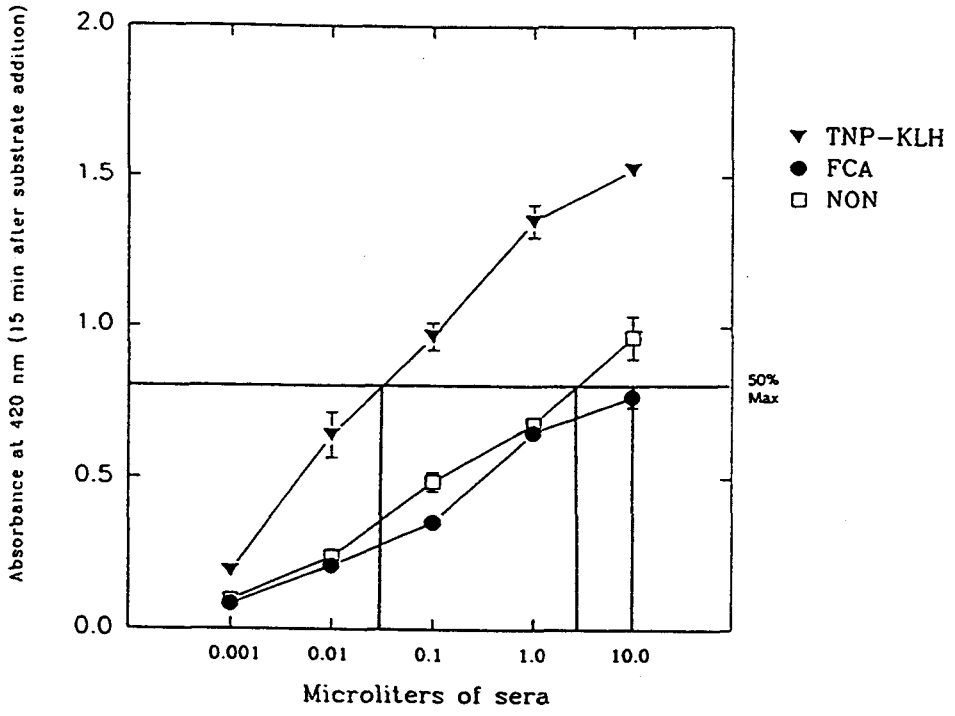

FIGURE 27
The titration curves for pooled trout sera are shown. The binding to TNP-BSA is approximately 100 times higher in TNP-KLH-injected animals than in animals injected with FCA (FCA), or non-injected controls (NON). The line depicting 50% maximum absorbance has been drawn in.

ENZYME-LINKED IMMUNOSORBENT ASSAY USING URCHIN COELOMIC FLUIDS

Urchin coelomic fluids were assayed by an ELISA similar to that used for the trout. Fluids from urchins injected with TNP-KLH in FCA were compared to those from FCA- and non-injected urchins. Eighteen animals, six per treatment group, were tested. An implication of specific binding to TNP was observed in six animals. In these preliminary results, the EBP's were similar to those of the *Oncorhynchus sp.* probed with polyclonal sera. Only one of the six animals had been injected with TNP-KLH. Twelve animals in this experiment did not appear to possess molecules specific for TNP.

Coelomic fluids from urchins injected with TNP-KLH adsorbed onto bentonite were examined for the presence of TNP-specific binding molecules. Thirty animals, 10 per treatment, were assayed. Preliminary results showed four of the thirty animals' coelomic fluids bound TNP at a higher level than BSA. One of these animals had been injected with bentonite, one was non-injected, and two had been injected with TNP-KLH on bentonite.

A third experimental group of urchins received TNP-LPS injections. When their coelomic fluids were examined for TNP-binding ability and the values were compared to those from control urchins, eleven of the twenty-four coelomic fluids tested bound TNP at a significantly higher level than BSA. Of those, seven were from non-injected control animals.

TABLE 5
URCHIN ELISA RESULTS

AG	NON	FCA/ BEN	TNP	TOT	X^2	X^2 if $p \leq .05$
TNP-KLH	3/6 (2)	2/6 (2)	1/6 (2)	6/18	1	5.99
TNP-KLH/ BEN	1/10 (1.3)	1/10 (1.3)	2/10 (1.3)	4/30	1.96	5.99
TNP-LPS	7/8 (3.6)	2/8 (3.6)	2/8 (3.6)	11/24	4.6	5.99

The results of three experiments are summarized in this table. The experiments differed in the type of antigen. All were TNP-haptenated molecules. Seventy-two urchins were divided into treatment groups: non-injected (NON), Adjuvant- or bentonite-injected, (FCA/BEN), or TNP-injected (TNP). Coelomic fluids from these animals were tested for TNP-specific binding molecules in an ELISA assay. The table shows the number of animals in each group whose coelomic fluids exhibited an ELISA binding pattern (EBP) indicative of the presence of TNP-specific molecules. The expected number of animals producing the EBP, based on normal variance, is also shown in parentheses. Since the differences between the observed and expected values are not significant in any of the experiments according to the X^2 test, it can be concluded that the results are not due to treatment effects.

Table 5 depicts the data from all of the urchin ELISA's. The table shows the observed number of urchins in each group which demonstrated the EBP that implies the production of a specific TNP-binding molecule. Expected values have also been calculated and are presented in parentheses below the observed values. For each experiment a chi squared value was calculated. The number of EBP- positive animals does not differ significantly from that which would be expected in a normal population. This implies that the production of molecules specific for TNP was not due to the treatment.

The high number of non-injected animals which exhibited an EBP characteristic of specific molecule production in the TNP-LPS experiment was unusual. Further analysis by ELISA investigated the specificities and titers in one set of urchins, TNP-LPS Set 4. When TNP-BSA-, LPS- and BSA-binding were compared in a dilution series, binding patterns were identical for each molecule (Figure 28). This suggests that the interactions mediated by the coelomic fluid are non-specific. The titer of this nonspecific molecule was five times higher in the control urchin coelomic fluid. Coelomic fluid protein concentrations did not differ significantly among treatment groups (see Appendix C).

TITRATIONAL ANALYSIS OF TNP-BSA-BINDING MOLECULES IN COELOMIC FLUID FROM NON-INJECTED, FCA-INJECTED, AND TNP-LPS-INJECTED URCHINS.

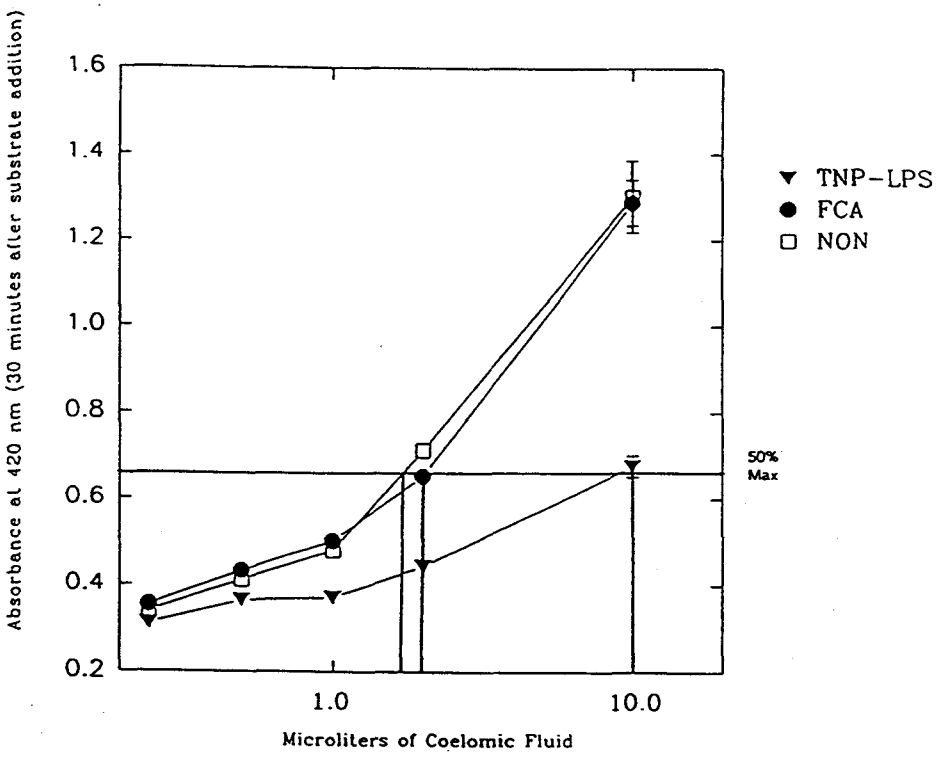

FIGURE 28a
Three titration curves are compared. In each graph, the binding capacity of coelomic fluid from urchins injected with TNP-LPS, adjuvant (FCA) and non-injected controls are compared. The difference between the graphs is the coating antigen used. a) The coating antigen is TNP-BSA. b) Coating antigen is LPS. c) Coating antigen is BSA. Since the levels of binding are nearly identical regardless of the coated antigen, the binding can be considered nonspecific. The line depicting 50% maximum absorbance has been drawn in.

TITRATIONAL ANALYSIS OF LPS-BINDING MOLECULES IN COELOMIC FLUID FROM NON-INJECTED, FCA-INJECTED, AND TNP-LPS-INJECTED URCHINS.

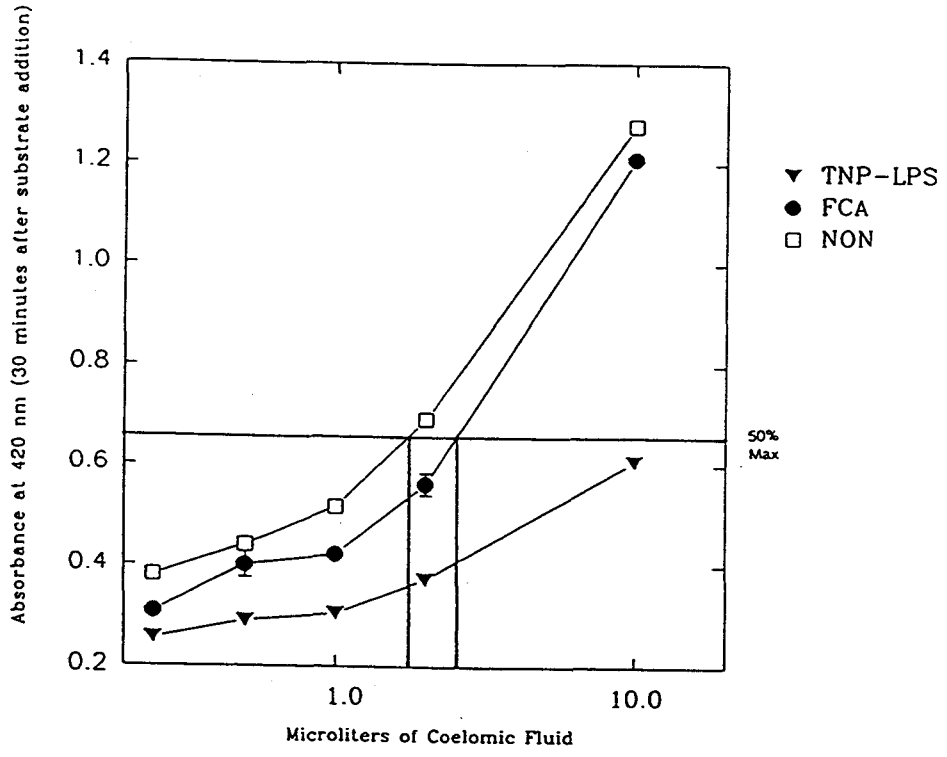

FIGURE 28b
Three titration curves are compared. In each graph, the binding capacity of coelomic fluid from urchins injected with TNP-LPS, adjuvant (FCA) and non-injected controls are compared. The difference between the graphs is the coating antigen used. a) The coating antigen is TNP-BSA. b) Coating antigen is LPS. c) Coating antigen is BSA. Since the levels of binding are nearly identical regardless of the coated antigen, the binding can be considered nonspecific. The line depicting 50% maximum absorbance has been drawn in.

TITRATIONAL ANALYSIS OF BSA-BINDING MOLECULES IN COELOMIC FLUID FROM NON-INJECTED, FCA-INJECTED, AND TNP-LPS-INJECTED URCHINS.

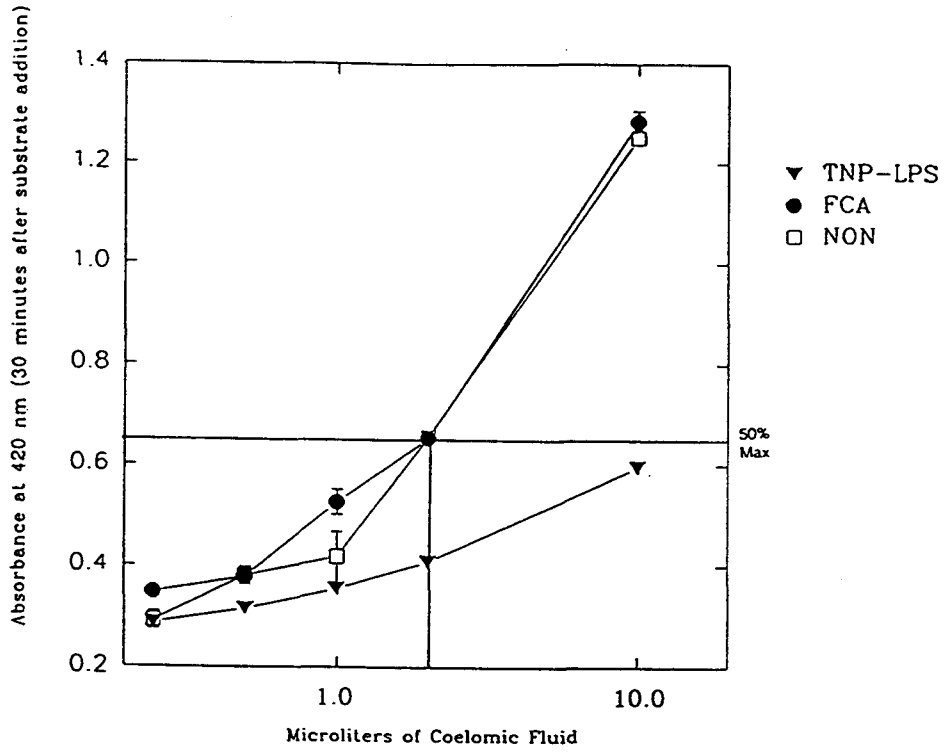

FIGURE 28c
Three titration curves are compared. In each graph, the binding capacity of coelomic fluid from urchins injected with TNP-LPS, adjuvant (FCA) and non-injected controls are compared. The difference between the graphs is the coating antigen used. a) The coating antigen is TNP-BSA. b) Coating antigen is LPS. c) Coating antigen is BSA. Since the levels of binding are nearly identical regardless of the coated antigen, the binding can be considered nonspecific. The line depicting 50% maximum absorbance has been drawn in.

DISCUSSION

Echinoderm axial organ cells have been implicated as mediators of host defense (Leclerc, *et al.*, 1980). However, the suggestion that axial ogan cells produce specific antigen-binding molecules in response to antigenic challenge (Brillouett, 1984) remains controversial. In the experiments reported in this chapter, axial organ cells from TNP-injected *Strongylocentrotus purpuratus* were probed for TNP-specific receptors. Coelomic fluids from the same animals were assayed for the presence of secreted TNP-specific molecules. Immunocytes and immune sera from *Onchorynchus mykiss* confirmed that an adaptive immune response could be detected with the chosen assays.

Splenic leukocytes from all eight injected rainbow trout secreted antibody molecules specific for TNP. This was evidenced by TNP-SRBC agglutination which was inhibitable by TNP-glycine. Splenic leukocytes from seven of the eight animals were seen to form rosettes with TNP-sRBC's. This response is characteristic of antibody producing cells (APC's). Only 2 of the eight FCA-injected animals had rosette forming cells, and none of the non-injected controls showed evidence of APC's.

Evidence of specific membrane receptors for TNP was not discovered in *S. purpuratus*: TNP-sRBC's did not bind axial organ cells in the characteristic rosette formation. On the other hand, hemagglutination was observed, but it was neither TNP-glycine inhibitable nor dependent upon previous antigen exposure.

Non-labeled sRBC's, which experienced the same labeling procedure without the addition of TNP, were not agglutinated as readily as the labeled erythrocytes. This suggests that the TNP molecule facilitates the agglutination of the erythrocytes in the presence of axial organ cells. This could have occurred if the binding of the TNP hapten to proteins in the erythrocyte cell membrane altered protein configuration, thus revealing epitopes which were not exposed on the surfaces of the unlabeled sRBC's.

Serum antibody specific for TNP was detected in all of the TNP-KLH-injected trout. It was detected strongly by the WARR-mAb to trout IgM, and was also evident when the primary antibody was polyclonal. Control animals lacked the specific response to TNP measured by this ELISA.

Urchin coelomic fluid was analyzed for the presence of specific TNP-binding molecules in a protocol modified from the trout ELISA's. The modifications were aimed at increasing the sensitivity of the assay, and included an increase in concentration of the coelomic fluid to be tested, an increase in the incubation time for the coelomic fluid step to allow maximum interactions between the coelomic fluid and the coating antigens, and removal of EDTA from the washing buffer and diluent, since it is not known whether calcium plays a role in any specific binding.

The number of urchins whose coelomic fluid exhibited the ELISA binding pattern (EBP) indicating specific binding to TNP fell within the range of normal variation. This is in direct contrast to the trout. In addition, one set of urchin fluids were further analyzed and did not confirm the specific EBP when titrated

against TNP, LPS and BSA. The identical binding patterns to all three of these molecules strongly suggests a non-specific interaction.

The data presented do not support the hypothesis that echinoid axial organ cells respond to antigenic challenge in a specific, inducible manner. There are several reasons why TNP-specific recognition molecules may not have been detected in the ELISA. The titer of the putative receptor may be below the sensitivity of the assay used in this system, and non-specific binding may have overshadowed any specific interactions. In an effort to optimize the physiological conditions of the buffers, as well as decrease the non-specific binding, an ELISA was performed (with TNP-LPS urchin set 4). The blocking BSA was increased three fold and the diluent for the coelomic fluid was osmotically corrected for urchin conditions. This modification in protocol lowered the nonspecific binding, but did not reveal the presence of specific TNP-binding molecules. Further examination of buffering sytems, including the addition of calcium may reveal specific binding.

The lack of specific binding by the axial organ cells in the immunocytoadherence assay may have been due to the condition of the TNP-sRBC's. The mammalian erythrocytes were adjusted to urchin osmolarity in stepwise washings. The sRBC's appeared compromised and TNP molecules may have been hidden from the axial organ cells by the infoldings of the erythrocyte membranes. In future experiments, a synthetic TNP carrier, such as latex or polyacrylamide beads, may prove to be preferable.

The TNP antigen was presented to the urchins in three different forms in these experiments. The first, TNP-KLH, was identical to that used to elicit an

adaptive immune response in trout. The second, TNP-KLH adsorbed onto bentonite, provided a particulate form of TNP which may have been processed more efficiently by the phagocytic cells of the urchins. The third antigen, TNP-LPS, is considered a vertebrate T-cell independent antigen because it does not require the interaction of B and T lymphocytes for the production of antibody. It was hoped that one of the three antigens would initiate a specific immune response in *S. purpuratus*, but this was not seen. Perhaps the urchins were sampled when the immune response was not maximal, and thus, the response could not be detected, or perhaps the urchins do not have the capacity to respond to molecules such as TNP, and natural antigens, such as gram negative bacteria should be used.

CHAPTER 4

GENERAL DISCUSSION

The axial organ lies along the oral-aboral axis of an echinoderm. It is situated at the point where the fluid directing systems of the animal converge. Its irregular contractions and glandular appearance have interested researchers for hundreds of years, yet the function of the organ has not been established. In the experiments presented here, the potential role of the echinoid axial organ in immunity was addressed. Previous work had suggested that axial organs of echinoids (Millott, 1969) and asteroids (Leclerc, *et al.*, 1980) respond to immune challenge. The descriptions of response ranged from general organ swelling following urchin injury, to suggestions that starfish axial organ cell subpopulations include cells resembling vertebrate lymphocytes, both in their responses to mitogens and in their production of antibody-like molecules after exposure to antigen.

The possibilities that this enigmatic organ functions in echinoderm immunity and that the cells of the organ respond to antigenic challenge in a specific manner led to the formation and testing of two hypotheses: I. The axial organ

cell subpopulations in *Strongylocentrotus purpuratus* would change quantitatively in response to antigenic challenge. II. The axial organ cells from *S. purpuratus* would produce molecules capable of specifically binding an injected antigen.

Evaluation of the first hypothesis required the identification of cell subpopulations in the axial organ. Eight cell types were identified based on morphological characteristics. Four types were unique to the axial organ, and four resembled cells found in the coelomic fluid. The variation in percentages of cell types between individual urchins was very high, and no significant changes in subpopulations were evident after antigenic challenges despite the use of several types of antigens, exposure methods and schedules, and sampling times.

The second hypothesis would have been supported by the presence of either antigen-specific cell surface receptors or antigen-specific secreted molecules, so analysis included examination of the axial organ cell membranes as well as coelomic fluid. There was no evidence of cell-bound or secreted, specific antigen receptors in *S. purpuratus*.

There have been reports (from isolated labs) which claim specific immune responses in representatives of several invertebrate phyla (echinoderms-Leclerc, *et al.*, 1980; insects-Karp and Rheins, 1980; 1988; annelids-Laulan, *et al.*, 1985). These authors suggest that the invertebrates studied are capable of adaptive immune responses characteristic of the vertebrates. But their results remain to be independently confirmed by other labs. The data presented in this work are in agreement with the current ideas on the evolution of the vertebrate immune system.

It has been argued that the invertebrates are unable to respond specifically to antigenic challenge (Klein, 1989; Sima and Vetvicka, 1990; Marchalonis and Schluter, 1990; Smith and Davidson, 1992). Several reasons have been proposed. 1) Invertebrates do not possess the structural specialization for a specific, anticipatory response. 2) Antibodies to immunoglobulins have not detected homologous molecules in invertebrates, and where investigated, no rearranging genes have been found. 3) Previous reports of adaptive responses, based on accelerated secondary antigen clearance, or graft rejection, may have been due to activation of non-specific responses.

According to Klein (1989), one possible basis for an animal's ability to mount a specific, anticipatory response to the myriad of antigens in its environment would be the possession of responsive cells, each of which is equipped with an array of antigen receptors as diverse as the universe of epitopes the animal encounters. Upon contact with antigen, the cell would then switch off production of the unnecessary receptors and increase production of the required one. A second alternative would be the possession of responsive cells, each carrying a single antigen receptor. In this case, the animal would need cells equal in number to the variety of epitopes it may encounter. When the antigen was bound by a cell, the cell would proliferate and increase the number of antigen-specific molecules.

Klein reasons that the first alternative is impractical. Each cell would be required to display thousands of antigen receptors in sufficient quantity to provide optimum conditions for epitope binding. The second alternative requires that, as a population, the immune cells must bear all possible receptors, and as

individuals, the cells must be capable of extensive proliferation and a long life span for the development of memory. This is characteristic of the adaptive response of the vertebrates. In vertebrates, these responses require specialized hemopoietic organs, or at least germinal centers. Such structures are rarely found in invertebrates.

No evidence of rearranging immunoglobulin genes has been found in the echinoderms (personal communication from E. Davidson to Marchalonis and Schluter, 1990), nor have molecules been found in echinoderm coelomic fluid which react with either antibodies to immunoglobulin of primitive vertebrates or antibodies against synthetic pepetide segments corresponding to the joining region of immunogloblin (Marchalonis and Schluter, 1990).

Finally, there has been an interest in the reinterpretation of data that originally was used to indicate adaptive immune function in invertebrates based on accelerated secondary respones. It is now suggested that what some researchers interpreted as specific memory may have been due to highly activated nonspecific components which, upon subsequent immune challenge, led to an accelerated secondary response (Klein, 1989; Smith and Davidson, 1992).

The phylogenetic origins of the adaptive immune response remain unknown. The results of the work reported in this thesis support the theories which suggest that the multigene complex necessary for the formation of antibodies did not evolve until after the echinoderm and chordate ancestors diverged (Marchalonis and Schluter, 1990).

Although it could not be concluded that the axial organ functions in a specific

immune capacity, the cellular descriptions presented here extend the earlier work of Bachmann and Goldschmid (1978), Anteunis, *et al.* (1985), and Welsch and Rehkamper (1987). No analysis of separated echinoid axial organ cell subpopulations have been published, and no reports exist of efforts to determine membrane carbohydrate composition of axial organ cells. In addition, the discovery of a previously undescribed subpopulation of Cell Type 3, which may be coelomocyte precursors, is novel. The confirmation of an ontogenic relationship between the red spherule cell and this subpopulation could imply a hemopoietic role for the axial organ.

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APPENDICES

APPENDIX A

BUFFERS

1. Cacodylate Buffer (0.28 M)

   38.6 grams Cacodylic Acid (mw 138 kDa)

   1 liter dH_2O

   pH to 7.0, sterile filter

2. 5X Modified Barbital Buffer (MBB)

   1 vial Barbital Buffer (0.05 moles sodium barbital, 0.01 moles barbital, Sigma)

   0.083 g $CaCl_2$ (anhydrous)

   0.508 g $MgCl_2 \cdot 6H_2O$

   42.5 g NaCl

   pH 7.5

   Bring up to 1 liter with dH_2O. Stir over low heat until dissolved. Dilute to 1X MBB as needed. Autoclave or sterile filter

3. Fish Phosphate Buffered Saline (PBS)

0.204 g NaH_2PO_4

1.18 g Na_2HPO_4

9.0 g NaCl

pH 7.4, 312 mOSM

Bring up to 1 L with dH_2O

4. Fish RPMI-1640

1 vial RPMI 1640 media (with L-glutamine, without sodium bicarbonate,Sigma)

2.6 g HEPES

pH 7.4

Bring up to 1 liter with dH_2O

Add gentamycin (100 μg/ml). Sterile filter.

Add Fetal Bovine Serum to 10%.

5. Urchin RPMI-1640

1 vial RPMI 1640 media (with L-glutamine, without sodium bicarbonate, Sigma)

2.6 g HEPES

18.1 g NaCl

pH 7.6, 830 mOSM

Bring up to 1 liter with dH_2O.

Add gentamycin (100 μg/ml). Sterile filter

Add Fetal Bovine Serum to 10%

6. Anticoagulant (Bertheussen, 1978)

3 mM Caffeine

2 mM TAME (p-tosyl-L-arginine methyl ester)

50 mM β Mercaptoethanol

Add to Instant Ocean

pH 7.6

7. ELISA Coating Buffer

1.53 g Na_2CO_3

2.93 g $NaHCO_3$

0.2 g NaN_3

pH 9.6

Dissolve in 1 liter distilled water

Store at 4°C for not longer than 2 weeks

8. Tris Buffered Saline (TBS)

6.07 g Trizma Base

0.37 g disodium EDTA

8.7 g NaCl

pH 8.0

Bring up to 1 liter with dH_2O

9. Tween Tris Buffered Saline (TTBS)

Add 0.1% Tween 20 to TBS

10.ELISA Substrate Solution

Add 0.1 ml of the following to each well

200 μl of 10 mg/ml ABTS (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)

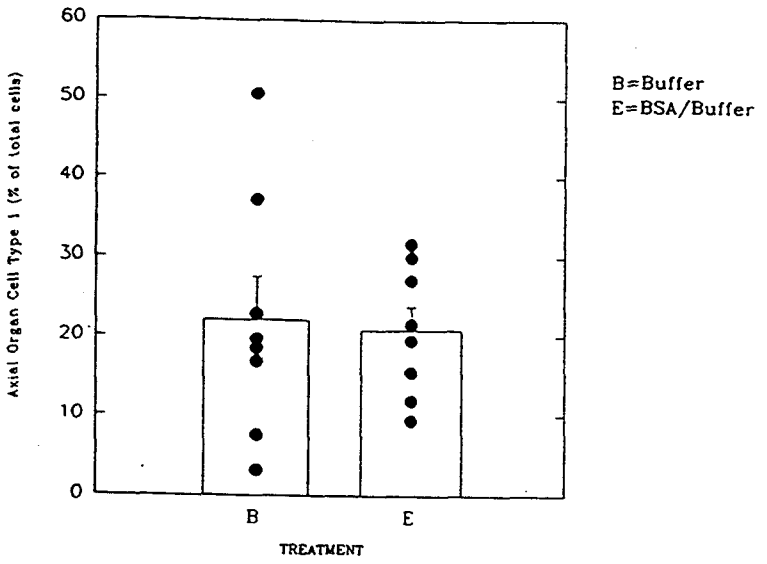
10 μl 30% H_2O_2

10 ml Citric acid buffer (0.2 g citric acid in 100 ml dH_2O, pH 4)

APPENDIX B

AXIAL ORGAN CELL TYPE DATA

EXPERIMENT 1: Axial Organ Cell Type 1



EXPERIMENT 1: Axial Organ Cell Type 3

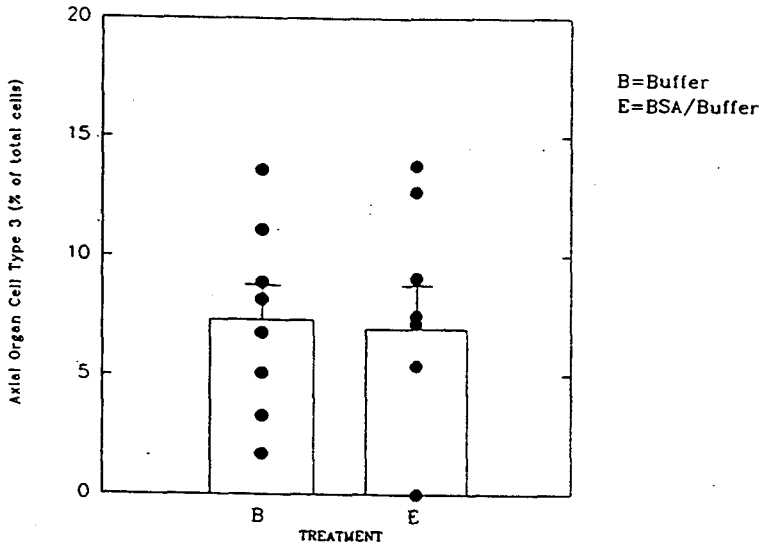
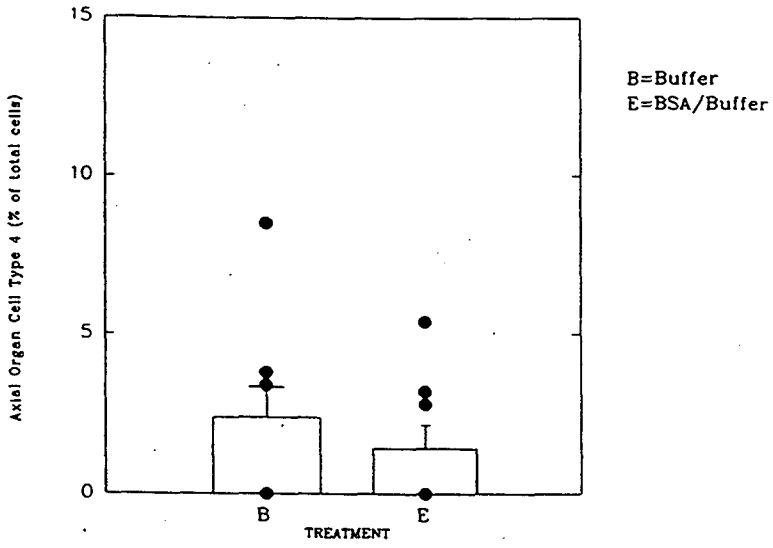


FIGURE 29a
The urchins in this experiment received a single injection of BSA (E) or were injected with Instant Ocean (B). Each graph depicts the data from a different cell type. No significant differences in the means were evident for Cell Types 1 and 3-8. The data for Cell Type 2 are presented in Chapter 2.

EXPERIMENT 1: Axial Organ Cell Type 4



EXPERIMENT 1: Axial Organ Cell Type 5

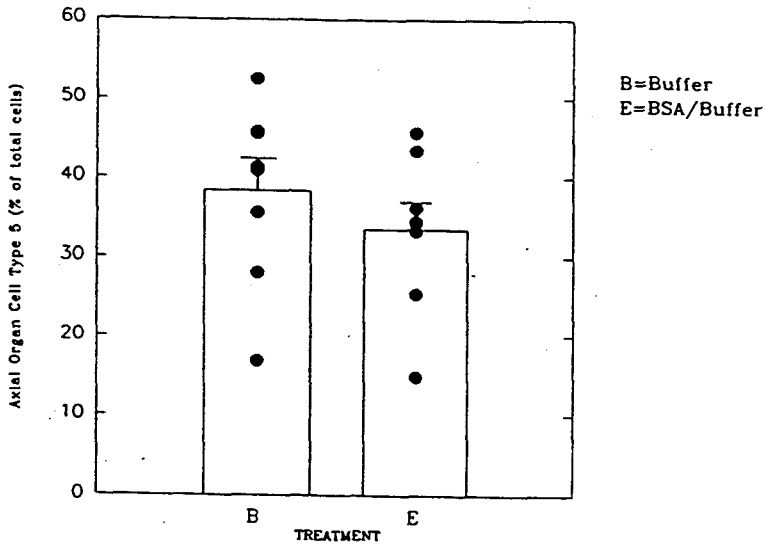
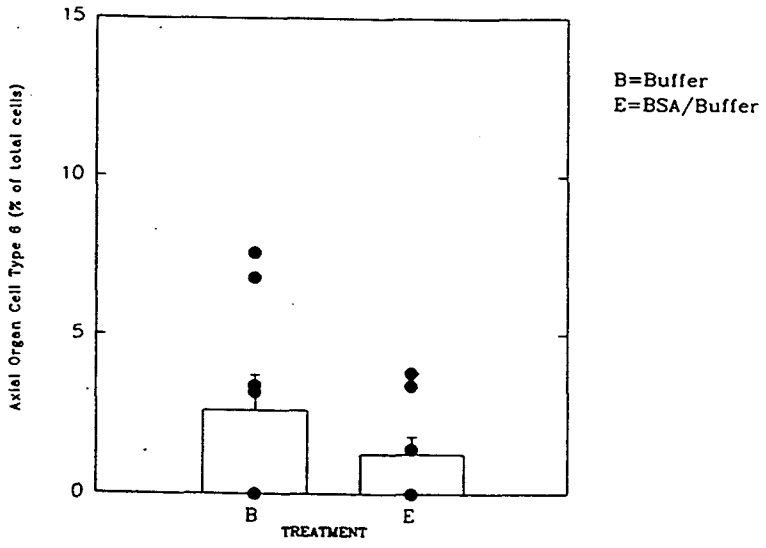


FIGURE 29b
The urchins in this experiment received a single injection of BSA (E) or were injected with Instant Ocean (B). Each graph depicts the data from a different cell type. No significant differences in the means were evident for Cell Types 1 and 3-8. The data for Cell Type 2 are presented in Chapter 2.



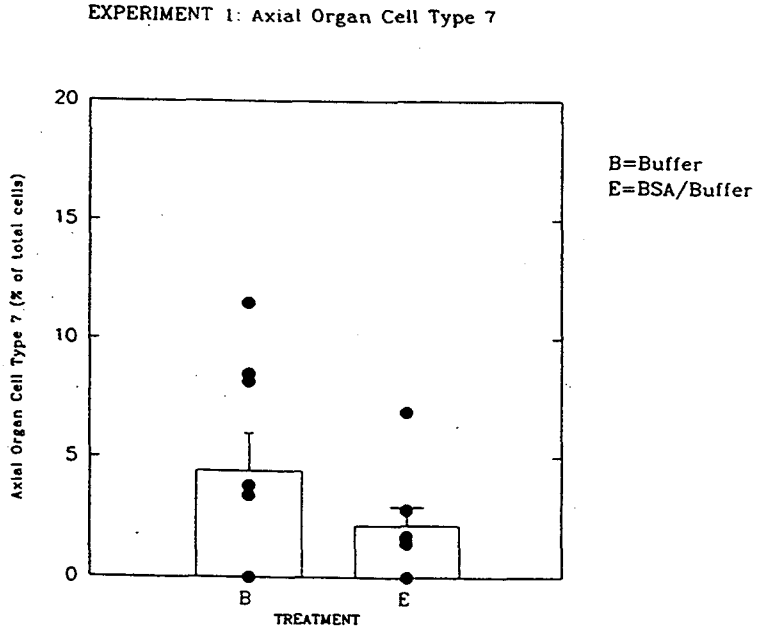
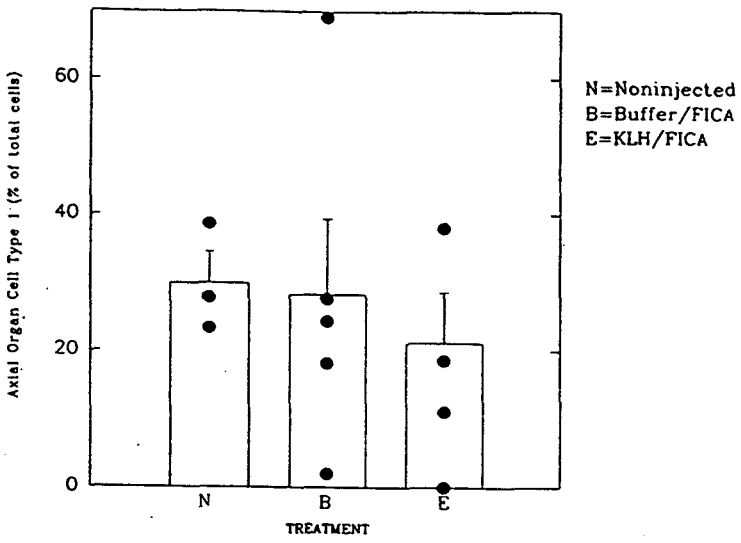
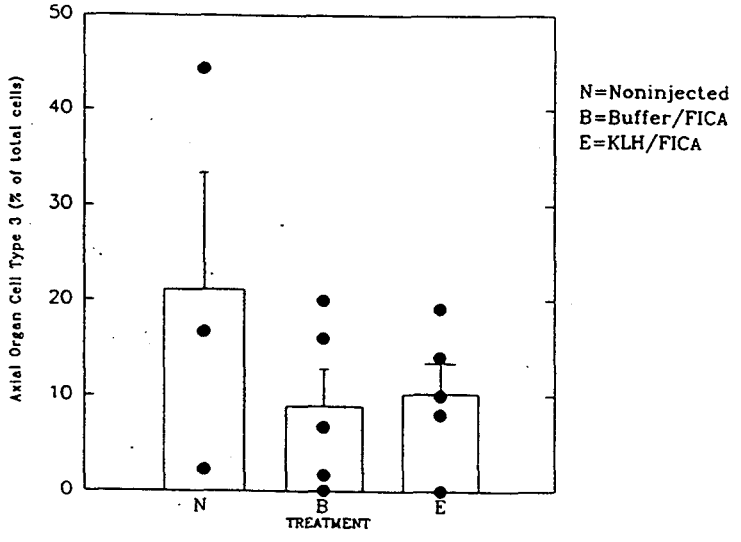


FIGURE 29c
The urchins in this experiment received a single injection of BSA (E) or were injected with Instant Ocean (B). Each graph depicts the data from a different cell type. No significant differences in the means were evident for Cell Types 1 and 3-8. The data for Cell Type 2 are presented in Chapter 2.

EXPERIMENT 2: Axial Organ Cell Type 1



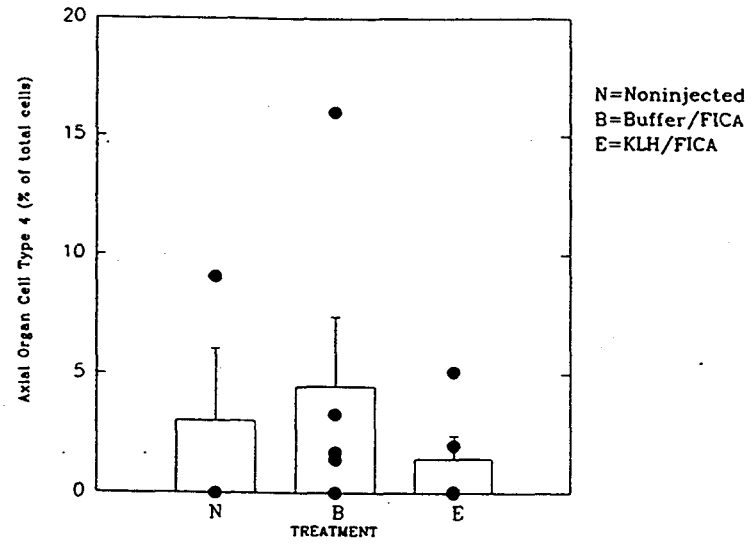
EXPERIMENT 2: Axial Organ Cell Type 3



FIGU

Animals in this experiment received three injections of KLH emulsified in FICA (E), or Instant Ocean emulsified with FICA (B). A third group was not injected (N). Axial organs were removed and cell percentages quantified. These graphs depict the data from each cell type individually. The differences in the means were not significant. The data for Cell Type 2 are presented in Chapter 2.

EXPERIMENT 2: Axial Organ Cell Type 4



EXPERIMENT 2: Axial Organ Cell Type 5

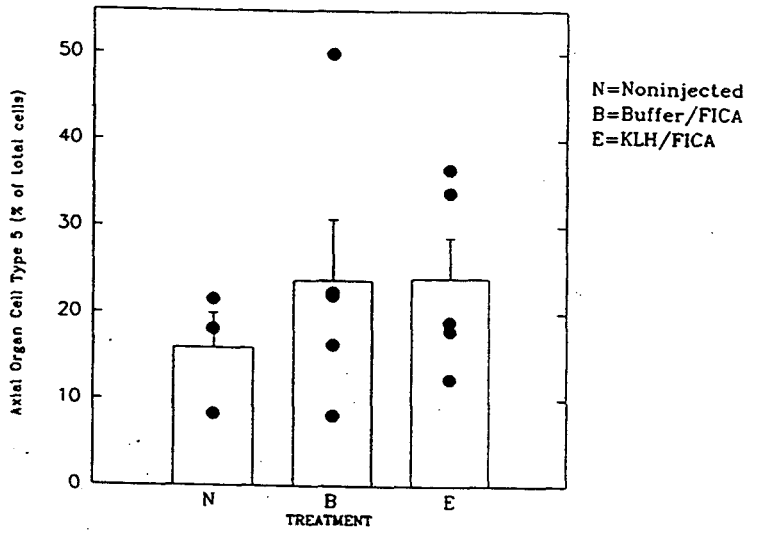
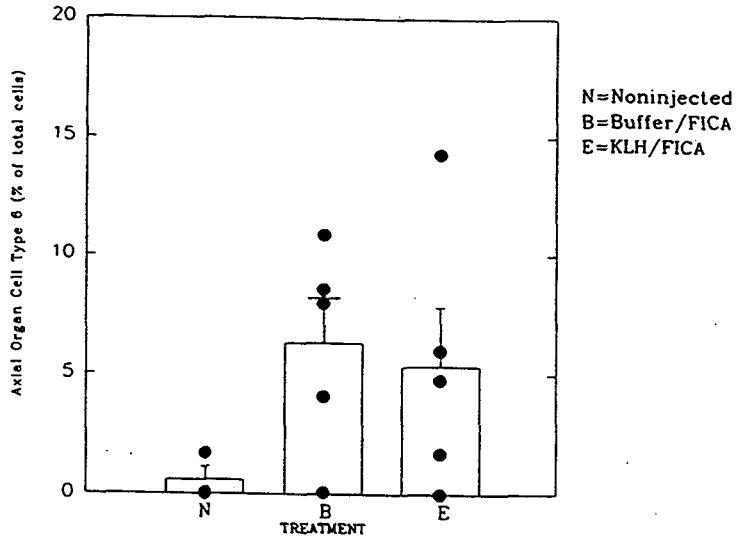


FIGURE 30b
Animals in this experiment received three injections of KLH emulsified in FICA (E), or Instant Ocean emulsified with FICA (B). A third group was not injected (N). Axial organs were removed and cell percentages quantified. These graphs depict the data from each cell type individually. The differences in the means were not significant. The data for Cell Type 2 are presented in Chapter 2.

EXPERIMENT 2: Axial Organ Cell Type 6



EXPERIMENT 2: Axial Organ Cell Type 7

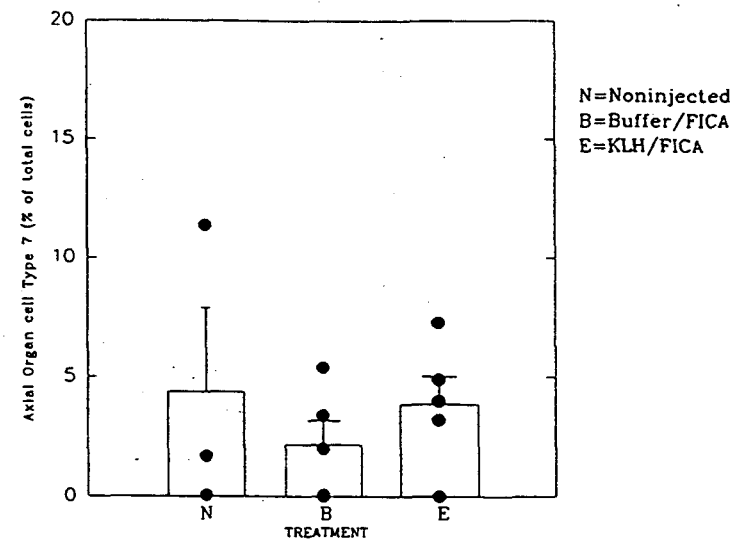


FIGURE 30c
Animals in this experiment received three injections of KLH emulsified in FICA (E), or Instant Ocean emulsified with FICA (B). A third group was not injected (N). Axial organs were removed and cell percentages quantified. These graphs depict the data from each cell type individually. The differences in the means were not significant. The data for Cell Type 2 are presented in Chapter 2.

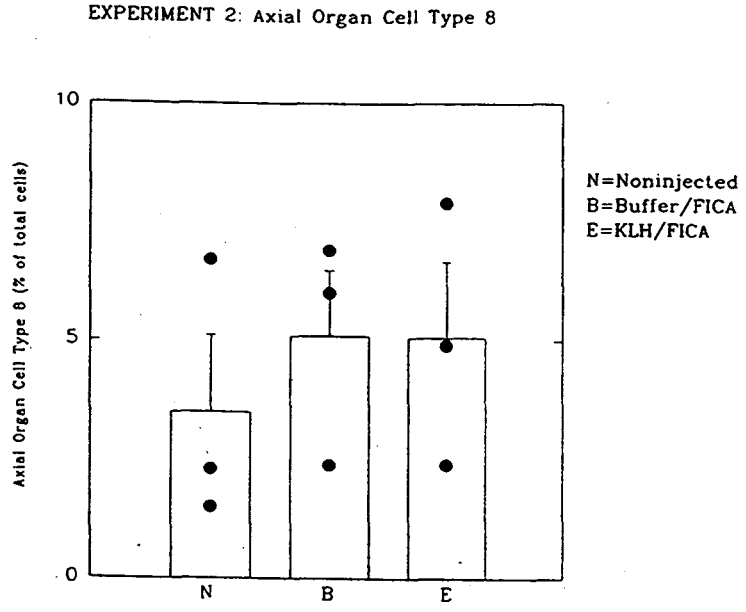
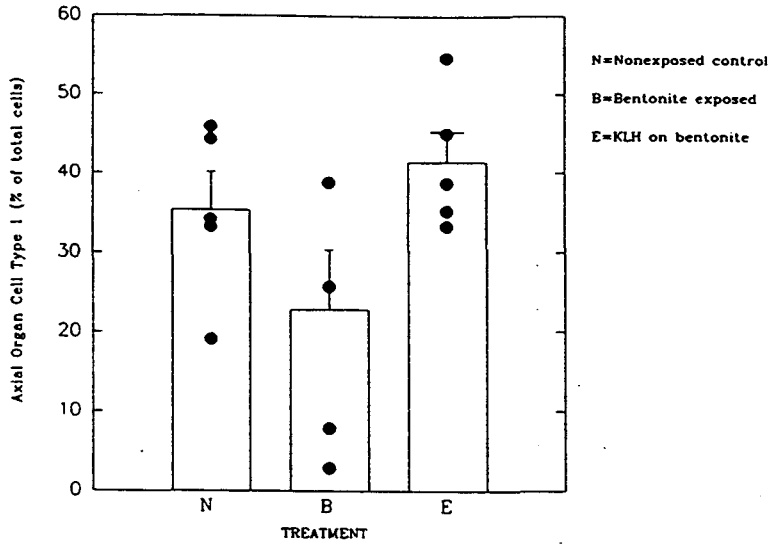


FIGURE 30d
Animals in this experiment received three injections of KLH emulsified in FICA (E), or Instant Ocean emulsified with FICA (B). A third group was not injected (N). Axial organs were removed and cell percentages quantified. These graphs depict the data from each cell type individually. The differences in the means were not significant. The data for Cell Type 2 are presented in Chapter 2.

EXPERIMENT 3: Axial Organ Cell Type 1



EXPERIMENT 3: Axial Organ Cell Type 3

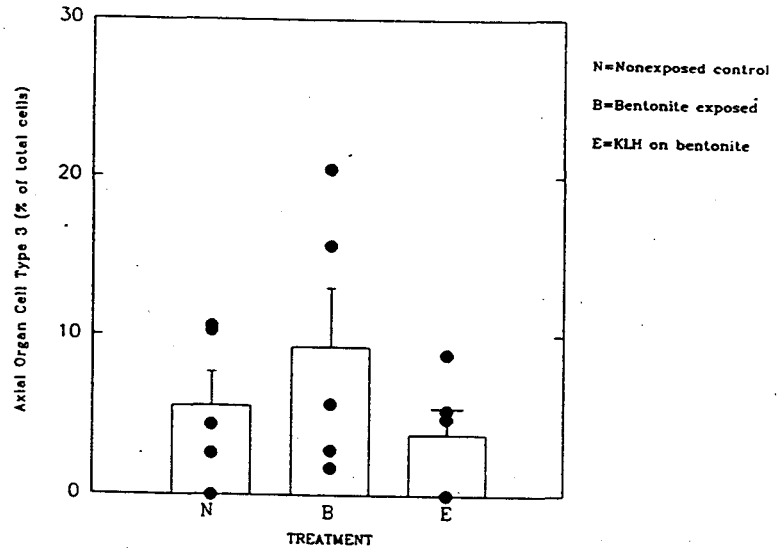
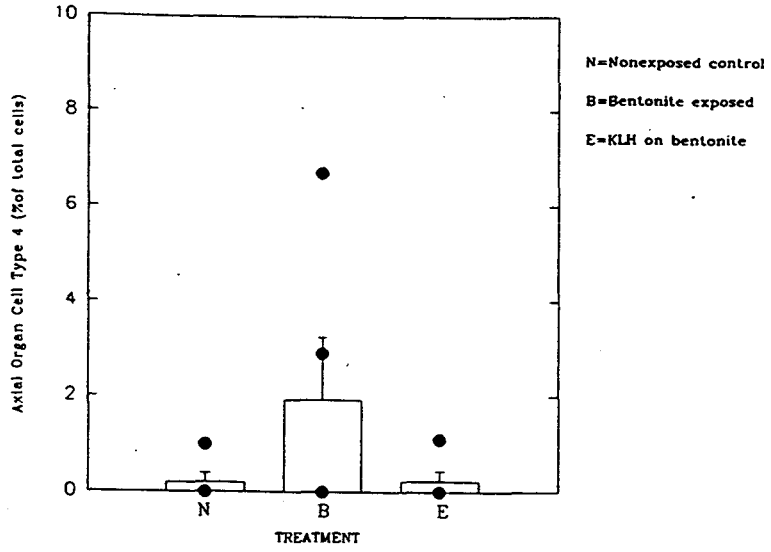


FIGURE 31a
Urchins in the experimental treatment group were exposed to KLH adsorbed onto bentonite (E) while control urchins received either plain bentonite (B) or were not exposed. The method of exposure was a combination of immersion and injection. Data for each cell type is plotted on a separate graph. Differences in the means were not significant. The data for Cell Type 2 are presented in Chapter 2.

EXPERIMENT 3: Axial Organ Cell Type 4



EXPERIMENT 3: Axial Organ Cell Type 5

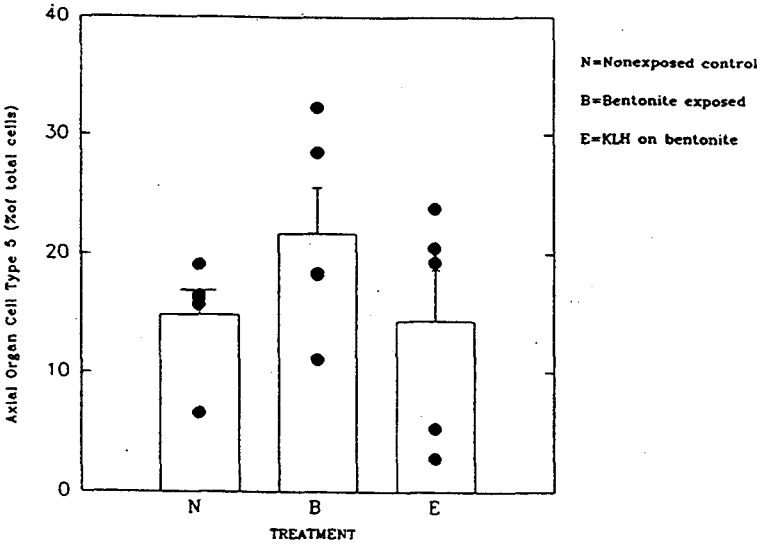
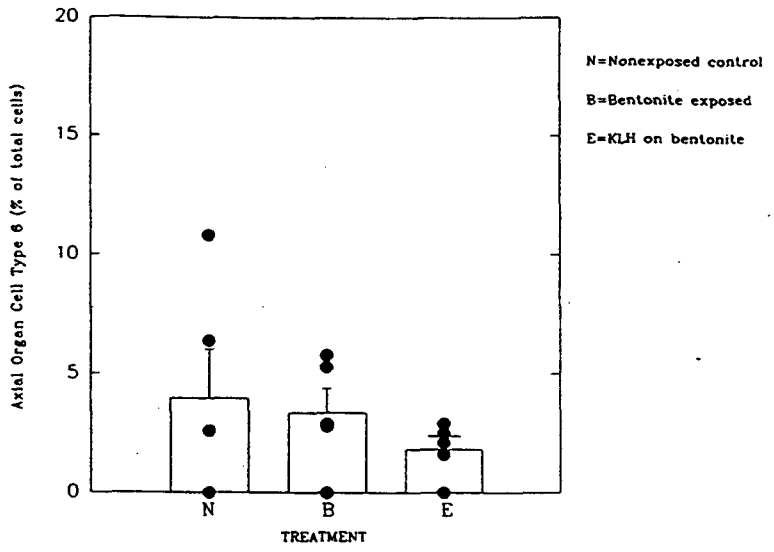


FIGURE 31b
Urchins in the experimental treatment group were exposed to KLH adsorbed onto bentonite (E) while control urchins received either plain bentonite (B) or were not exposed. The method of exposure was a combination of immersion and injection. Data for each cell type is plotted on a separate graph. Differences in the means were not significant. The data for Cell Type 2 are presented in Chapter 2.

EXPERIMENT 3: Axial Organ Cell Type 6



EXPERIMENT 3: Axial Organ Cell Type 7

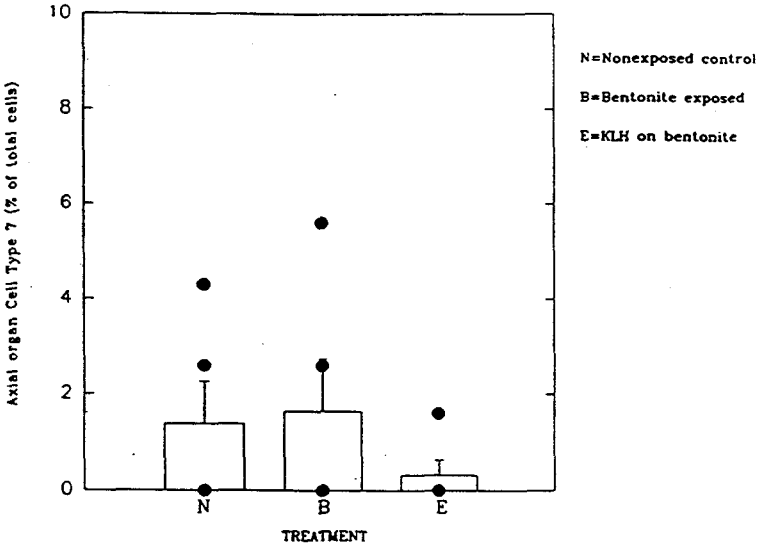


FIGURE 31c
Urchins in the experimental treatment group were exposed to KLH adsorbed onto bentonite (E) while control urchins received either plain bentonite (B) or were not exposed. The method of exposure was a combination of immersion and injection. Data for each cell type is plotted on a separate graph. Differences in the means were not significant. The data for Cell Type 2 are presented in Chapter 2.

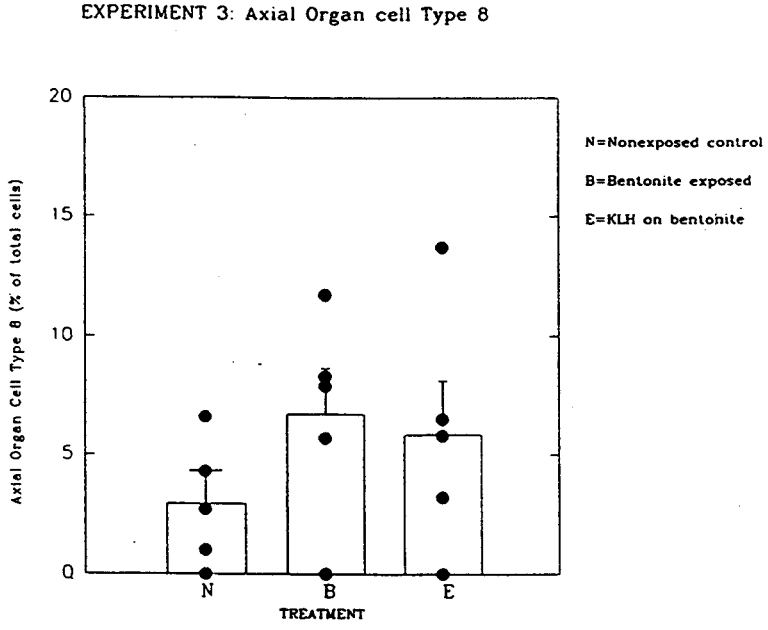
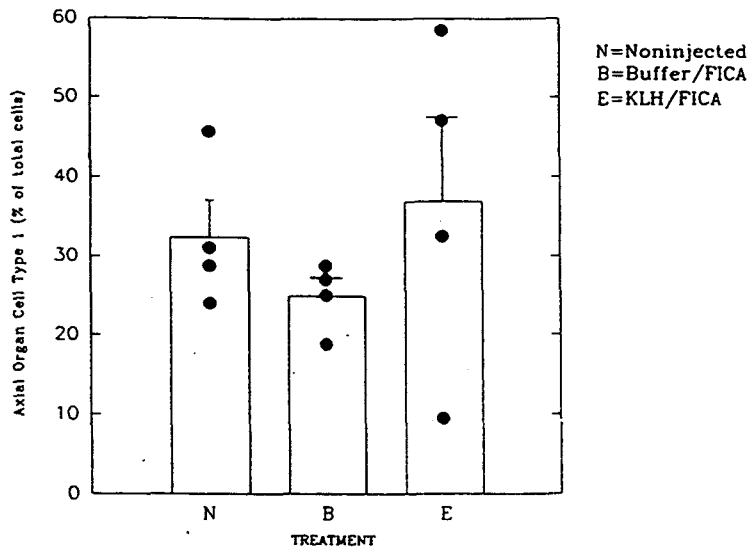


FIGURE 31d
Urchins in the experimental treatment group were exposed to KLH adsorbed onto bentonite (E) while control urchins received either plain bentonite (B) or were not exposed. The method of exposure was a combination of immersion and injection. Data for each cell type is plotted on a separate graph. Differences in the means were not significant. The data for Cell Type 2 are presented in Chapter 2.

EXPERIMENT 4: Axial Organ Cell Type 1



EXPERIMENT 4: Axial Organ Cell Type 3

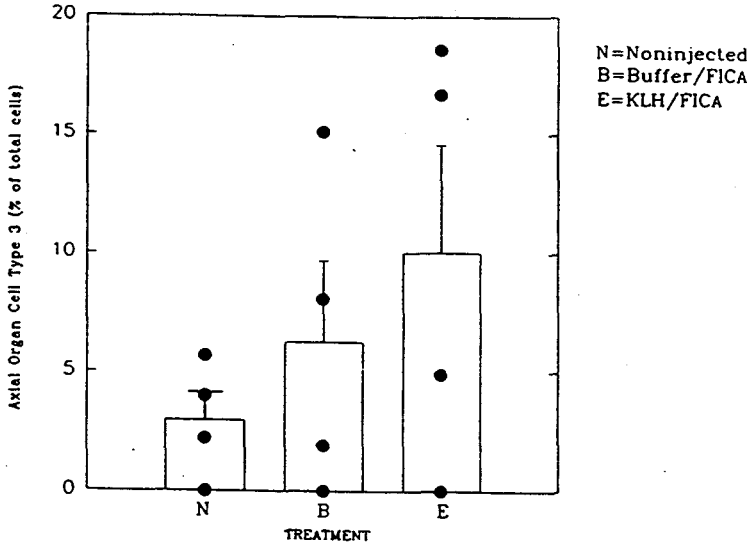
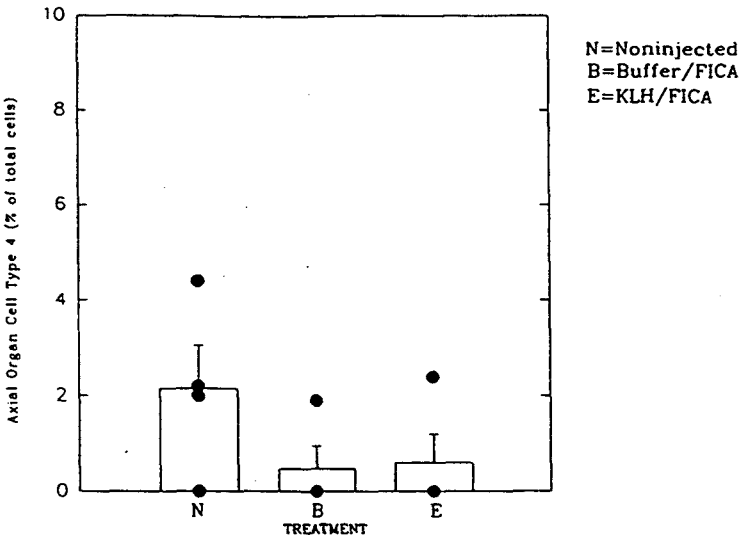


FIGURE 32a
Animals in this experiment received extended exposure to KLH resulting from six injections followed by a seven week period before sampling. Animals were injected with KLH in FICA (E), FICA (B), or remained non-injected (N). Data from each cell type is graphed separately. Differences in the means were not significant. The data for Cell Type 2 are presented in Chapter 2.

EXPERIMENT 4: Axial Organ Cell Type 4



EXPERIMENT 4: Axial Organ Cell Type 5

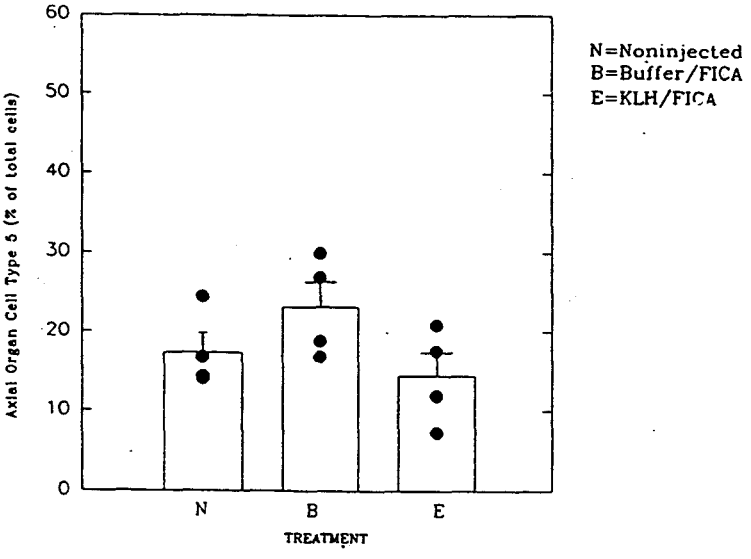
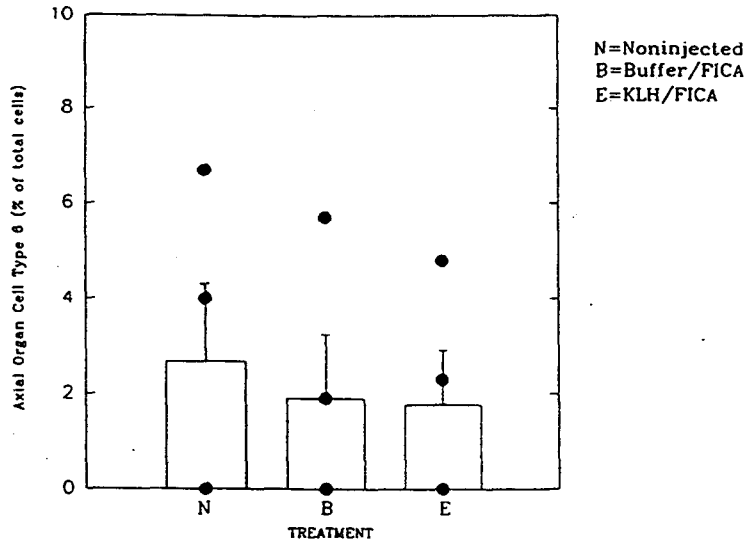


FIGURE 32b
Animals in this experiment received extended exposure to KLH resulting from six injections followed by a seven week period before sampling. Animals were injected with KLH in FICA (E), FICA (B), or remained non-injected (N). Data from each cell type is graphed separately. Differences in the means were not significant. The data for Cell Type 2 are presented in Chapter 2.

EXPERIMENT 4: Axial Organ Cell Type 6



EXPERIMENT 4: Axial Organ Cell Type 7

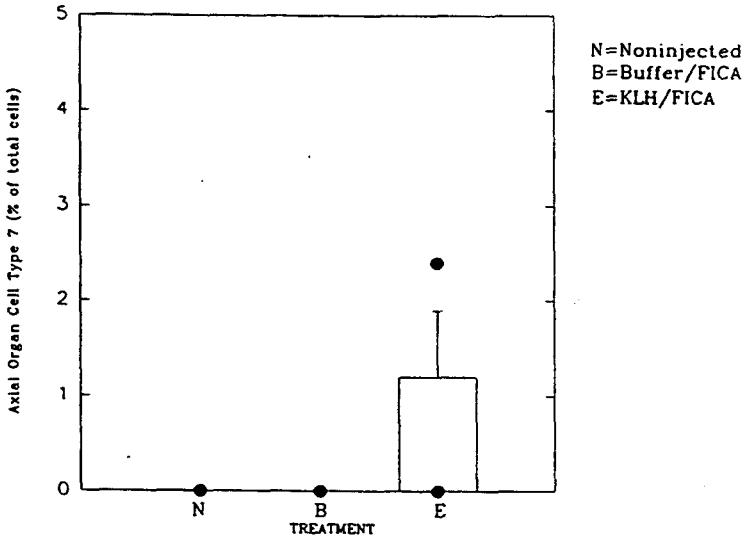


FIGURE 32c
Animals in this experiment received extended exposure to KLH resulting from six injections followed by a seven week period before sampling. Animals were injected with KLH in FICA (E), FICA (B), or remained non-injected (N). Data from each cell type is graphed separately. Differences in the means were not significant. The data for Cell Type 2 are presented in Chapter 2.

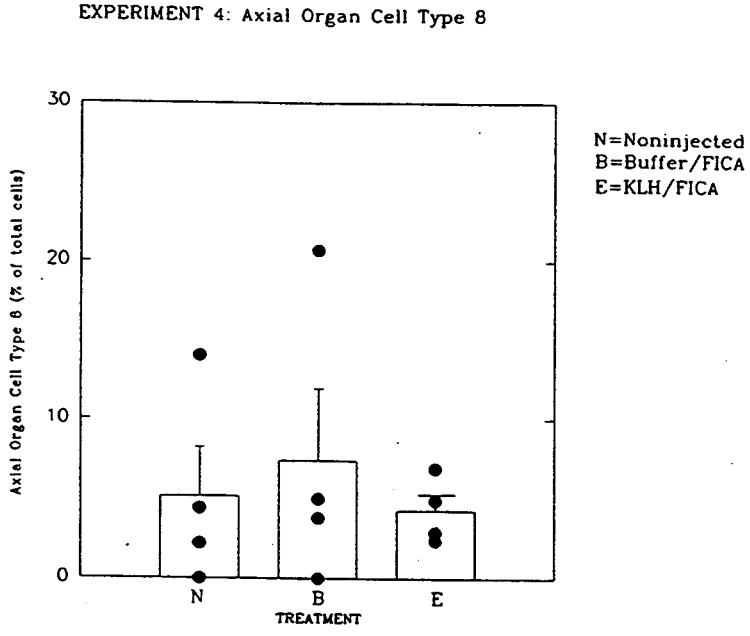


FIGURE 32d
Animals in this experiment received extended exposure to KLH resulting from six injections followed by a seven week period before sampling. Animals were injected with KLH in FICA (E), FICA (B), or remained non-injected (N). Data from each cell type is graphed separately. Differences in the means were not significant. The data for Cell Type 2 are presented in Chapter 2.

APPENDIX C

TROUT ELISA DATA

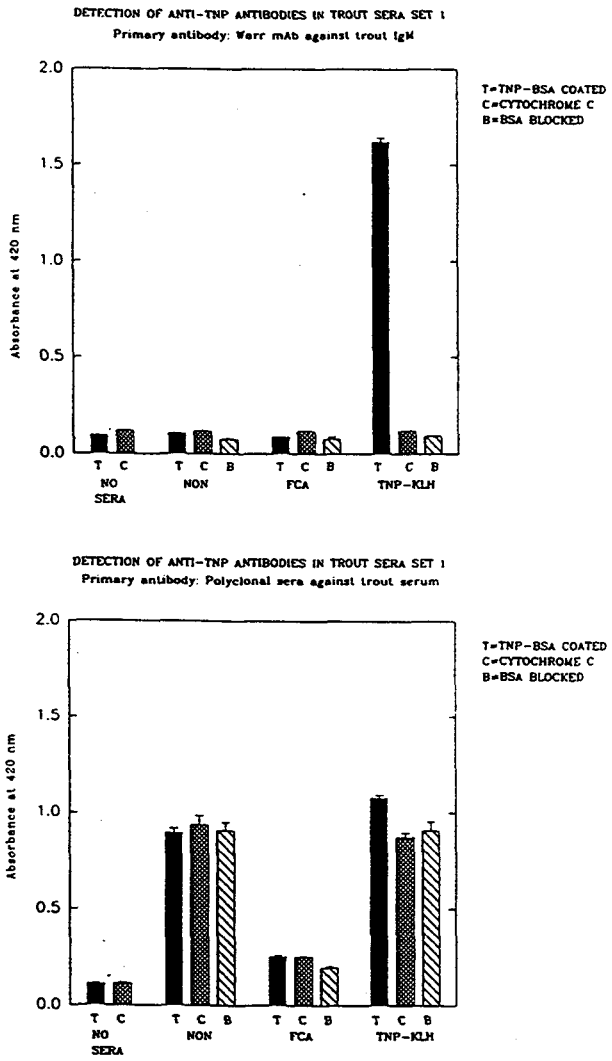


FIGURE 33a

Each pair of graphs depicts the binding of identical trout sera from animals injected with TNP-KLH in FCA (TNP-KLH), FCA (FCA), or from non-injected controls (NON). The plate wells were coated with TNP-BSA (T), cytochrome c (C), or were blocked with BSA (B). The difference between the graphs is the primary antibody used to detect trout serum binding. The upper graph shows the binding of specific trout antibodies to TNP, since the primary probe is a monoclonal antibody against trout immunoglobulin. The lower graph shows trout sera probed with polyclonal rabbit sera against general trout serum proteins. The background binding is much higher in the lower graph but the ELISA binding pattern (EBP) indicative of the presence of specific molecules against TNP, can be seen in all of the TNP-KLH-injected trout. Absorbances were read 15 minutes after substrate addition.

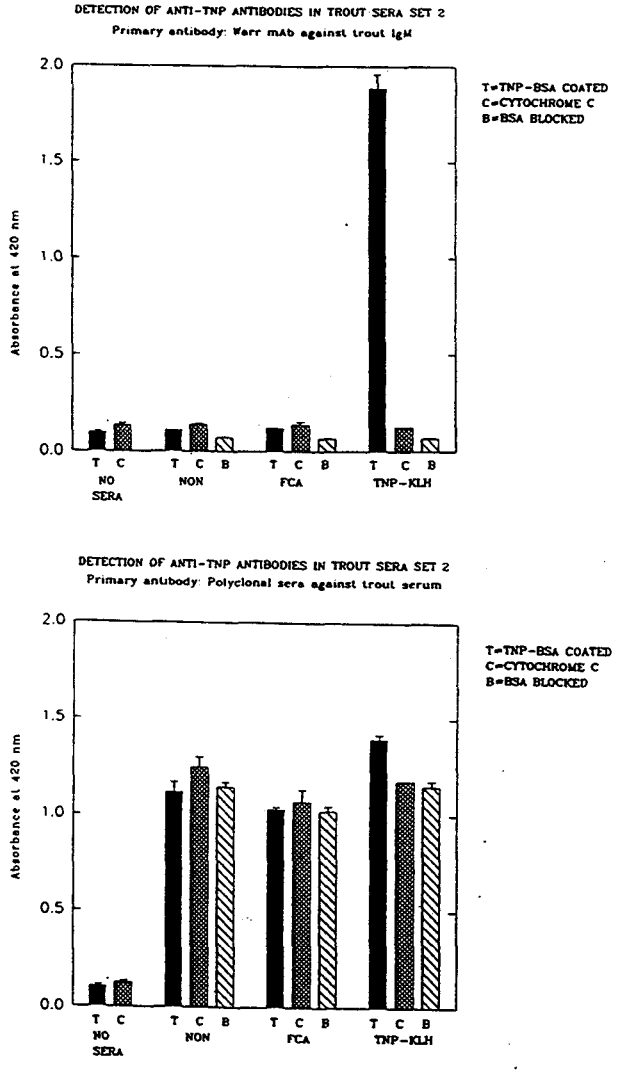


FIGURE 33b
Each pair of graphs depicts the binding of identical trout sera from animals injected with TNP-KLH in FCA (TNP-KLH), FCA (FCA), or from non-injected controls (NON). The plate wells were coated with TNP-BSA (T), cytochrome c (C), or were blocked with BSA (B). The difference between the graphs is the primary antibody used to detect trout serum binding. The upper graph shows the binding of specific trout antibodies to TNP, since the primary probe is a monoclonal antibody against trout immunoglobulin. The lower graph shows trout sera probed with polyclonal rabbit sera against general trout serum proteins. The background binding is much higher in the lower graph but the ELISA binding pattern (EBP) indicative of the presence of specific molecules against TNP, can be seen in all of the TNP-KLH-injected trout. Absorbances were read 15 minutes after substrate addition.

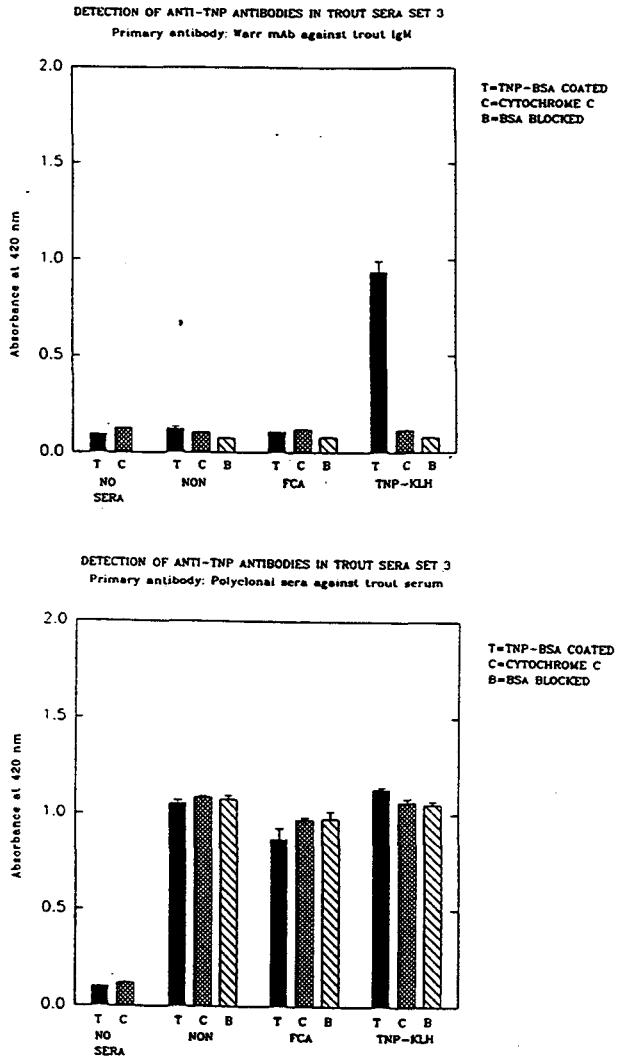


FIGURE 33c

Each pair of graphs depicts the binding of identical trout sera from animals injected with TNP-KLH in FCA (TNP-KLH), FCA (FCA), or from non-injected controls (NON). The plate wells were coated with TNP-BSA (T), cytochrome c (C), or were blocked with BSA (B). The difference between the graphs is the primary antibody used to detect trout serum binding. The upper graph shows the binding of specific trout antibodies to TNP, since the primary probe is a monoclonal antibody against trout immunoglobulin. The lower graph shows trout sera probed with polyclonal rabbit sera against general trout serum proteins. The background binding is much higher in the lower graph but the ELISA binding pattern (EBP) indicative of the presence of specific molecules against TNP, can be seen in all of the TNP-KLH-injected trout. Absorbances were read 15 minutes after substrate addition.

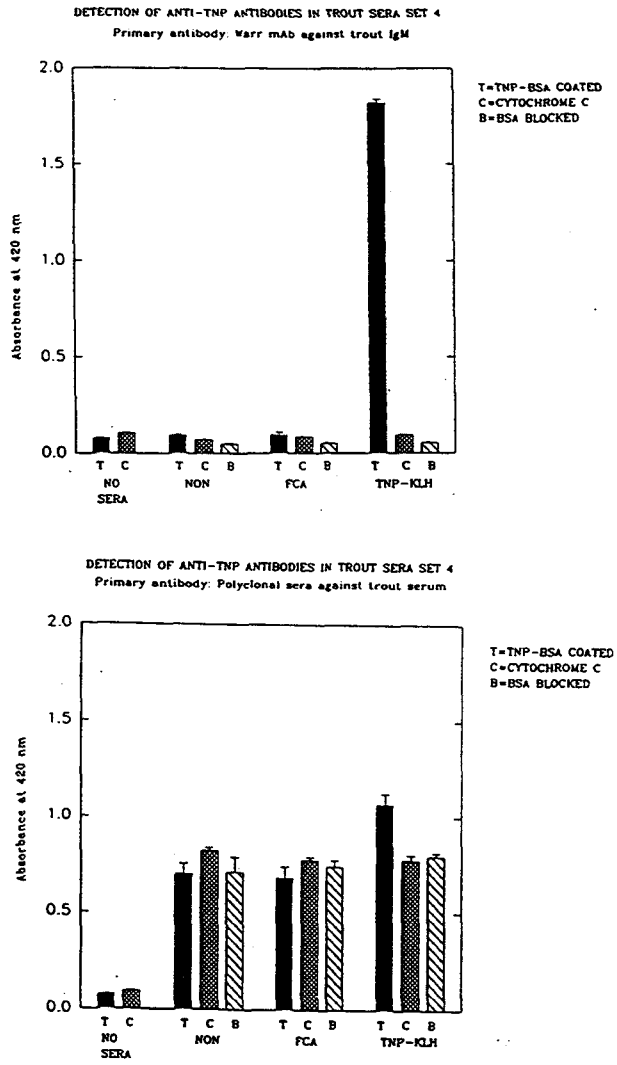


FIGURE 33d

Each pair of graphs depicts the binding of identical trout sera from animals injected with TNP-KLH in FCA (TNP-KLH), FCA (FCA), or from non-injected controls (NON). The plate wells were coated with TNP-BSA (T), cytochrome c (C), or were blocked with BSA (B). The difference between the graphs is the primary antibody used to detect trout serum binding. The upper graph shows the binding of specific trout antibodies to TNP, since the primary probe is a monoclonal antibody against trout immunoglobulin. The lower graph shows trout sera probed with polyclonal rabbit sera against general trout serum proteins. The background binding is much higher in the lower graph but the ELISA binding pattern (EBP) indicative of the presence of specific molecules against TNP, can be seen in all of the TNP-KLH-injected trout. Absorbances were read 15 minutes after substrate addition.

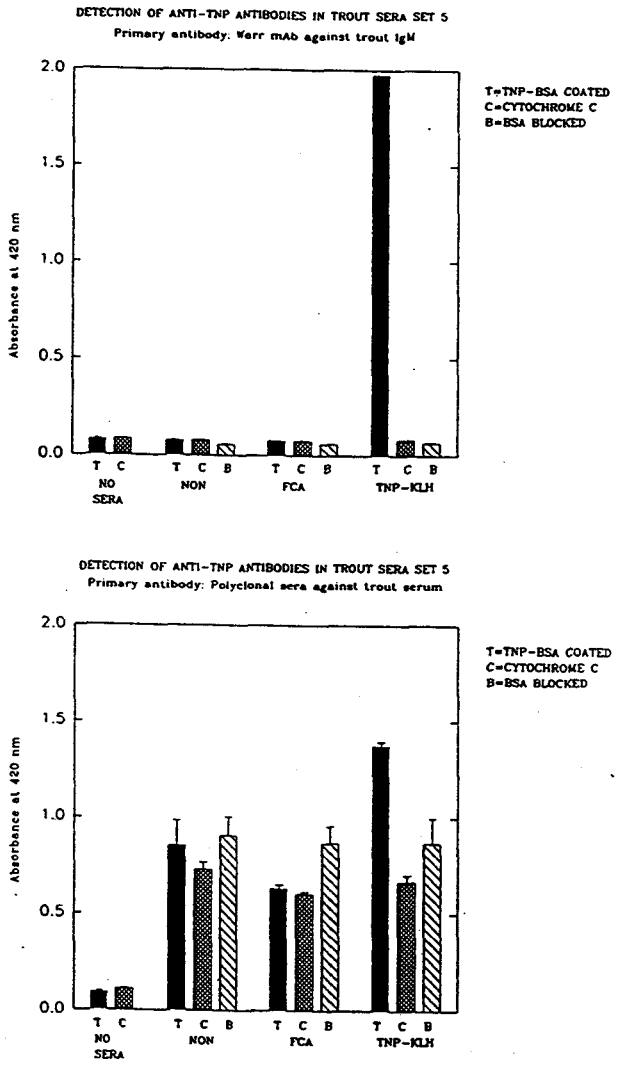


FIGURE 33e

Each pair of graphs depicts the binding of identical trout sera from animals injected with TNP-KLH in FCA (TNP-KLH), FCA (FCA), or from non-injected controls (NON). The plate wells were coated with TNP-BSA (T), cytochrome c (C), or were blocked with BSA (B). The difference between the graphs is the primary antibody used to detect trout serum binding. The upper graph shows the binding of specific trout antibodies to TNP, since the primary probe is a monoclonal antibody against trout immunoglobulin. The lower graph shows trout sera probed with polyclonal rabbit sera against general trout serum proteins. The background binding is much higher in the lower graph but the ELISA binding pattern (EBP) indicative of the presence of specific molecules against TNP, can be seen in all of the TNP-KLH-injected trout. Absorbances were read 15 minutes after substrate addition.

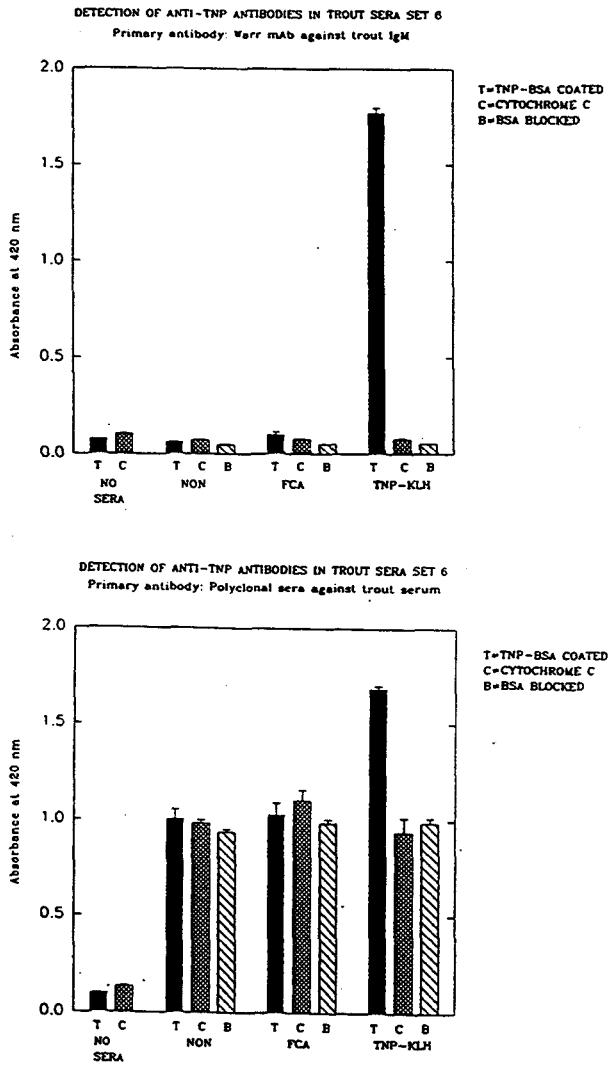


FIGURE 33f

Each pair of graphs depicts the binding of identical trout sera from animals injected with TNP-KLH in FCA (TNP-KLH), FCA (FCA), or from non-injected controls (NON). The plate wells were coated with TNP-BSA (T), cytochrome c (C), or were blocked with BSA (B). The difference between the graphs is the primary antibody used to detect trout serum binding. The upper graph shows the binding of specific trout antibodies to TNP, since the primary probe is a monoclonal antibody against trout immunoglobulin. The lower graph shows trout sera probed with polyclonal rabbit sera against general trout serum proteins. The background binding is much higher in the lower graph but the ELISA binding pattern (EBP) indicative of the presence of specific molecules against TNP, can be seen in all of the TNP-KLH-injected trout. Absorbances were read 15 minutes after substrate addition.

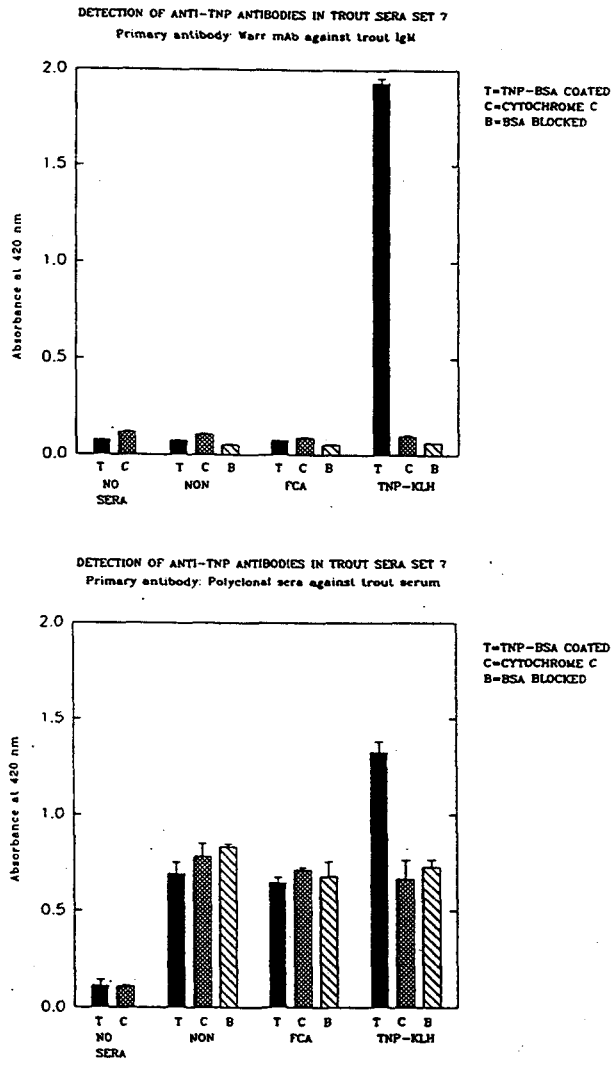


FIGURE 33g

Each pair of graphs depicts the binding of identical trout sera from animals injected with TNP-KLH in FCA (TNP-KLH), FCA (FCA), or from non-injected controls (NON). The plate wells were coated with TNP-BSA (T), cytochrome c (C), or were blocked with BSA (B). The difference between the graphs is the primary antibody used to detect trout serum binding. The upper graph shows the binding of specific trout antibodies to TNP, since the primary probe is a monoclonal antibody against trout immunoglobulin. The lower graph shows trout sera probed with polyclonal rabbit sera against general trout serum proteins. The background binding is much higher in the lower graph but the ELISA binding pattern (EBP) indicative of the presence of specific molecules against TNP, can be seen in all of the TNP-KLH-injected trout. Absorbances were read 15 minutes after substrate addition.

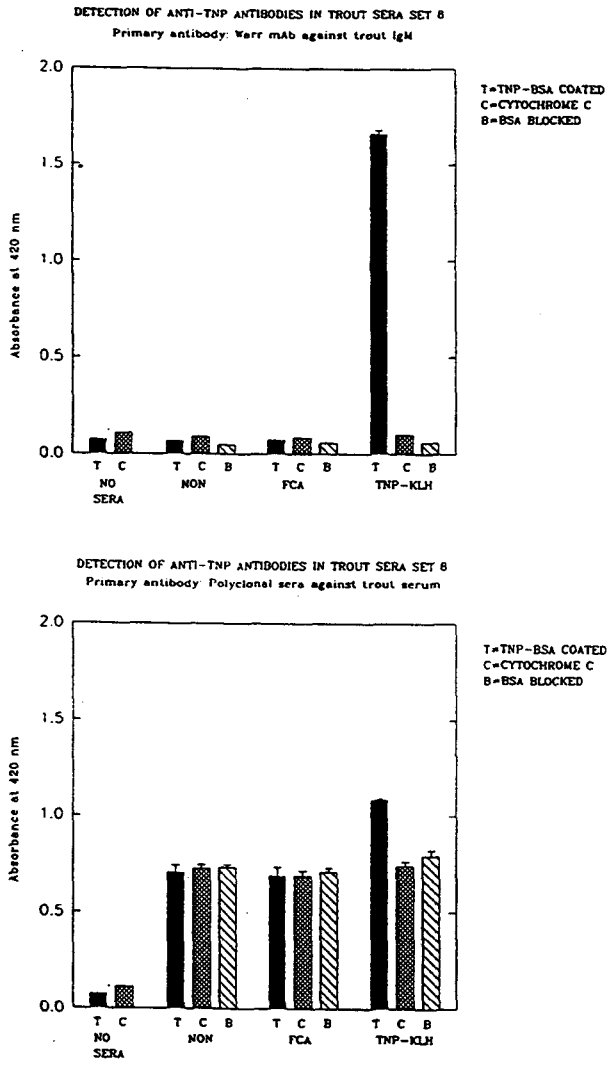


FIGURE 33h
Each pair of graphs depicts the binding of identical trout sera from animals injected with TNP-KLH in FCA (TNP-KLH), FCA (FCA), or from non-injected controls (NON). The plate wells were coated with TNP-BSA (T), cytochrome c (C), or were blocked with BSA (B). The difference between the graphs is the primary antibody used to detect trout serum binding. The upper graph shows the binding of specific trout antibodies to TNP, since the primary probe is a monoclonal antibody against trout immunoglobulin. The lower graph shows trout sera probed with polyclonal rabbit sera against general trout serum proteins. The background binding is much higher in the lower graph but the ELISA binding pattern (EBP) indicative of the presence of specific molecules against TNP, can be seen in all of the TNP-KLH-injected trout. Absorbances were read 15 minutes after substrate addition.

APPENDIX D

URCHIN ELISA DATA

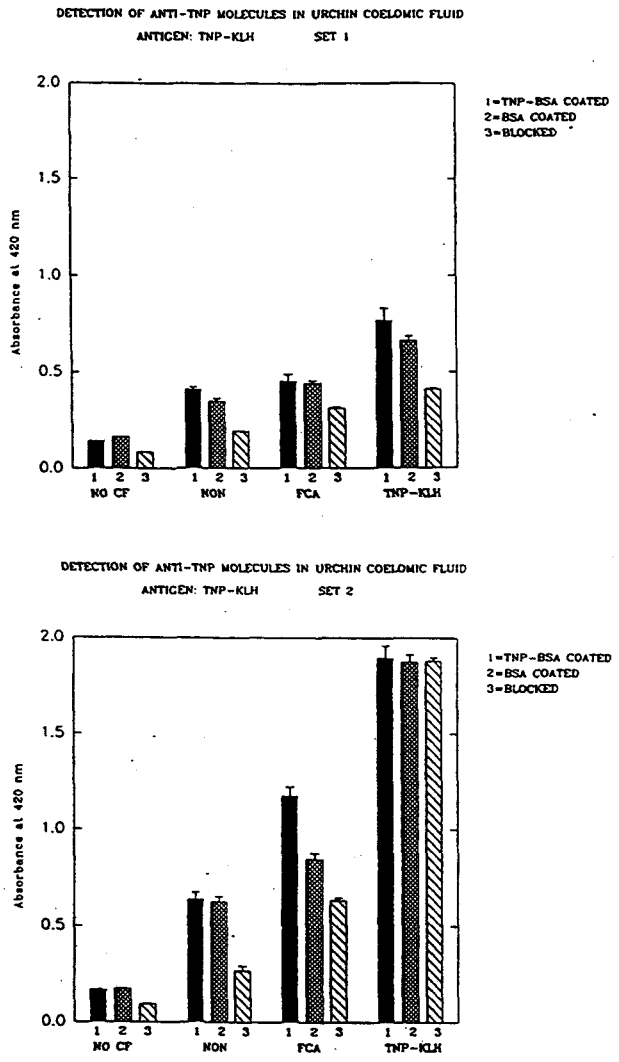


FIGURE 34a
Coelomic fluids from urchins injected with TNP-KLH in FCA or FCA, and from non-injected controls were added to three replicate ELISA plate wells coated with TNP-BSA or BSA, or which were blocked with BSA. Coelomic fluid binding was measured with polyclonal sera against general coelomic fluid proteins. Control wells which did not receive coelomic fluid were included. Each graph represents a set of three animals, one from each treatment group. An example of the ELISA binding pattern (EBP) indicative of the poduction of a specific molecule against TNP can be seen expressed by the FCA-injected animal in set 2. The EBP's were not dependent on treatment (X^2 test). Absorbances were read 35 minutes after substrate addition.

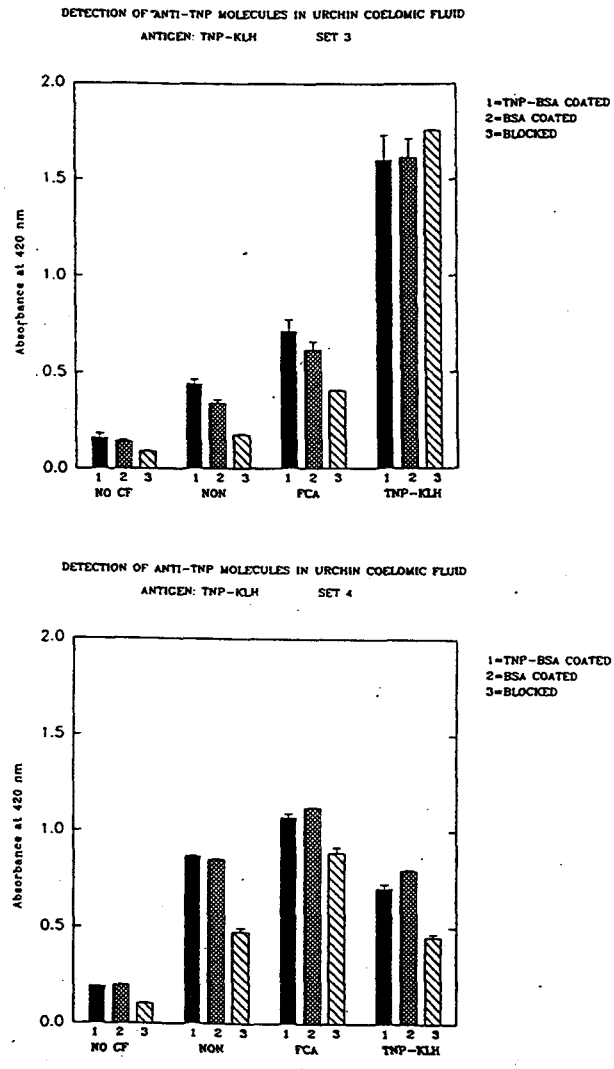


FIGURE 34b
Coelomic fluids from urchins injected with TNP-KLH in FCA or FCA, and from non-injected controls were added to three replicate ELISA plate wells coated with TNP-BSA or BSA, or which were blocked with BSA. Coelomic fluid binding was measured with polyclonal sera against general coelomic fluid proteins. Control wells which did not receive coelomic fluid were included. Each graph represents a set of three animals, one from each treatment group. An example of the ELISA binding pattern (EBP) indicative of the production of a specific molecule against TNP can be seen expressed by the FCA-injected animal in set 2. The EBP's were not dependent on treatment (X^2 test). Absorbances were read 35 minutes after substrate addition.

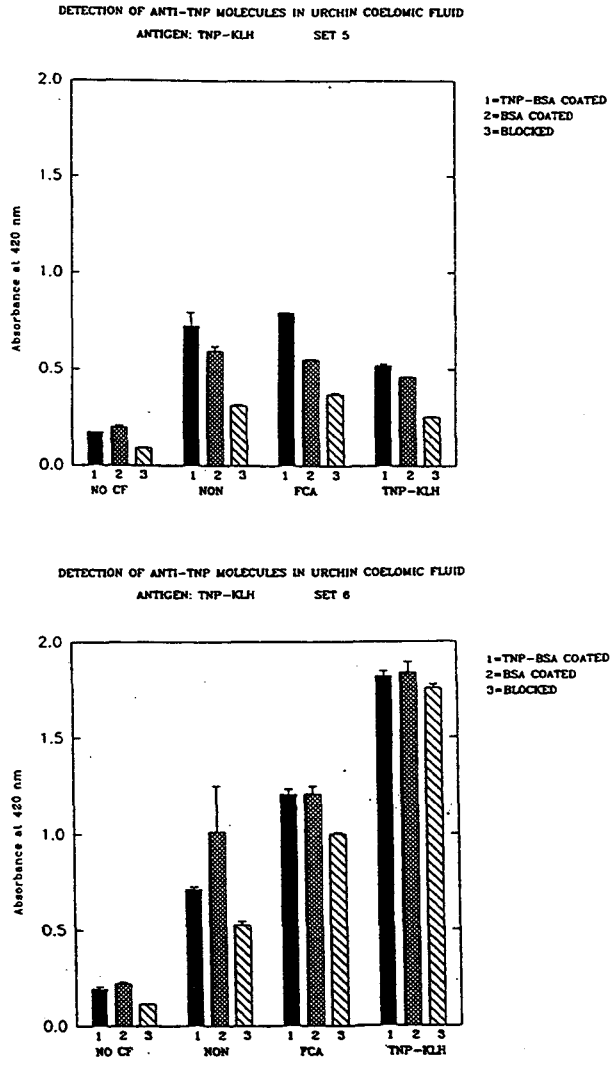


FIGURE 34c
Coelomic fluids from urchins injected with TNP-KLH in FCA or FCA, and from non-injected controls were added to three replicate ELISA plate wells coated with TNP-BSA or BSA, or which were blocked with BSA. Coelomic fluid binding was measured with polyclonal sera against general coelomic fluid proteins. Control wells which did not receive coelomic fluid were included. Each graph represents a set of three animals, one from each treatment group. An example of the ELISA binding pattern (EBP) indicative of the production of a specific molecule against TNP can be seen expressed by the FCA-injected animal in set 2. The EBP's were not dependent on treatment (X^2 test). Absorbances were read 35 minutes after substrate addition.

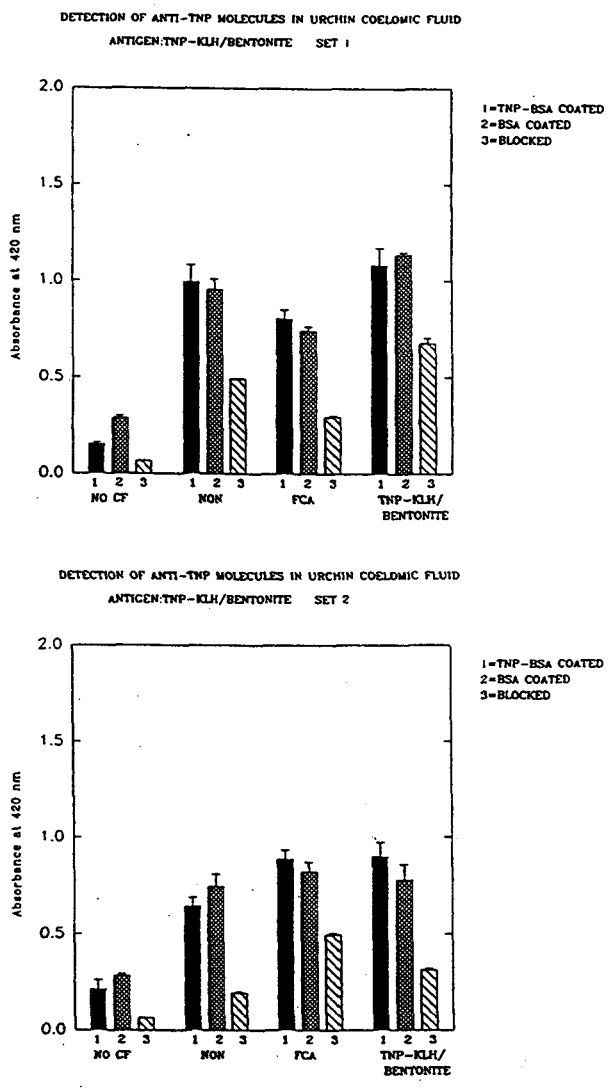


FIGURE 35a
Coelomic fluids from urchins injected with TNP-KLH adsorbed onto bentonite, bentonite alone, and from non-injected controls were added to three replicate ELISA plate wells coated with TNP-BSA or BSA, or which were blocked with BSA. Coelomic fluid binding was measured with polyclonal sera against general coelomic fluid proteins. Control wells which did not receive coelomic fluid were also present. Each graph represents a set of three animals, one from each treatment group. An example of the ELISA binding pattern (EBP) indicative of the production of a specific molecule against TNP can be seen expressed by the TNP-KLH/Ben injected animal in set 3. The presence of EBP's was not dependent on treatment (X^2 test). Absorbances were read 35 minutes after substrate addition.

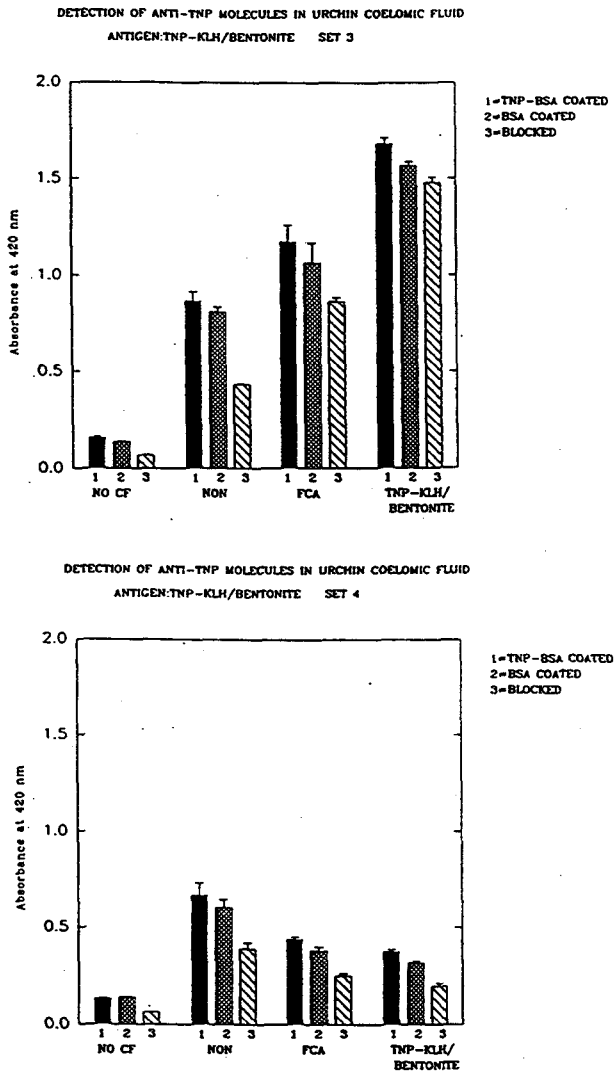


FIGURE 35b
Coelomic fluids from urchins injected with TNP-KLH adsorbed onto bentonite, bentonite alone, and from non-injected controls were added to three replicate ELISA plate wells coated with TNP-BSA or BSA, or which were blocked with BSA. Coelomic fluid binding was measured with polyclonal sera against general coelomic fluid proteins. Control wells which did not receive coelomic fluid were also present. Each graph represents a set of three animals, one from each treatment group. An example of the ELISA binding pattern (EBP) indicative of the production of a specific molecule against TNP can be seen expressed by the TNP-KLH/Ben injected animal in set 3. The presence of EBP's was not dependent on treatment (X^2 test). Absorbances were read 35 minutes after substrate addition.

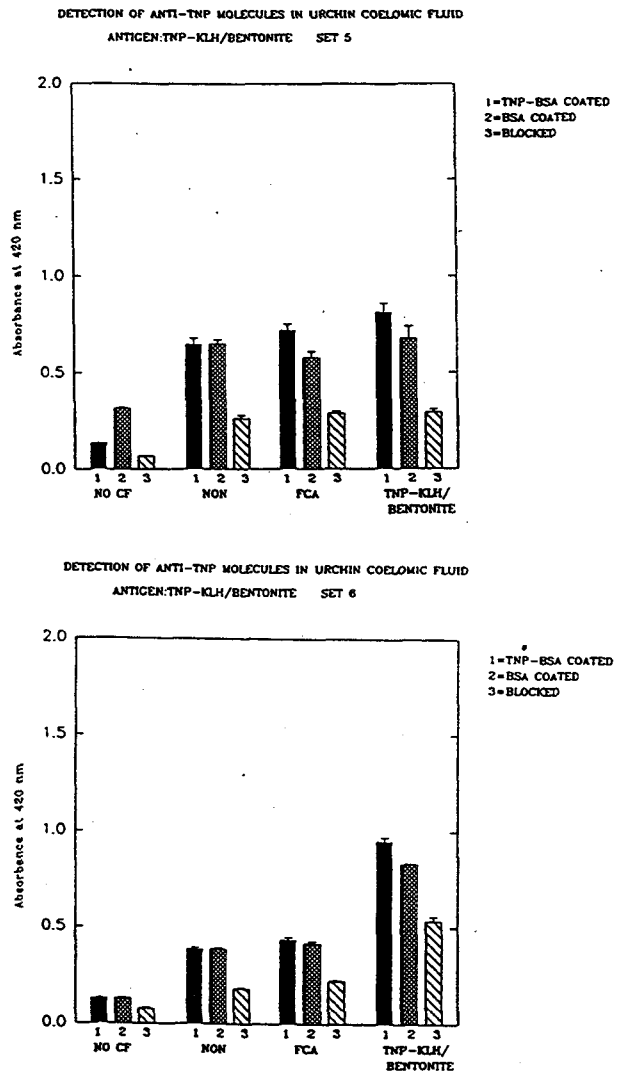


FIGURE 35c
Coelomic fluids from urchins injected with TNP-KLH adsorbed onto bentonite, bentonite alone, and from non-injected controls were added to three replicate ELISA plate wells coated with TNP-BSA or BSA, or which were blocked with BSA. Coelomic fluid binding was measured with polyclonal sera against general coelomic fluid proteins. Control wells which did not receive coelomic fluid were also present. Each graph represents a set of three animals, one from each treatment group. An example of the ELISA binding pattern (EBP) indicative of the production of a specific molecule against TNP can be seen expressed by the TNP-KLH/Ben injected animal in set 3. The presence of EBP's was not dependent on treatment (X^2 test). Absorbances were read 35 minutes after substrate addition.

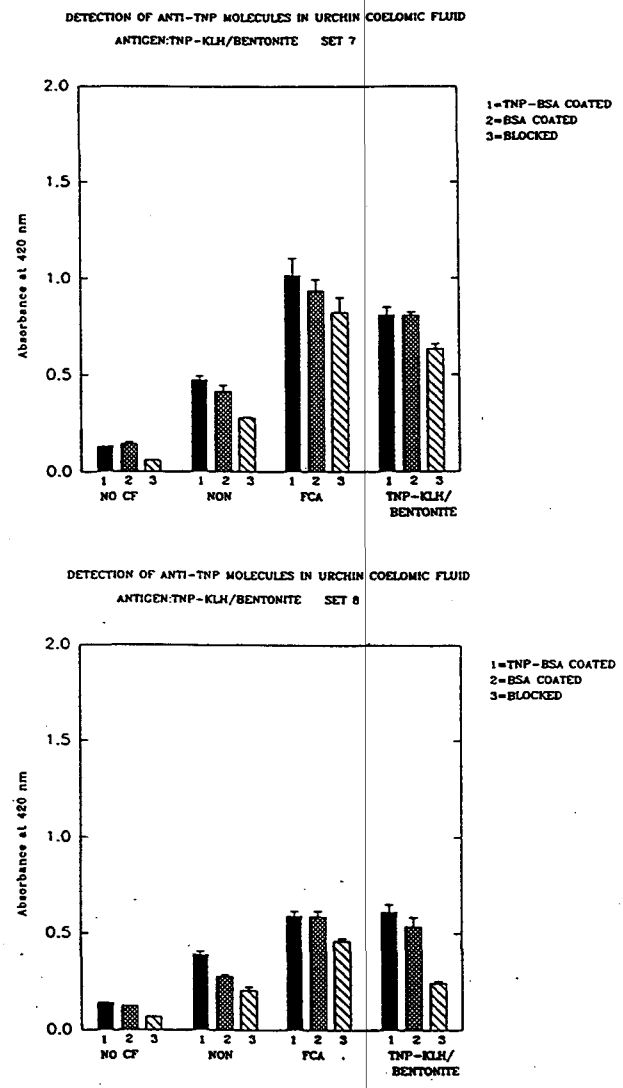


FIGURE 35d
Coelomic fluids from urchins injected with TNP-KLH adsorbed onto bentonite, bentonite alone, and from non-injected controls were added to three replicate ELISA plate wells coated with TNP-BSA or BSA, or which were blocked with BSA. Coelomic fluid binding was measured with polyclonal sera against general coelomic fluid proteins. Control wells which did not receive coelomic fluid were also present. Each graph represents a set of three animals, one from each treatment group. An example of the ELISA binding pattern (EBP) indicative of the production of a specific molecule against TNP can be seen expressed by the TNP-KLH/Ben injected animal in set 3. The presence of EBP's was not dependent on treatment (X^2 test). Absorbances were read 35 minutes after substrate addition.

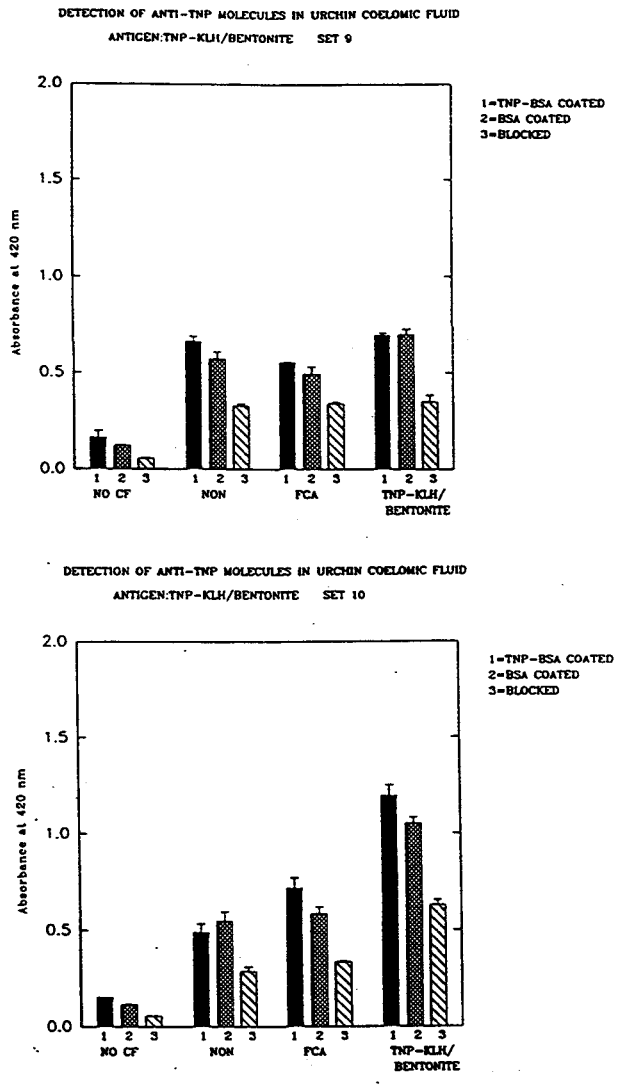


FIGURE 35e

Coelomic fluids from urchins injected with TNP-KLH adsorbed onto bentonite, bentonite alone, and from non-injected controls were added to three replicate ELISA plate wells coated with TNP-BSA or BSA, or which were blocked with BSA. Coelomic fluid binding was measured with polyclonal sera against general coelomic fluid proteins. Control wells which did not receive coelomic fluid were also present. Each graph represents a set of three animals, one from each treatment group. An example of the ELISA binding pattern (EBP) indicative of the production of a specific molecule against TNP can be seen expressed by the TNP-KLH/Ben injected animal in set 3. The presence of EBP's was not dependent on treatment (X^2 test). Absorbances were read 35 minutes after substrate addition.

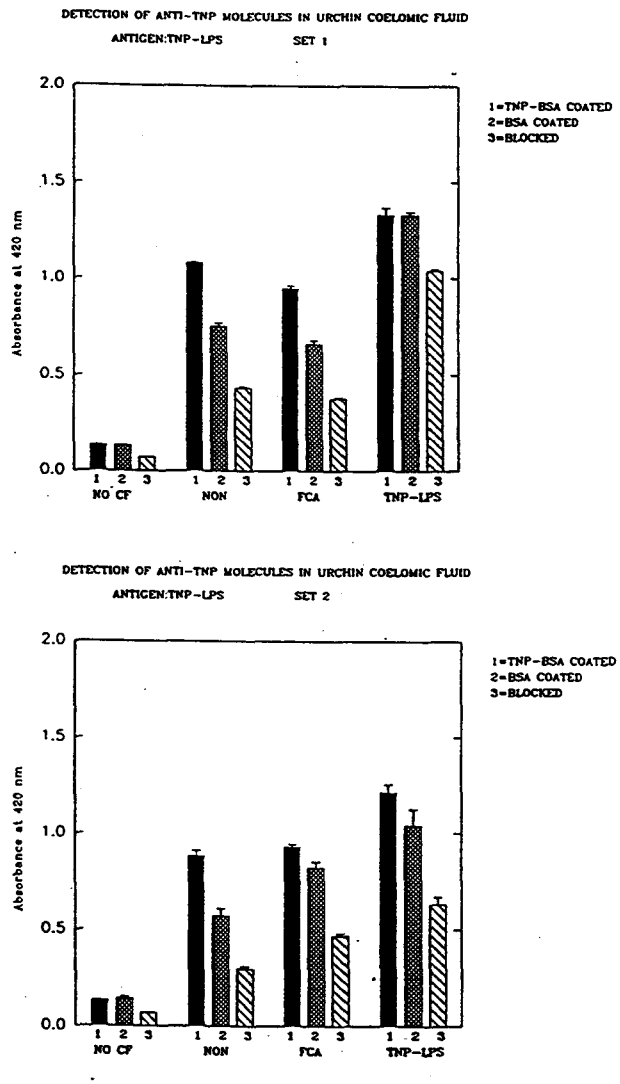


FIGURE 36a
Coelomic fluids from urchins injected with TNP-LPS in FCA, or FCA, and from non-injected controls were added to three replicate ELISA plate wells coated with TNP-BSA or BSA, or which were blocked with BSA. Coelomic fluid binding was measured with polyclonal sera against general coelomic fluid proteins. Control wells which did not receive coelomic fluid were also present. Each graph represents a set of three animals, one from each treatment group. An example of the ELISA binding pattern (EBP) indicative of the production of a specific molecule against TNP can be seen expressed by the non-injected animals in every set. The presence of EBP's was not dependent on treatment (X^2 test). Absorbances were read 35 minutes after substrate addition.

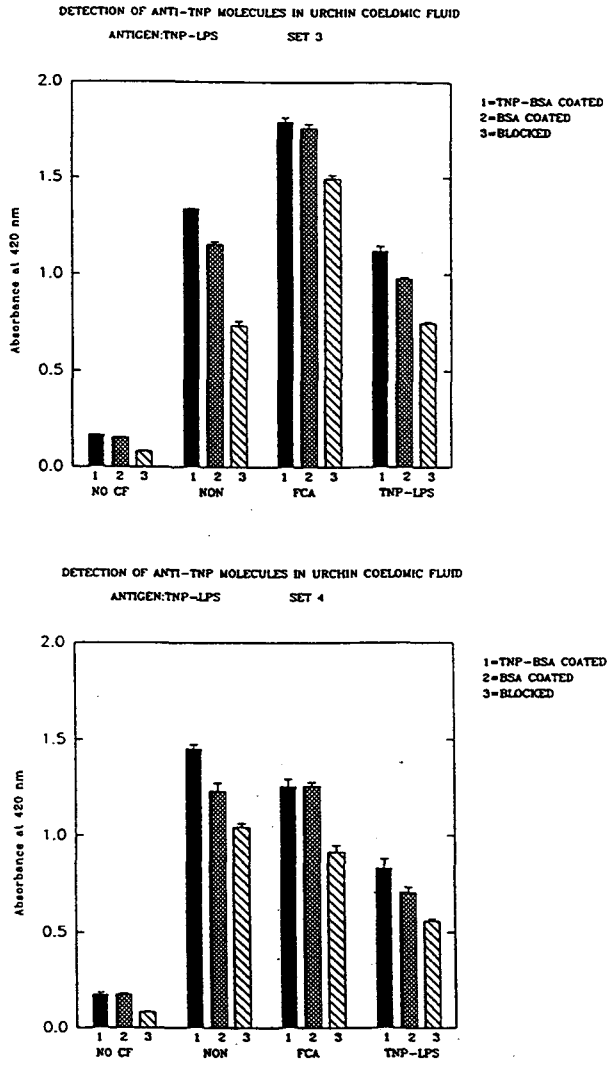


FIGURE 36b
Coelomic fluids from urchins injected with TNP-LPS in FCA, or FCA, and from non-injected controls were added to three replicate ELISA plate wells coated with TNP-BSA or BSA, or which were blocked with BSA. Coelomic fluid binding was measured with polyclonal sera against general coelomic fluid proteins. Control wells which did not receive coelomic fluid were also present. Each graph represents a set of three animals, one from each treatment group. An example of the ELISA binding pattern (EBP) indicative of the production of a specific molecule against TNP can be seen expressed by the non-injected animals in every set. The presence of EBP's was not dependent on treatment (X^2 test). Absorbances were read 35 minutes after substrate addition.

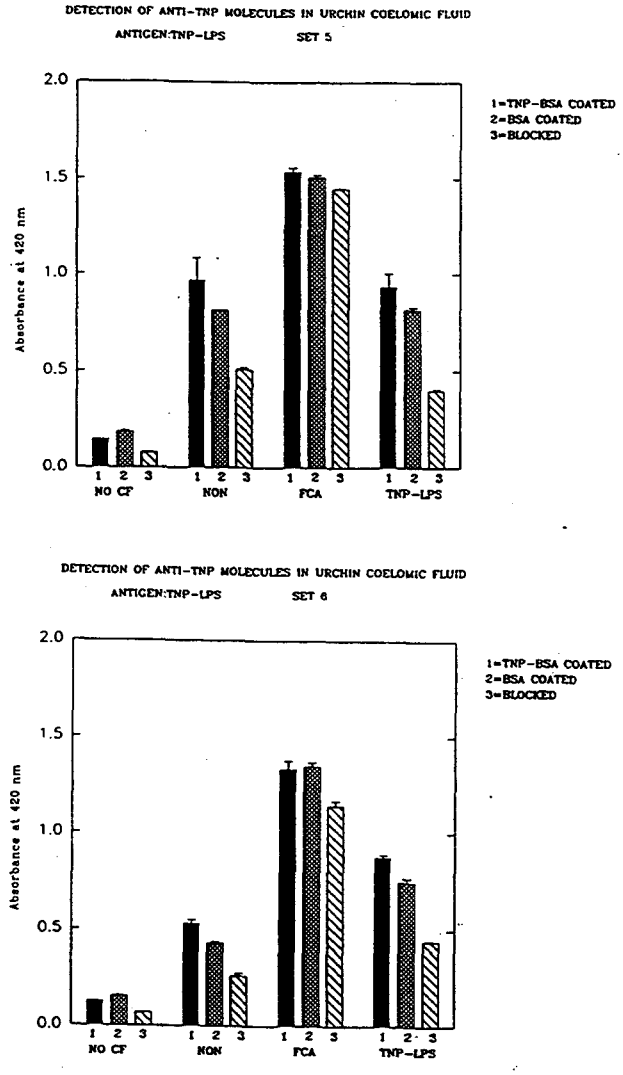


FIGURE 36c
Coelomic fluids from urchins injected with TNP-LPS in FCA, or FCA, and from non-injected controls were added to three replicate ELISA plate wells coated with TNP-BSA or BSA, or which were blocked with BSA. Coelomic fluid binding was measured with polyclonal sera against general coelomic fluid proteins. Control wells which did not receive coelomic fluid were also present. Each graph represents a set of three animals, one from each treatment group. An example of the ELISA binding pattern (EBP) indicative of the production of a specific molecule against TNP can be seen expressed by the non-injected animals in every set. The presence of EBP's was not dependent on treatment (X^2 test). Absorbances were read 35 minutes after substrate addition.

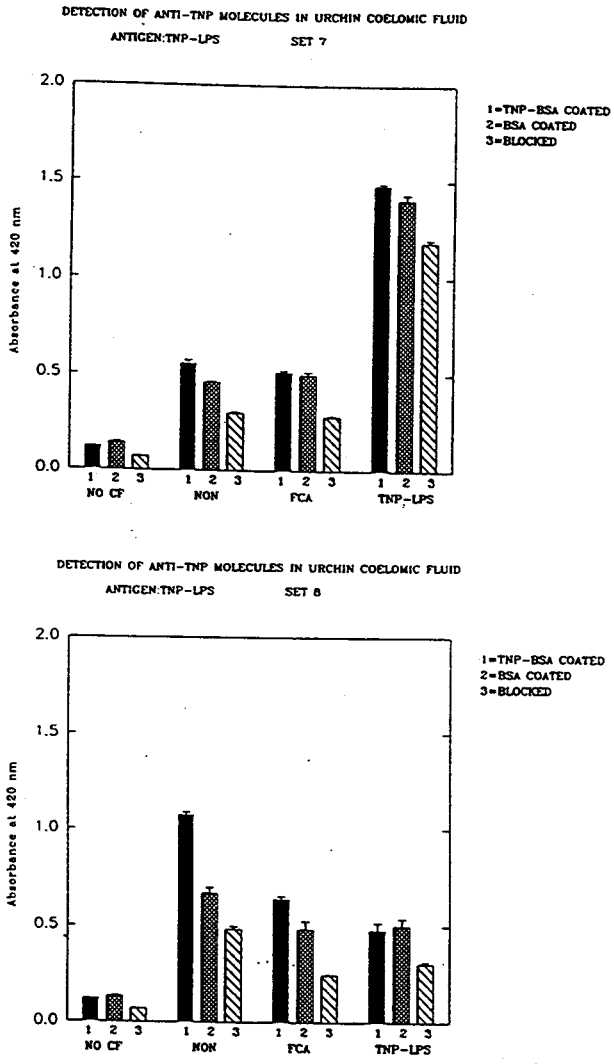


FIGURE 36d
Coelomic fluids from urchins injected with TNP-LPS in FCA, or FCA, and from non-injected controls were added to three replicate ELISA plate wells coated with TNP-BSA or BSA, or which were blocked with BSA. Coelomic fluid binding was measured with polyclonal sera against general coelomic fluid proteins. Control wells which did not receive coelomic fluid were also present. Each graph represents a set of three animals, one from each treatment group. An example of the ELISA binding pattern (EBP) indicative of the production of a specific molecule against TNP can be seen expressed by the non-injected animals in every set. The presence of EBP's was not dependent on treatment (X^2 test). Absorbances were read 35 minutes after substrate addition.

TABLE 6

PROTEIN CONCENTRATIONS OF COELOMIC FLUID
FROM URCHINS IN TNP-LPS EXPERIMENT

SET	CONCENTRATION (μg/ml)		
	NON-INJ	**FCA-INJ**	**TNP-LPS-INJ**
1	520	480	240
2	180	520	370
3	340	270	300
4	260	210	220
5	370	210	220
6	150	300	240
7	280	125	370
8	370	260	240
AVERAGE	**308.7 ± 41.8**	**296.8 ± 48.2**	**267.5 ± 26.1**

Urchins were injected with TNP-LPS in FCA (TNP-LPS-INJ), adjuvant alone (FCA-INJ), or were non-injected controls (NON-INJ). Nine weeks later, coelomic fluids were collected and protein concentrations were measured using BCA Reagents (Sigma). Average concentrations were compared among treatment groups and the differences were not significant.