Channel Catfish, *Ictalurus punctatus*, T-Lymphocyte Mitogenic Responses to Outer Membrane Proteins and LPS of the Gram Negative Rod Bacterium, *Edwardsiella ictaluri*

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CHANNEL CATFISH, *ICTALURUS PUNCTATUS*, T-LYMPHOCYTE MITOGENIC RESPONSES TO OUTER MEMBRANE PROTEINS AND LPS OF THE GRAM NEGATIVE ROD BACTERIUM, *EDWARDSIELLA ICTALURI*

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Although the immune system of the channel catfish has been studied for many years now, the role of the channel catfish T-lymphocytes is still poorly understood. There are several efforts underway to develop a vaccine against *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish. Current vaccines can induce a specific antibody response which confers some protection; but it is not 100% effective. An understanding of the cellular, as well as the humoral, immune response is needed to develop an effective vaccine. In addition, identifying the outer membrane proteins that induce an antibody and cell-mediated response will allow investigators to develop a vaccine against *E. ictaluri*. In this project, the channel catfish T-lymphocyte mitogenic responses to outer membrane proteins of *E. ictaluri* were examined. It was shown that 10 μg of protein was sufficient to induce a significant proliferative response. There was a significant mitogenic response to *E. ictaluri* outer membrane proteins which occurred in the range of 54-66 hours in channel catfish pre-exposed to *E. ictaluri* and in the range of 66-78 hours in control channel catfish. Channel catfish T-cells were also shown to respond to outer membrane proteins of *Escherichia coli* and *E. ictaluri* to a similar

extent. Proliferative assays involving *E. coli* and *E. ictaluri* lipopolysaccharide resulted in a weak mitogenic response or no response at all.

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Introduction

Channel Catfish Immune System Overview

The immune system of the channel catfish, *Ictalurus punctatus* (Figure 1), is capable of eliciting both a humoral and cell-mediated response. In response to an invading pathogen channel catfish B-lymphocytes produce specific antibodies against the pathogen (Miller et al., 1985). The antibodies bind and target the pathogen for destruction via phagocytosis by neutrophils and macrophages. Specific antibody in the presence of complement can initiate bactericidal activity and serve in the recruitment of macrophages. Channel catfish neutrophils are capable of nonspecific phagocytosis (Ainsworth, 1990). Channel catfish macrophages phagocytose and digest bacteria non-specifically, process and present antigens, and produce and secrete cytokines. Although the presence of morphologically identifiable thymus tissue in a variety of fish species is well established (Botham and Manning, 1981; Gorgollen, 1983; Finstad et al., 1964; Tatner and Manning, 1982; Tamura, 1978), the functional role(s) of cells from such tissue remains to be elucidated. The role of T-lymphocytes in catfish immunity is unclear, although their assistance is probably required for the development of protective immunity (Sizemore el al., 1984).

Edwardsiella ictaluri and Enteric Septicemia of Catfish (ESC)

Edwardsiella ictaluri is a gram-negative rod (0.5x1.3 μm) that is able to survive in catfish and in pond bottom mud (Plumb and Quinlan, 1986). *E. ictaluri* is the causative agent of enteric septicemia of catfish (ESC), and is the leading cause of bacterial mortality in cultured channel catfish in the United States (Hawke, et al., 1979). *E. ictaluri* is most frequently isolated from the brain and kidneys of catfish with enteric septicemia of catfish (Areechon, 1982; Eisenmann, 1986).

Figure 1. Channel Catfish, *Ictalurus punctatus*. Catfish currently accounts for about one half of the aquaculture production in the United States (photo by E.R. Degginger).

Enteric Septicemia of Catfish (ESC) Pathology

The *E. ictaluri* disease complex can occur clinically as acute or chronic ESC (MacMillan, 1985). Acute ESC is the more frequent manifestation of clinical disease (Newton et al., 1989). Acute ESC develops about 4-6 days after exposure to *E. ictaluri* and is characterized grossly by cutaneous petechial hemorrhage and ulceration at the base of the fins and tissues surrounding the nares. Outbreaks of acute septicemic disease occur during the summer months when the water temperatures are between 20 and 30°C. Non-ictalurid warm water fish appear to be resistant to *E. ictaluri* (Plumb and Sanchez,1983). Chronic ESC develops 3-4 weeks after exposure and is most frequently characterized by dorsocranial swelling and ulceration, granulomatous olfactory neuritis/perineuritis, and meningoencephalitis involving the olfactory bulbs, olfactory tracts and olfactory lobes of the brain. These observations have suggested that the olfactory and intestinal mucosa may be the sites of entry of *E. ictaluri* into catfish (Newton, et al., 1989). Systemic infection is disseminated from the brain. Internally, petechial haemorrhages are spread over the viscera and there is generally a haemorrhagic ascites (Miyazaki and Plumb, 1985). Histologically the principal features are those of any bacterial septicemia: focal necrosis of spleen, kidney and liver, and generalized hemorrhage, but it is particularly characterized by the development of true abscesses which are not typical of many fish diseases. Melanomacrophage centers are destroyed, and there is a high frequency of melanin-containing leukocytes in engorged vessels (Plumb et al,. 1986). The pathogenesis of ESC is not completely understood but it is believed catfish that survive ESC epizootics become carriers and serve as the primary resevoir for *E. ictaluri.*

One of the most economically important diseases involving channel catfish, ESC is hard to control. The high morbidity and mortality associated with ESC and it's rapid onset in the spring and fall make it a major threat to catfish farming (MacMillan, 1985). Severity of reported ESC cases range from low (5%) to severe (50%) mortality, which results in a loss of millions of dollars in fish, feed and treatment to the channel catfish industry (Plumb, 1988). ESC occurs in channel catfish of all ages (Plumb, 1988).

Channel Catfish Antibody and the Humoral Response to *E. ictaluri*

E. ictaluri infection is capable of inducing a specific antibody response in channel catfish. A number of assays have been employed demonstrating significant serum levels of antibodies to *E. ictaluri* including agglutination (Saeed and Plumb, 1987) and ELISA (Waterstat, et al., 1989; Klesius, et al., 1991). Channel catfish immunoglobulin is distributed in a wide variety of tissues including kidney, spleen, liver and intestine (Klesius, 1992). Channel catfish antibody is called IgM because of the similarities of it's structural properties to mammalian IgM. Channel catfish immunoglobulin has a tetrameric structure with a relative mass of about 700 kDa consisting of polypeptide H and L chains of 70 kDa and 21 kDa to 24 kDa, respectively (Lobb and Clem, 1983). The antibody possesses eight identical antigenic binding sites. Varying in carbohydrate content, the antibody has 2 isotypes of L chains and possibly up to 4 isotypes of H chains (Lobb, et al., 1984). It has considerable size heterogeneity, due to the unusual arrangement and numbers of H chain cysteine residues that form the disulfide bridges linking the antibody subunits (Ghaffari and Lobb, 1989). The only difference between catfish mucosal antibody and serum antibody is that mucosal antibody lack accessory proteins or J chains (Lobb, 1984). In mammals, the function of the J chain is to facilitate the formation of the subunits of IgA and IgM

into their appropriate polymeric structure. Channel catfish antibody has been shown to activate complement. Ourth and Bachinski (1987) demonstrated that both classical and alternative complement pathways were activated. Complement, together with specific antibodies, play a bactericidal role against catfish pathogens. Complement activation is an important means of defense against gram negative bacteria. The alternate pathway provides some degree of non-specific protection against some gram negative bacteria. The presence and amount of sialic acid on the surface of the gram negative bacteria determines whether the alternative complement pathway is effective in destruction of the bacteria. Bactericidal activity is strongest against bacteria lacking sialic acid on their surface and weakest against those bacteria that have large amounts of surface sialic acid, like *E. ictlarui.*

Channel Catfish Cell-Mediated Response to *E. ictaluri*

Cellular responses to *E. ictaluri* in channel catfish have also been demonstrated. Channel catfish neutrophils are capable of phagocytizing *E. ictaluri*, although not as readily as other bacterial species (Ainsworth and Dexiang, 1990). In addition, Waterstat, et al. (1991) used phagocytic and bactericidal assays to show the extracellular killing by neutrophils was more effective than it's intracellular killing ability. The ability of neutrophils to phagocytize *E. ictaluri* is dependent on opsonins and serum components (Scott, et al., 1981).

Macrophages also play an important role in protective immunity against *E. ictaluri.* Channel catfish possess both mobile and fixed macrophages that are morphologically and functionally similar to mammalian monocytes. Macrophages play three major roles in the catfish defense against *E. ictaluri*: (a) non-specific phagocytosis, processing and specific presentation of *E. ictaluri* antigens on their surface, (b) destruction of *E. ictaluri* by non-specific phagocytosis, and

(c) cytokine production and release, which allows channel catfish immune system cells to function more proficiently and communicate with each other (Ellsaesser and Clem, 1994).

It is hypothesized that the proteins located in the outer lipid membrane of *E. ictaluri* are the antigenic agent responsible for inducing a cell-mediated response. It has been previously shown that *E. ictaluri* outer membrane proteins could be resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) into ten bands ranging in molecular weight from 71 kDa to 19.5 kDa (Newton, et al., 1990). Proteins located in the outer membrane of *E. ictaluri* are the primary antigens that evoked an immune response (Klesius and Horst, 1991). The outer membrane of many gram-negative organisms is the site of several virulence factors including fimbriae (Peterson and Quie, 1981), lipopolysaccharide (Ratzen, 1979), and outer membrane proteins (Orskov, 1979). Outer membrane proteins (OMP) prepared with sodium N-lauryl sarcocinate (SLS) from 33 *E. ictaluri* isoates were examined by SDS-PAGE and their profiles compared. Ten bands were identified in all isolates ranging from 71 kDa to 19.5 kDa in molecular weight. One major 35-kd protein band comprises most of the protein content of the outer membrane. Differences exist among isolates in the amount of protein within minor OMP bands. *E. ictaluri* ATCC 33202 contains larger quantities of the 38.5 and 37 kDa proteins than did the other isolates (Newton, et al., 1990).

Another outer membrane antigen, lipopolysaccharide (LPS), causes the catfish to have a high susceptibility to this bacterium. These complex molecules are of particular interest because of their pathophysiological properties, i.e., they induce endotoxin shock (Ribi et al., 1979), pyrogenicity (Galanos et al., 1972), macrophage activation (Weinberg et al., 1978), B-lymphocyte mitogenicity (Chiller et al.,1973), interferon production (Feingold et al., 1970), and tumor

regression (Ribi et al., 1975). They consist of three chemically distinct regions: *(i)* an O-specific polysaccharide of repeating di-, tri-, tetra-, or penta-saccharide units; *(ii)* a polysaccharide core characterized by the presence of 2-keto-3-deoxy-octulosonic acid (KDO; 3-deoxy-D-mannose-2-octulosonic acid [2-keto-3-deoxy - D-mano-octonic acid]), and a heptose sugar; and *(iii)* lipid A which is usually composed of an acylated and phosphorylated glucosamine dimer. The structures of LPS from numerous bacterial species have been studied and show both common and distinct features (Imoto et al., 1983). LPS isolated from *E. ictaluri* evokes an immune response when injected into channel catfish (Saeed and Plumb, 1986), and can be serologically detected in the serum of immunized fish (Saeed and Plumb, 1987).

Antigen Processing and Presentation

Antigen processing and presentation are among the central events involved in the induction of immune responses to thymus-dependent antigens (Chain et al., 1988). The processing of antigen is a primary transduction event where in antigens are taken up by accessory cells and modified to a state which subsequently interact with major histocompatibility complex (MHC) molecules (Unanue, 1984; Chestnut and Grey, 1985; Chain et al., 1988) to form a bimolecular complex that is now recognizable by T-cells (Babbitt et al., 1985; Buus et al., 1986; Ashwell et al., 1988; Berzofsky et al., 1988). Unlike B-cells, T-cells recognize "processed" antigen in association with "self" MHC and not just the antigen itself. The requirement for accessory cells (monocytes) during fish *in vitro* immune responses can be replaced by exogenous IL-1 (Miller et al., 1985; Clem et al., 1985; Ellsaesser, 1989). The employment of isolated leukocyte subpopulations demonstrated that both catfish B (Ig+) lymphocytes and monocytes are efficient antigen presentors of hemocyanins (Vallejo et al., 1990).

Influencing the Channel Catfish Immune System

There are a variety of factors influencing the immune response of channel catfish to pathogens like *E. ictaluri*. Changes in water temperature, seasonal changes, stress, and diet are significant in the way they affect the channel catfish immune system.

A decrease in water temperature results in a temporary suppression of the catfish immune system. The rate and degree of water temperature change determine the extent of immunosuppression. After adapting to the water change, the normal function of the catfish immune system returns. Bly and Clem (1991) studied the effect that very low water temperatures had on the catfish immune system. They found that a rapid drop in water temperature from 23 to 11°C over a 24-hour period suppresses both B and T cell function as measured by *in vitro* responses to mitogens. Mitogen-induced proliferation of B cells recovers after 3 weeks of acclimation of fish to 11°C when measured *in vitro* at 27°C. Antibody production *in vitro* at 27°C is initially suppressed by decreased water temperature, but partially recovers after the fish are acclimated to 11°C for about 5 weeks. Proliferation of T cells in response to mitogens require a 2 week longer period of acclimation of fish to 11°C.

This decrease in water temperature to 11°C induces a temporary change in the ratio of blood cell subpopulations. T cells increase 80% and B cells decrease 10%. A normal T/B cell ratio is recovered after 3 to 5 weeks of acclimation of the fish to 11°C. The decreased water temperature has no effect on the total number of lymphocytes. The fish become anemic at the lower water temperature and remain so for 14 weeks or longer. The number of neutrophils are not affected.

Seasonal outbreaks of ESC caused by *E. ictaluri* may occur because of the effect of water temperature on immunity. Fall and spring are the most prevalent

seasons for outbreaks of ESC because the changes in water temperatures are the greatest during these seasons and they occur rapidly. Recently, yearling fish have been shown to be dormantly infected with *E. ictaluri* and immune to challenge infections (Klesius, 1992). Perhaps, a rapid change in water temperature suppresses immunity of these carrier fish and they develop ESC. Susceptible fish become infected from the diseased carriers and the ESC outbreak increases in it's size and intensity (Klesius, 1992).

Stress-induced immunosuppression in catfish is produced by handling, transport and cortisol administration. Ellsaesser and Clem (1986) reported that handling and transport results in hematological and immunological changes in catfish. The immunosuppressive changes last about one week. Handling and transport produces a reduction in the numbers of both circulating T and B cells. The number of neutrophils increase in peripheral blood while the macrophage populations remain unchanged in response to this stressor. Bly et al, (1990) showed that handling and transport suppress mitogen-induced lymphocyte proliferation and antibody responses to both T-dependent and independent antigens. They also showed that the loss of immune reactivity is not caused by suppressor cells or accessory cells and the increased number of neutrophils, found after stress, is not the cause of immunosuppression of T and B cell responses to mitogens.

Cortisol administration by injection also induces hematological and immunological changes in catfish that are similar to the characteristics caused by handling and transport (Ellsaesser and Clem, 1987). Within 18 hours after cortisol administration the numbers of circulating blood lymphocytes decreases and the number of neutrophils increase. The mitogen-induced lymphocyte proliferation is transiently suppressed.

The effect of diet on the immune response in catfish has not received much attention, but it was found thatÜ¥e□=À

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mg/kg) enhances the killing of *E. ictaluri* by catfish macrophages and higher levels of vitamin E (2500 mg/kg) suppresses killing of *E. ictaluri* by the macrophages.

Channel Catfish Vaccinology

A major goal of studying immune responses in channel catfish is to find a vaccine for *E. ictaluri.* A good vaccine should induce a sufficient humoral and cell mediated response that would result in the 100% destruction of the bacteria. After developing a vaccine, an effective delivery system must be determined. The routes of vaccine administration are injection, immersion bath (with or without hyperosmotic immersion), and oral with food. Injection is usually by the intraperitoneal route using killed whole cells. It is time consuming, labor intensive, and practiced in limited situations which are usually small scale. Immersion of catfish in an antigen bath is one of the most practical methods of large scale immunization. Bath immersion is also done in hyperosmotic conditions, using various chemicals to increase the uptake of antigen from the bath. These chemicals are used for various periods of time from minutes to an hour at different concentrations. Another method of large scale immunization is incorporation of antigen in catfish feed. However, oral immunization is most often used to booster primary immunization rather than stimulate the primary antibody response to antigen in feed. Finally, controlled release of antigen by encapsulation of antigen, in various media, is considered a promising method of oral vaccine delivery. Liposomes, microbeads, microcapsules, and oils are examples of encapsulation media for fish vaccines (Klesius, 1992).

Lobb (1987) showed when catfish are immunized by bath immersion, antibody is present more consistently in the cutaneous mucus than in serum. The antibody present in the mucus is structurally identical to antibody in the serum. It was concluded that bath immersion results in production of mucosal antibody, and it's production is independent of serum antibody. This indicates that immersion may not be the most effective method of producing systemic antibody responses in the catfish (Klesius, 1992).

Live and killed vaccines are commonly employed when immunizing channel catfish. Killed vaccines can be subdivided into whole cell, disrupted cell and, semi-purified cell preparations. Live vaccines can also be subdivided into modified, attenuated or genetically transformed carriers (Klesius, 1992).

A whole cell vaccine utilizes heat or formalin to kill the bacteria turning it into a polyvalent immunogen called a bacterin. Bacterins represent a large number of the vaccines that are approved for use in food animals such as channel catfish. Bacterins stimulate antibody responses, and boosters are needed for long-lasting, high titer responses. Since cell-mediated immunity is not stimulated by bacterins, protection is limited to responses mediated by antibody (Klesius, 1992).

Disruption of bacterial cells by sonication or pressure cell provides a greater number of antigenic components. Disrupted bacterial cell vaccines produce antibody responses: not cell-mediated immune responses. Generally, better protection is not associated with the delivery of a large number of antigens, and this kind of vaccine is less frequently employed than the whole cell bacterin.

Semi-purified cell vaccines assume that microbes have a single protective antigen. Isolating this protective antigen and using it in immunization elicits a humoral response. Again, cell-mediated immunity is not produced by this type of vaccine (Klesius, 1992).

Live vaccines stimulate both cell-mediated and humoral immunity. However, live vaccines that contain virulent organisms, for use in food animals, are not approved. Genetic modification of virulence had led to the development of live vaccines that are avirulent. Avirulent bacteria are produced by mutation using radiation or chemical mutagens. Modified live vaccines replicate in the host and antigenic stimulation is usually longer than for killed vaccines. A novel idea for a live vaccine is genetic transformation of a nonpathogenic microbe that will replicate and express the recombinant gene in the host. Protection is provided by antibody produced against the recombinant protein antigen. The traditional approaches to the development of an ESC vaccine include killed whole bacterial cells, killed disrupted bacterial cells, and semi-purified and purified bacterial cell components (Klesius, 1992).

Objectives

The objective of the proposed research was to determine if outer membrane proteins of *E. ictaluri* produce T-lymphocyte mitogenic responses. Specific cells, involved in the cell-mediated immune response, were isolated from channel catfish previously exposed to *E. ictaluri* and those never exposed to *E. ictaluri*. These T-lymphocytes and macrophages/monocytes were placed into culture with outer membrane protein preparations from *E. ictaluri*. ^{3}H-thymidine, a deoxy-nucleic acid was then added to the culture and the T-lymphocyte mitogenic response of incorporating ^{3}H-thymidine into T-lymphocyte daughter cell DNA was determined by liquid scintillation spectroscopy. The ^{3}H-thymidine incorporated in daughter T-cells originating from channel catfish previously exposed to *E. ictaluri* was compared with ^{3}H-thymidine incorporated in daughter T-cells originating from channel catfish that had never been exposed to *E. ictaluri*.

Significance

A major goal of investigative efforts into ESC and the channel catfish immune system is the development of a vaccine against ESC. An effective vaccine could save the catfish industry millions of dollars presently lost to mortality, feed and treatment. The results of this study will contribute to the development of a more effective vaccine based on a better understanding of those mechanisms which induce protective immunity. In addition, more insight into the channel catfish T-lymphocyte and it's functional significance in channel catfish immunity will be gained. Very little is known about the channel catfish T-lymphocyte at present. By establishing and becoming familiar with a system designed to examine the *in vitro* interactions of catfish T-lymphocytes and specific antigens, other aspects of channel catfish T-lymphocyte biology can be investigated. Examples of future investigations include the isolation and characterization of channel catfish T-cell receptors, the interactions between monocytes and T-lymphocytes, and the isolation and characterization of cytokines.

Materials and Methods

Bacteria

Edwardsiella ictaluri, ATCC 33202 (ATCC, Bethesda, MD) were maintained on brain heart infusion agar (Difco Laboratories, Detroit, MI) immersed in sterile mineral oil at 26°C. *E. ictaluri* isolated from the brains of pre-exposed catfish and *E. ictaluri* cultures were positively identified by performing a gram stain for morphological characteristics and by performing a series of biochemical and enzymatic test. Results were compared to those of *E. ictaluri* found in *Bergey's Manual of Determinative Bacteriology Ninth Edition* (1994).

Channel catfish, *Ictalurus punctatus*

Channel catfish, never exposed to *E. ictaluri*, were obtained from local farm ponds and transported back to the lab in a 30-gallon, aerated Coleman cooler and were used as control channel catfish. Fish were held in 50–gallon glass tanks at room temperature for 4 weeks to overcome the effects of transport stress (Ellsaesser and Clem, 1986). They were fed to satiation 3 times/week. *E. ictaluri*-exposed channel catfish were collected from the Frankfort Fish Hatchery in Frankfort, Kentucky by either hatchery harvesting or hook and line. These channel catfish were transported back to the lab the same way as control channel catfish. Channel catfish were bled in the lab on the same day that cell separation was performed. Channel catfish were anesthetized before bleeding.

Monoclonal antibodies

Murine monoclonal antibodies (mAb) to channel catfish immunoglobulin (mAb 9E1) and to a channel catfish T-cell marker (mAb 13C10) have been described previously (Sizemore, et al., 1984; Ellsaesser, et al., 1988), and were kindly provided by Dr. William Clem of the Department of Microbiology,

University of Mississippi Medical Center, Jackson, MS. Specificity of the monoclonal antibodies was checked by enzyme linked immunosorbent assay (ELISA).

Enzyme Linked ImmunoSorbent Assay (ELISA)

The following protocol is for the indirect method for assay of antibody (Voller et al., 1979). The enzyme labeled antibody used was sheep anti-mouse IgG (M-8642-Sigma, St. Louis, MO) conjugated with horse radish peroxidase and the substrate used was *o*-Phenylenediamine (OPD). Microtiter wells were coated with 50µl of 2.0 mg/ml concentrations of mAb-9E1(9E1), mAb-13C10 (13C10), or Normal Catfish Serum (NCFS) in Phosphate Buffered Saline (PBS). The microtiter plate was then allowed to incubate overnight at 4°C. The solution was knocked out of the wells into the sink. The wells were then blocked for 1 hour with 200µl of 3.0% Bovine Serum Albumin (BSA) in PBS at room temperature. In blocking, BSA, a smaller protein the antibody, makes it's way into crevices formed by antibody and coats the areas of the petri-dish that antibody did not cover. This prevents subsequent antibody from binding the petri-dish and altering the results. The wells were knocked out and washed three times with 250 µl PBS containing 0.05% Tween-20/well. A squeeze bottle was used to facilitate speed. The wells were then incubated at room temperature for two hours on a rotary shaker with 100 µl of the appropriately diluted α-mouse IgG isotype (IgG, IgG1, IgG2a, IgG2b, and IgG3), PBS, Normal Mouse Serum (NMS), and monoclonal antibodies 9E1 or 13C10. The wells were knocked out and washed three times with the PBS/Tween-20 solution. The wells were allowed to incubate with 100 µl of a 1:1000 dilution of anti-mouse IgG with horse radish peroxidase conjugate in PBS for two hours at room temperature. The wells were emptied and washed six times with the PBS/Tween-20 solution. The wells were then incubated with OPD

in citrate buffer (Add 2 µl of H_2O_2 to 10 ml of 0.1 M citrate buffer, pH 5.0, containing 5 mg of OPD), containing H_2O_2 for 30 minutes at room temperature in the dark. The reaction was then stopped by adding 50 µl of 2.5 M H_2SO_4 for 30 minutes. The wells were read by an automatic microtiter plate reader (Dynatech), the results were recorded, and specificity was determined.

Preparation of bacterial outer membrane proteins

One liter of brain heart infusion broth (Difco Laboratories, Detroit, MI) was inoculated with *Edwardsiella ictaluri* and incubated at 28°C with moderate shaking for 12-18 hours. Cells were collected by centrifugation at 12,000*g* for 20 minutes at room temperature, and the supernatant was discarded. The pellet was washed once by suspension in 25 ml of 10 mM HEPES buffer, pH 7.4 (Sigma Chemical Co., St. Louis, MO). Washed cells were resuspended in 10 ml of 10mM HEPES buffer, then sonicated four times for 15 seconds while cooling in an ice bath. Intact cells and large debris were removed by centrifugation at 1,700*g* for 20 minutes. The supernatant was retained, then the total membrane preparation was harvested from the supernatant by centrifugation at 100,000*g* for 60 minutes at 4° C. The pellet was resuspended in 1 ml of 2% sodium lauryl sarcosinate (SLS; Sigma) in 10 mM HEPES buffer for 30 minutes at 24°C. The detergent insoluble fraction was harvested by centrifugation at 100,000*g* for 60 minutes at 4°C and resuspended in 4 ml of distilled water (Barenkamp, et al., 1981). Protein concentration was determined by using a Bradford's standard assay.

Bradford's standard assay for determination of protein concentration

Bovine Serum Albumin was used as the protein standard. Several dilutions of protein standard containing 20 µl, 40 µl, 60 µl, 80 µl, 100 µl, 120 µl, 140 µl, 200 µl and 250 µl/ml. One-hundred µl of protein standards and appropriately diluted unknown samples were dispersed in clean, dry test tubes. To prepare a

"blank", 100 µl of phosphate-buffered saline (PBS) was placed into the test tube. Five ml of diluted dye reagent (80 ml H_2O and 20 ml Bio-Rad dye reagent) was added to each test tube. Each test tube was then carefully vortexed to avoid excess foaming. After a period of 10 minutes the blank tube, protein standards, and unknown samples were analyzed by spectroscopy for their ability to absorb light at a wavelengh of 595nm (OD_{595}). The blank test tube was used to calibrate the spectroscope to zero absorbance or 100% transmittance. Standard curves for each assay were used to determine protein concentration.

Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE technique of Laemmli (1970) was used on outer membrane protein preparations to show that proteins were separated individually from the membrane lipid bilayer. Briefly, the gel sandwich was assembled into the clamp assembly and then into the gel casting stand as directed by the BioRad Mini-Protein II Dual Slab Cell Instruction Manual. Ten ml of fresh 10% (weight/volume) sodium dodecyl sulfate (SDS) and 1 ml of 10% (weight/volume) ammonium persulfate (AP) was prepared. The AP was kept on ice until used. Ten ml of 10% resolving gel solution was prepared. Immediately after the addition of the AP and TEMED, the gel solution was dispensed in between the glass plates using a pasteur pipet. The gel was then overlaid with distilled water. The gel was allowed to polymerize for 1 hour. Five ml of 4% stacking gel solution was prepared, but the AP and TEMED were not added at this time. After the resolving gel had polymerized, the distilled water was discarded from the top of the gel. The comb was inserted between the glass plates until there was a very slight space between the notches of the comb and the shorter glass plate; this allowed air to escape when the stacking gel was poured. The AP and TEMED were added to the stacking gel solution. The stacking gel solution was immediately poured using a

Pasteur pipet. The comb was then pushed down in between the glass plates and polymerization continued for 45 minutes. After the stacking gel had polymerized, the comb was removed and the wells were rinsed with distilled water. Excess distilled water was then removed from the wells using a syringe. The gel sandwich was assembled into the electrophoresis apparatus as directed by the manufacturer's instructions. Running buffer was then poured into the upper and lower buffer chambers. Next, the sample to be electrophoresed was mixed with an equal volume of 2X sample treatment buffer. It was then boiled for 3 minutes, then placed on ice. Ten μg of total protein were loaded into each well. The original sample was concentrated or diluted so that 10 μg of protein per 25 μl of solution could be loaded into each well. The sample was loaded into the well using a 10-100 μl micropipetter. Five μl of high molecular weight markers were loaded into one well and 5 μl of low molecular weight markers were loaded into an adjacent well. The safety lid was placed onto the electrophoresis unit and the leads were attached to the power supply. The electrophoresis was run at 100 volts until the dye front enters the resolving gel, then the power was increased to 150 volts and run until the dye front entered the running buffer in the lower buffer chamber. The total run time was 1-1.5 hours. The power supply was turned off, the leads were disconnected, and the gel sandwich was disassembled. The gel was then notched in one corner and subjected to gel staining.

SDS-PAGE gel staining

Coomassie blue and silver staining techniques were used to develop protein bands that resulted from the migration of the different molecular weight proteins. In comassie blue staining, the notched gel was then placed in coomassie blue staining solution (0.25 g coomassie blue, 900 ml dH_2O, 100 ml glacial acetic acid) for 20 minutes with gentle agitation. The gel was then transferred to a destaining

solution (50 ml methanol, 875 ml dH_2O, 75 ml glacial acetic acid) overnight. After destaining, the gel was dried for a permanent record. In silver staining, before the gel can be stained it must be exposed to a series of washes and staining solutions must be prepared. The initial wash consisted of leaving the gel in methanol for 1-3 hours. The gel was then washed overnight in 2X distilled water. The gel then was allowed to soak for 1-3 hours in 50% methanol with 1 drop of 38% formaldehyde for every 100 ml of solution. Three staining solutions needed to be prepared at this point in the procedure. Solution A consist of dissolving 0.8 g silver nitrate in 4 ml 2X distilled water. Solution B is prepared by mixing 21 ml 0.36% NaOH and 1.4 ml ammonium hydroxide. To prepare solution C, solution A was added dropwise into solution B with constant stirring. The volume was then brought up to 100 ml with 2X distilled water. The gel was then rinsed several times with 2X distilled water. The gel was stained in solution C for 15 minutes with gentle agitation, then exposed to three 5 minute washes in 2X distilled water. At this point the gel was allowed to soak in developer (2.5 ml 1% citric acid, 0.25 ml of 38% formaldelyde, bring to 500 ml with distilled water) with agitation until bands appear, usually 10-30 minutes. When sufficient band intensity had developed the gel was rinsed in 2X distilled water and development was stopped by washing in 50% MeOH/10% acetic acid for 10 minutes. Gel was then dried for a permanent record.

Lipopolysaccharide extraction and purification

Lipopolysaccharide (LPS) was extracted from wet cells using the hot aqueous phenol procedure of Westphal and Jann (1965). For removal of nucleic acid contaminants, the LPS was treated with deoxyribonuclease and ribonuclease (Sigma Chemical Co., St. Louis, MO) as described by Stevens et al. (1980). LPS

extraction as described above was performed on cultures of *E. ictaluri* and *E. coli* in order to test channel catfish cell-mediated responses to these extracts.

Lymphocyte panning

Channel catfish peripheral blood leukocytes (PBLs) were separated into surface immunoglobulin (+) and surface immunoglobulin (-) populations by indirect panning (Buttke et al., 1983; Sizemore et al., 1984). Polystyrene Petri dishes were coated with 10 ml of a rabbit anti-mouse IgG (Organon-Teknika Corp., WestChester, PA) (0.2 mg/ml in phosphate buffer saline, PBS, pH 7.4) for 36 hours at 4°C. The antibody solution was aspirated and the plates were washed three times with sterile PBS. Catfish-RPMI (9 parts RPMI 1640, and 1 part distilled water with 10 mM HEPES, 1% L-glutamine, 100 U penicillin and 100 U streptomycin; CF-RPMI) containing 2% bovine calf serum and 0.1 mg normal rabbit serum/ml was added to the dishes for a 40 minute incubation at 22°C. Isolated channel catfish leukocytes (2×10^8/ml) were mixed with murine monoclonal antibody-producing hybridoma tissue culture supernatant 9E1, which is specific for channel catfish B-lymphocytes, at a final dilution of 1:3 for 30 minutes at 4°C with occasional mixing. The cells were washed twice in CF-RPMI and diluted to 6×10^7/ml in CF-RPMI and 10 ml of the cell suspension added to the rabbit anti-mouse IgG coated dishes. Upon incubating the dishes at 4°C for 60-90 minutes with gentle swirling every 15 minutes, the non-adherent cells (T-lymphocytes) were aspirated and retained and the fluid from one wash was saved (Ainsworth, et al., 1990). An indirect immunofluorescence assay, using T and B cell markers, was done each time the cells were separated to show homogeneity of the separated population.

Indirect immunofluorescence assay

A modification from Current Protocols in Immunology (1995) was used to perform the indirect immunofluorescence assay. Separated cells were resuspended in 5 ml of Hank's balanced salt solution (HBSS). Cells were washed 2X by centrifugation at 300 *g* in HBSS. The pellet obtained from the second wash was resuspended in mAb culture supernatant of either mAb 9E1 or mAb 13C10 for 60 minutes at room temperature. Cells were pelleted and washed twice. The cells were then resuspended and incubated at room temperature with rabbit anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) diluted to 1 µl/ml in HBSS. Cells were then washed 2X and resuspended in 1 ml of HBSS. Cells were mounted on microscope slides and analyzed for immunofluorescence by fluorescent microscope (Zeiss Inc., Germany). The total number of cells were counted and compared with the number of fluorescing channel catfish T or B cells.

Preparation of Micro-exudate coated plates

Sizemore et al. (1984) found that Baby Hamster Kidney (BHK) cells, a fibroblast cell line, secretes proteins that adhere to the bottom of a microtiter well. These adhesive proteins remain bound even after the fibroblasts are broken loose and removed from the well. When channel catfish peripheral blood leukocytes (PBL) are added to these wells and allowed to incubate, channel catfish macrophages bind these adhesive proteins. Remaining peripheral blood leukocytes can be washed out leaving only the bound channel catfish macrophages. Briefly, BHK cells were cultured in RPMI 1640 medium with 10% FBS, 1% Pen/Strep at 37°C in atmosphere of 7.5 % CO_2/95.5% humidity. They were allowed to grow to confluence on the bottom of a sterile tissue flask. They were then detached from the tissue flask with 0.25% trypsin, 0.30% EDTA, in RPMI 1640. The cells were harvested, washed two times, and a cell count was performed. Ten-thousand BHK

cells in 100 µl of culture medium were added to each well of a sterile 96-well microtiter plate. The BHK cells were then grown to confluence in the 96-well microtiter plate. The culture medium was decanted and the BHK cells were detached with 10 mM EDTA in PBS followed by repeated washes with PBS. The plates were stored at 4°C until they were to be used. Immediately before use, microexudate-coated wells were washed twice with CF-RPMI with 2% pooled catfish serum.

In vitro culture of channel catfish T-lymphocytes and monocytes

E. ictaluri-infected channel catfish T-lymphocytes were cultured with autologous peripheral blood monocytes in 96-well microtiter plates. Studies of channel catfish T-lymphocyte response to mitogens have shown that these responses are dependent on the presence of monocytes in the culture medium (Ellsaesser, et al., 1988). The peripheral blood monocytes were isolated and cultured *in situ* on baby hamster kidney cell (BHK; ATCC) microexudate-coated microtiter culture wells (Sizemore, 1984). One million channel catfish peripheral blood leukocytes, obtained from the 1.060-1.065 interface of the Percoll gradient, were applied to each well and allowed to adhere for 2-3 hours. The wells were extensively washed to remove nonadherent cells. To these cells 10^6 channel catfish T-lymphocytes in 1 ml of CF-RPMI supplemented with 10% human serum (Gibco) and 5% pooled catfish serum (cell culture medium; Faulman, et al., 1983) were added and cultured for 2 days at 27°C.

Lymphoproliferative assay systems

Channel catfish T-lymphocytes were assayed for their ability to incorporate ^{3}H-thymidine in response to stimulation by a variety of *E. ictaluri* outer membrane proteins. Cells were then cultured in the presence of 10 µg of protein from a total outer membrane protein preparation and pulsed with 10 µl of 0.5

μCi/ml of ^{3}H-thymidine (Amersham Corp., Arlington Heights, IL) 18 hours prior to harvesting by water lysis onto glass fiber filters. Channel catfish monocyte antigen processing and presentation time requirement involved culturing cells with 10 μg of protein from a total membrane protein preparation, pulsing it with 10 μl of 0.5 μCi/ml of ^{3}H-thymidine at 12 hour intervals, and harvesting the cells 18 hours later by water lysis onto glass fiber filters. ^{3}H-thymidine incorporation was assessed by liquid scintillation spectrometry (Bly and Clem, 1991). Controls included cultures of OMP fractions and naive channel catfish T- lymphocytes, and cultures of naive and previously infected channel catfish T-lymphocytes with the T-lymphocyte mitogen concanavalin A (Con A; Sigma, St. Louis, MO).

Trypan Blue Exclusion Test of Cell Viability

The dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, whereas dead cells do not posses intact cell membranes which will allow dye to enter the cell. In this test, a cell suspension is simply mixed with dye and then visually examined to determine whether cells take up or exclude dye. In the protocol presented here, a viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm. For each set of cells used in the lymphoproliferative assays, three wells were used to test cell viability on a daily basis between the time the cells were put into culture and the time that antigen or mitogen was added. Briefly, cells were harvested from wells and centrifuged for 5 minutes at 100 x g and the supernatant discarded. The cells were diluted to a final concentration of 5×10^{5} cells/ml of PBS. One part 0.4% trypan blue was mixed with one part cell suspension and the mixture was allowed to incubate for ~3 minutes at room temperature. Cells should be counted within 3 to 5 minutes of mixing with trypan

blue, as longer incubation periods will lead to cell death and reduced viability counts. A drop of the trypan blue/cell mixture was applied to a hemacytometer and placed on the stage of a binocular inverted microscope. The number of unstained cells were counted and cell viability was assessed (Current Protocols in Immunology, 1993).

Statistical analysis

All assays were conducted in triplicate, and statistical significance was determined by student t-test ($p<0.05$) at a 95% confidence level. Channel catfish T-lymphocyte proliferative responses to OMP were compared to basal mitogenic levels. T-lymphocytes of control and previously exposed channel catfish were also compared to measure differences in the response. Figure 2 illustrates the experimental design used to determine if outer membrane proteins of *E. ictaluri* produce channel catfish T-lymphocyte mitogenic responses.

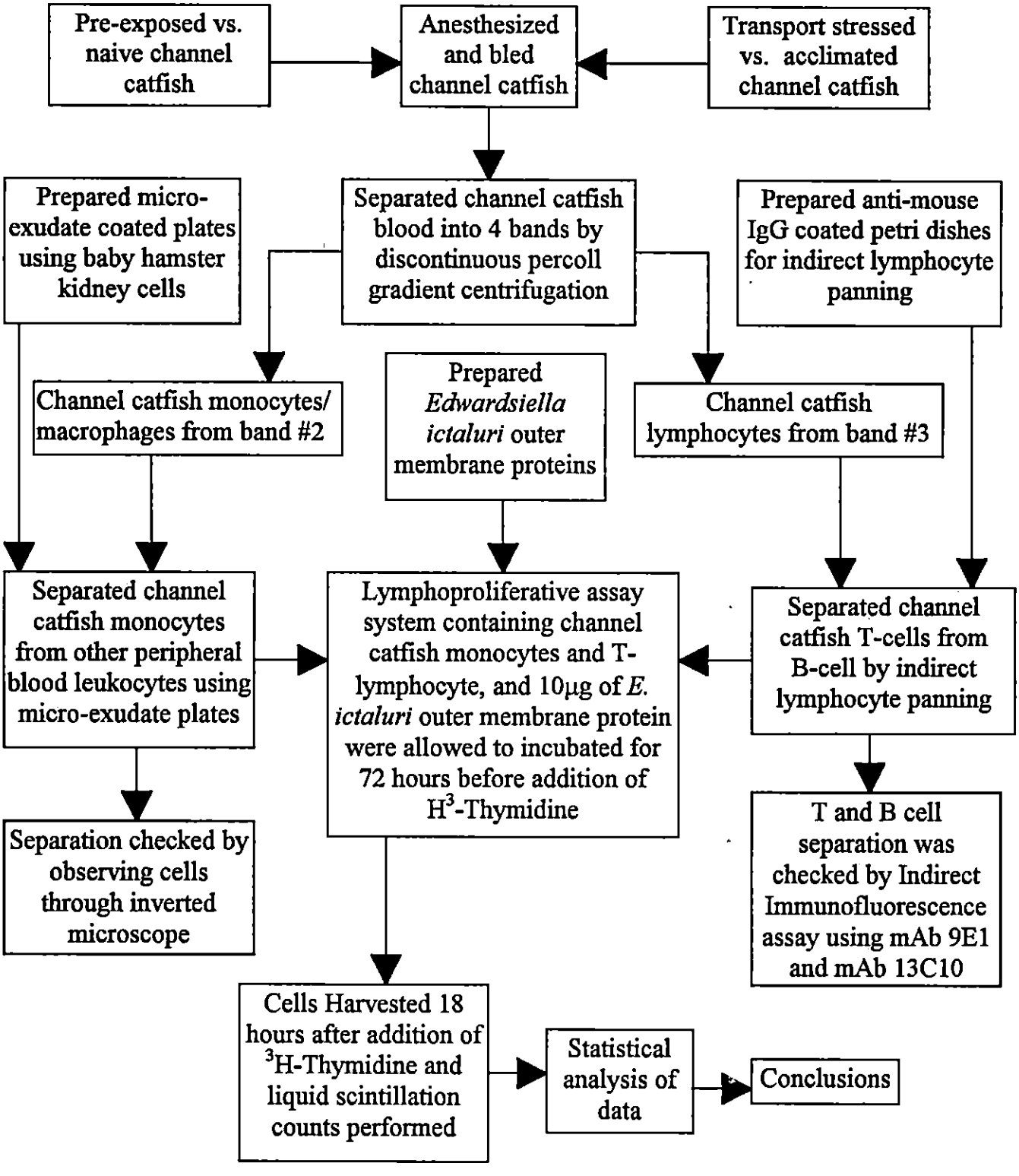


Figure 2. Experimental design to determine if channel catfish, *Ictalurus punctatus*, T-lymphocytes respond to outer membrane proteins of *Edwardsiella ictaluri*.

Results

Experimental bacteria was *Edwardsiella ictaluri*

Biochemical and enzymatic test results showed that bacterial proteins used in lymphoproliferative assays were from E. ictaluri. These test also showed that bacteria isolated from thebrain of previously exposed channel catfish was E. ictaluri. Biochemical and enzymatic test and test results are shown in Table I. The test results were located and compared with those of *E. ictaluri* found in Bergey's Manual for Determinative Bacteriology Ninth Edition (1994).

Table I. Biochemical and Enzymatic identification of *Edwardsiella ictaluri*. Isolation of *E. ictaluri* was accomplished on a blood agar plate.

Biochemical and Enzymatic Test	+ Phenotypic Result	Phenotypic Results of Biochemical and Enzymatic Test (+/-)
o-nitrophenol isopropylthiogalactopyranoside	yellow	-
Arginine Dihydrolase	red	-
Lysine Decarboxylase	red	-
Ornithine Decarboxylase	red	-
Citrate	blue	-
Hydrogen Sulfate	black deposit	-
Urease	red	-
Indole	red	-
Sodium Pyruvate/Creatine	red	-
Gelatin	diffusion	-
Glucose Metabolism	yellow	+
Other Sugar Metabolism	yellow	-
Nitrate Present	no bubbles	-
Nitrate Reduction (N_2 gas)	Bubbles	+

Anti-mouse IgG specifically binds mouse IgG's mAb 9E1 and mAb 13C10

Enzyme linked immunosorbant assay showed that anti-mouse IgG specifically binds all isotypes of mouse IgG, including IgG1, IgG2a, IgG2b, IgG3, mAb 9E1, and mAb 13C10. ELISA results were gathered by automatic microtiter plate reader measuring optical density at 495 nm. Although anti-mouse IgG binds mAb 9E1(OD_{495}=.415) is greater than the binding affinity between anti-mouse IgG and mAb 13C10 (OD_{495}=.166). Monoclonal Ab 9E1 and mAb 13C10 have a higher affinity for mouse IgG3 than all other isotypes of mouse IgG.

Outer membrane proteins were separated from each other during preparation

It has been previously shown that *E. ictaluri* outer membrane proteins could be resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) into ten bands (Newton et al., 1990). Figure 3 SDS-PAGE results show that outer membrane preparations used in this experiment were separated into ten bands of proteins.

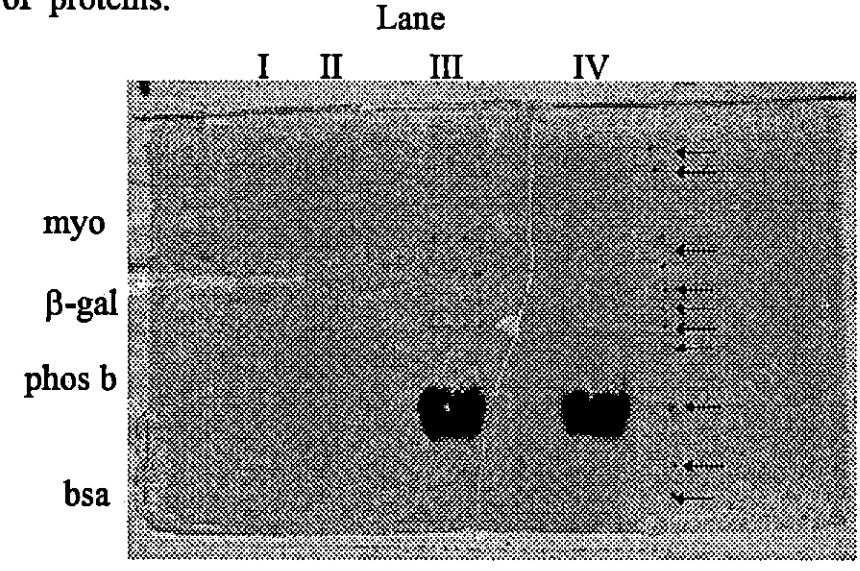


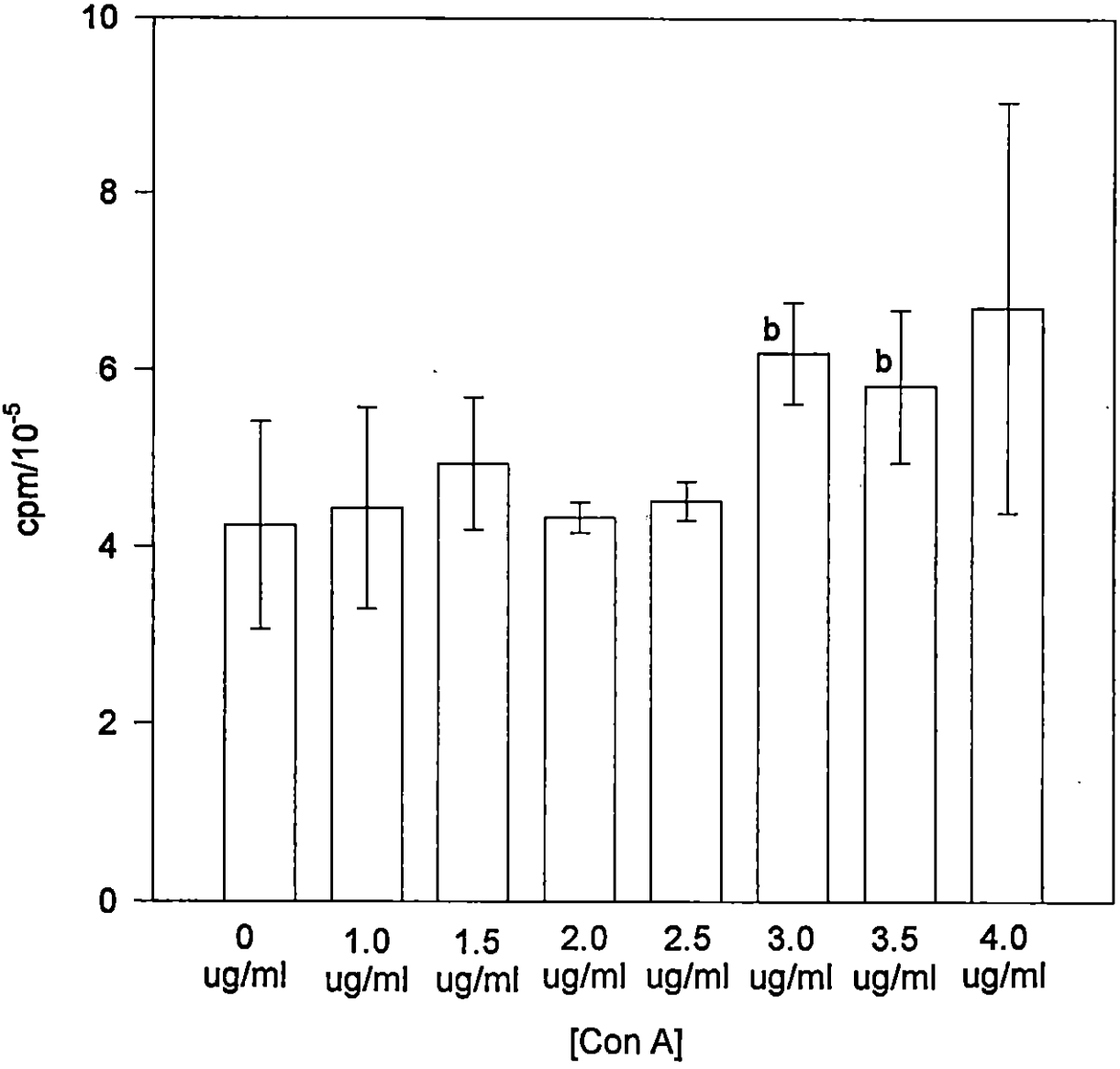
Figure 3. Silver Stained SDS-PAGE of *E. ictaluri* outer membrane protein prepatation showing separation into ten individual proteins. Lanes I and II, molecular weight standards; Lanes III and IV, *E. ictaluri* outer membrane preparations. Standards used were myosin (myo), β-galactosidase (β-gal), phosphotase B (phos B), and bovine serum albumin (bsa).

Channel catfish T-cells respond to T-cell mitogen, concanavalin A

Concanavalin A stimulated channel catfish T-lymphocytes to proliferate. The results of these responses are shown in Figure 4. The proliferative response of channel catfish T-cells to concanavalin A was higher than constitutive cell levels after the 18 hour incubation period in the presence of ^{3}H-thymidine. Stimulation with 10 µl of 1.0 µg/ml, 1.5 µg/ml, 2.0 µg/ml, or 2.5 µg/ml of concanavalin A did not show any significant difference in the proliferative response in comparison to constitutive cell levels (*p* values= 0.8614, 0.4625, 0.8883, and 0.6895, respectively). Increasing the amount of concanavalin A to 3.0 µg/ml, 3.5 µg/ml, or 4.0 µg/ml increased the proliferative response of channel catfish T-cells in comparison to basal level mitosis, but was not statistically significant with values of p= 0.0810, p=0.1757 and p=0.2712 respectively.

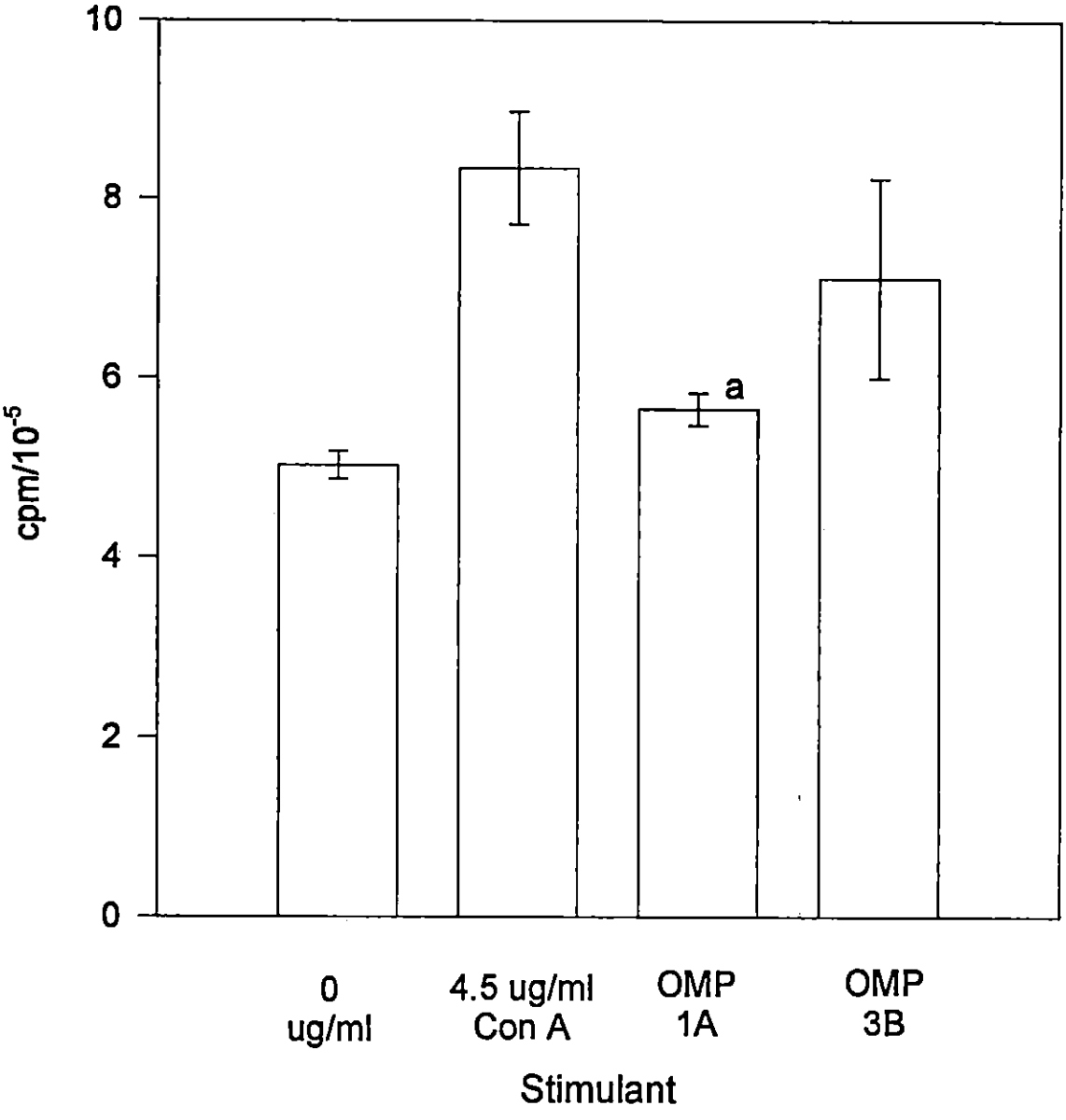
Channel catfish T-cells respond to crude outer membrane protein preparations

Channel catfish T-cells are induced to proliferate when stimulated with *E. ictaluri* outer membrane proteins as well as with concanavalin A. The results of these comparisons are shown in Figure 5. The proliferative response of channel catfish T-cells to *E. ictaluri* outer membrane protein (OMP) preparations 1A and 3B were higher than constitutive cell levels after the 18 hour incubation period in the presence of ^{3}H-thymidine. The proliferative response of channel catfish T-cells to OMP 1A was statistically higher than the basal levels of mitosis in unstimulated channel catfish T-cells with a *p* value=0.0273. Although the proliferative response to OMP 3B was higher than constitutive levels, it is not statistically significant (*p*=0.0879). Both channel catfish T-cell cultures stimulated with OMP 1A and OMP 3B had lower responses than the culture stimulated with 4.5 µg/ml of concanavalin A.



[b] 3.0 and 3.5 ug/ml had significant increases compared to 0 ug/ml, 2.0 and 2.5 ug/ml

Figure 4. Channel catfish T-cell responses to T-cell mitogen concanavalin A (Con A).



[a] OMP 1A had significant increase compared to media alone.

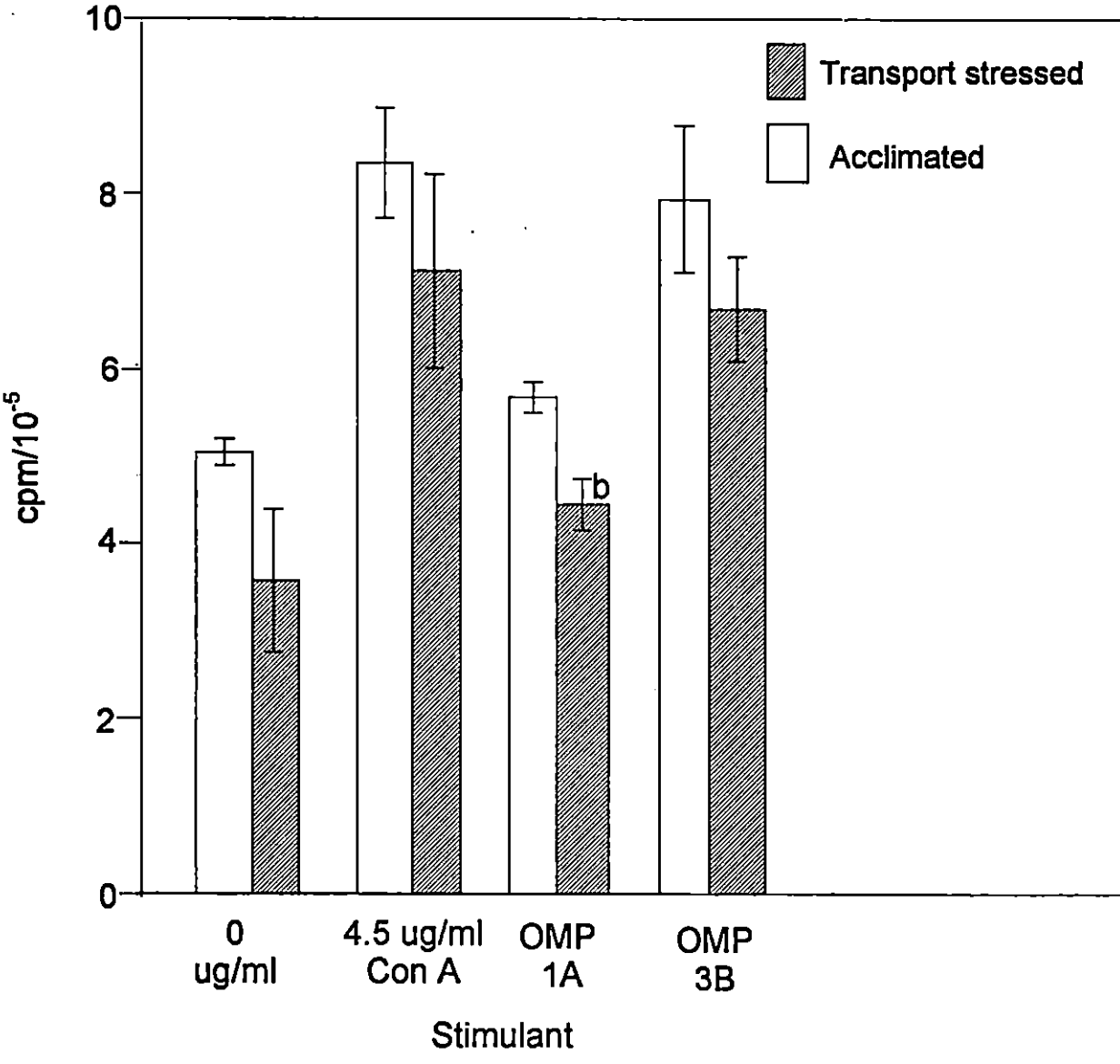
Figure 5. Pre-exposed Channel Catfish T-cell responses to *Edwardsiella ictaluri* outer membrane proteins (OMP) preparations 1A and 3B and concanavalin A (Con A).

Transport stress suppresses the channel catfish cell-mediated response to *E.ictaluri*

The proliferative response of channel catfish T-cells is reduced after exposing the fish to transport stress. A comparison of acclimated and transport stressed induced channel catfish T-lymphocyte mitogenic responses are shown in Figure 6. In comparison to acclimated channel catfish, transport stressed channel catfish had a significantly less mitogenic response to *E. ictaluri* OMP 1A (p=0.0035), but not to OMP 3B. In transport stressed channel catfish, the constitutive level of mitosis were less than acclimated channel catfish. The response of transport stressed channel catfish T-cells to both OMP 1A and OMP 3B was higher than constitutive levels, but the response T-cell response to OMP 1A was not statistically higher than constitutive levels (p=0.1280). The response to OMP 3B was statistically higher than constitutive levels, with a p value=0.0235. Although transport stressed channel catfish T-cell response to OMP 3B was higher than that of OMP 1A, it was not significantly higher (p=0.0435). Transport stressed channel catfish T-cell response to both OMPs were higher than that of constitutive levels, however their responses to these OMPs were either comparable or lower than basal levels of mitosis in acclimated channel catfish.

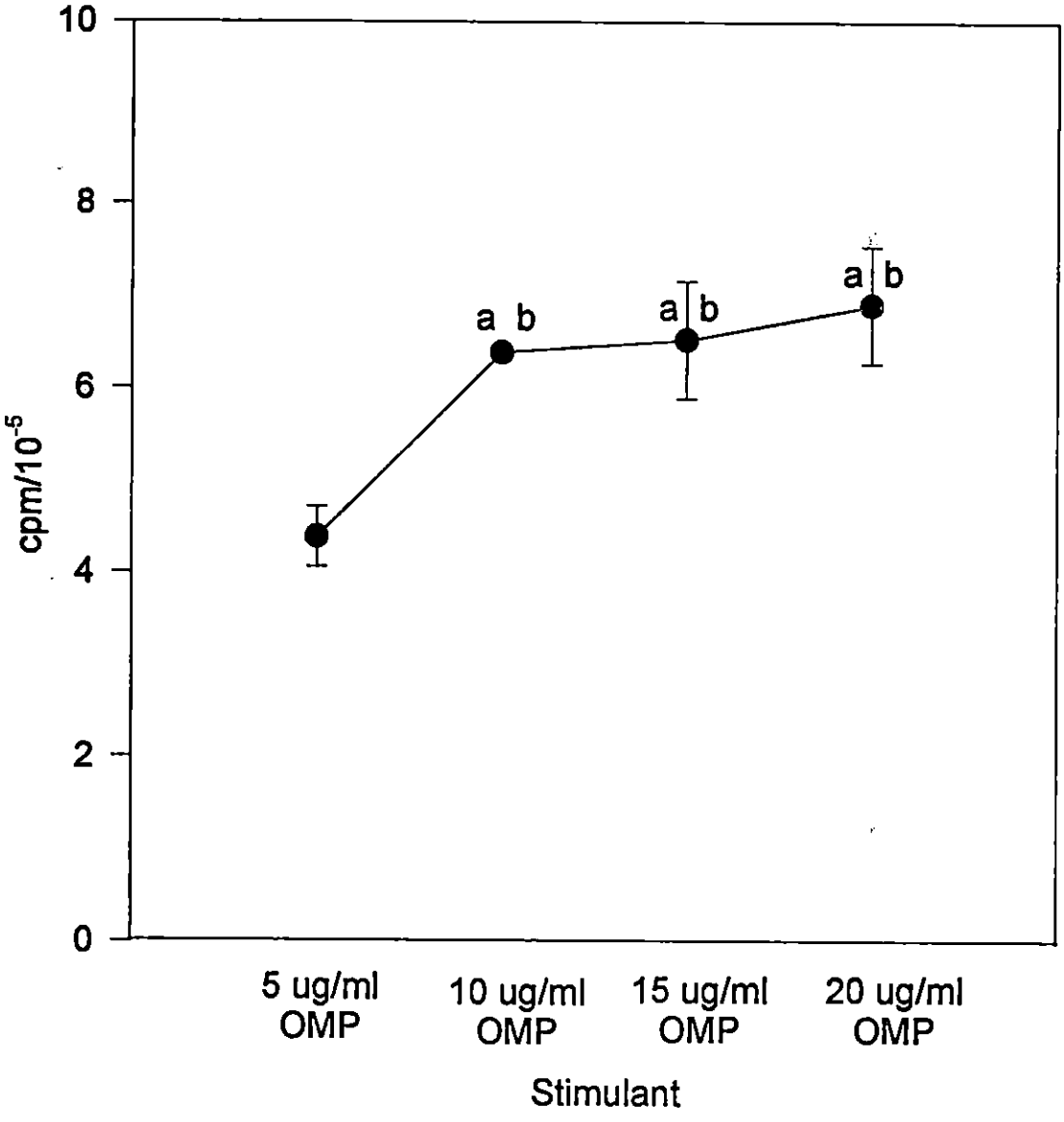
Mitogenic response increases as outer membrane protein concentrations increase

Channel catfish T-cell response to increasing protein concentrations increases with the amount of protein present. These data are shown in Figure 7. In comparison to basal mitogenic levels, channel catfish T-cells stimulated with 5.0 μg/well of OMP 3B did not induce a significant proliferative response (p=0.7362). On the other hand, when the *E. ictaluri* outer membrane protein preparation was increased to 10 μg/well, 15 μg/well and 20 μg/well, a significant increase occurred. The P values were: 10 μg/well (p=0/0093), 15 μg/well (p=0.0173), and 20 μg/well (p=0.0103). The proliferative response to OMP concentrations of



b significantly less than acclimated channel catfish when stimulated with OMP 1A.

Figure 6. Acclimated and transport stressed channel catfish T-cell responses to outer membrane proteins (OMP) preparations 1A and 3B of *E. ictaluri* and concanavalin A (Con A).



[a] significant increase compared to 0 ug/ml OMP.
[b] significant increase compared to 5 ug/ml OMP.

Figure 7. Channel catfish T-cell mitogenic responses to increasing concentrations of *E. ictaluri* outer membrane proteins (OMP).

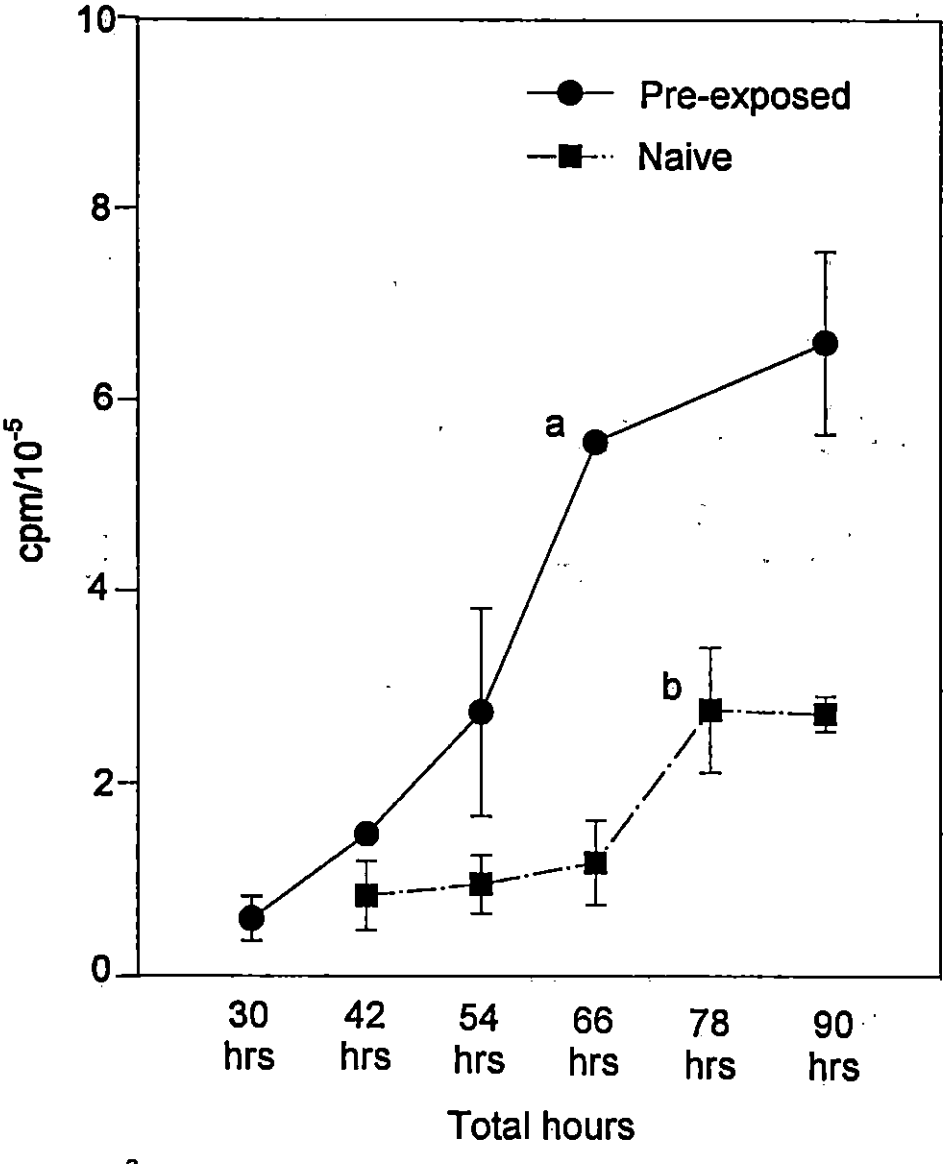
15 μg/well and 20 μg/well were higher than OMP concentration of 10 μg/well, but not significantly higher (p=0.7293 and p=0.2317). Ten μl of 4.5 ug/ml concanavalin A solution was used to show that channel catfish T-cell cultures were being stimulated as a positive control.

Pre-exposed channel catfish T-cells show a serious response between 54-66 hours

The time required for pre-exposed channel catfish macrophages to process and present *E. ictaluri* outer membrane proteins and induce a significant T-cell mitogenic response occurred at sometime in the range of 54-66 hours after addition of OMP. These data are shown in Figure 8. When pre-exposed channel catfish T-cells were stimulated with 10 μg of OMP 3B, the first scintillation count was taken at 30 total hours in order to give us a reference point. There was a statistically significant increase in the pre-exposed channel catfish T-cell response to OMP 3B at 42 total hours in comparison to the scintillation counts at 30 total hours (p=0.0029). There was not a statistically significant increase in the pre-exposed channel catfish T-cell response to OMP 3B twelve hours later at 54 total hours (p=0.1115). At 66 total hours the proliferative response of pre-exposed channel catfish T-cells increased significantly from the counts at 54 hours (p=0.0110), more than doubling in counts per minute within a twelve hours period. The proliferative response of pre-exposed channel catfish T-cell levels off after 66 total hours showing no further significant increases up to 90 total hours (p=0.1341).

Control channel catfish T-cells show a serious response between 66-78 hours

The time required for control (naive) channel catfish macrophages to process and present *E. ictaluri* outer membrane proteins and induce a significant T-cell response occurred of 66-78 hours after addition of OMP. These data are also shown in Figure 8. When control channel catfish T-cells were stimulated with



[a] time interval for significant increased in pre-exposed channel catfish.
[b] time interval for significant increase in naive channel catfish.

Figure 8. Time required for macrophages from channel catfish pre-exposed and naive (never exposed) to *E. ictaluri* to process *E. ictaluri* antigen and initiate a T-cell mitogenic response. *E. ictaluri* outer membrane protein was added at time 0, ^{3}H-thymidine was added at 12 hour intervals and harvested 18 hours later.

10 μg of OMP 3B, the first scintillation count was taken at 42 total hours. There was not a statistically significant increase in the control channel catfish T-cell response to OMP 3B at 54 total hours in comparison to the scintillation counts at 42 total hours (p=0.6882). There was not a statistically significant increase in the control channel catfish T-cell response to OMP 3B twelve hours later at 66 total hours (p=0.5071). At 78 total hours the proliferative response of control channel catfish T-cells increased significantly from the counts at 66 hours (p=0.0243), almost doubling with a twelve hours period. The proliferative response of control channel catfish T-cells levels off after 78 total hours showing no further significant increases up to 90 total hours (p=0.9189).

Pre-exposed channel catfish T-cells respond to *E. ictaluri* and *E. coli* with similar intensities of proliferation

The proliferative response of pre-exposed channel catfish T-cells to *E. coli* outer membrane proteins are higher than responses of pre-exposed channel catfish T-cells stimulated with *E. ictaluri* outer membrane protein. Both *E. coli* and *E. ictaluri* outer membrane protein stimulated pre-exposed channel catfish T-cell cultures showed a significant increase in proliferation when compared to basal mitotic T-cell levels with respective p values of p=0.0200 and p=0.0037. This data is shown in Figure 9. Although stimulated cultures were significantly higher than basal mitotic levels and *E. coli* induced a higher response than *E. ictaluri* there was not a statistically significant difference between *E. coli* and *E. ictaluri* stimulated pre-exposed channel catfish T-cell cultures (p=0.4961).

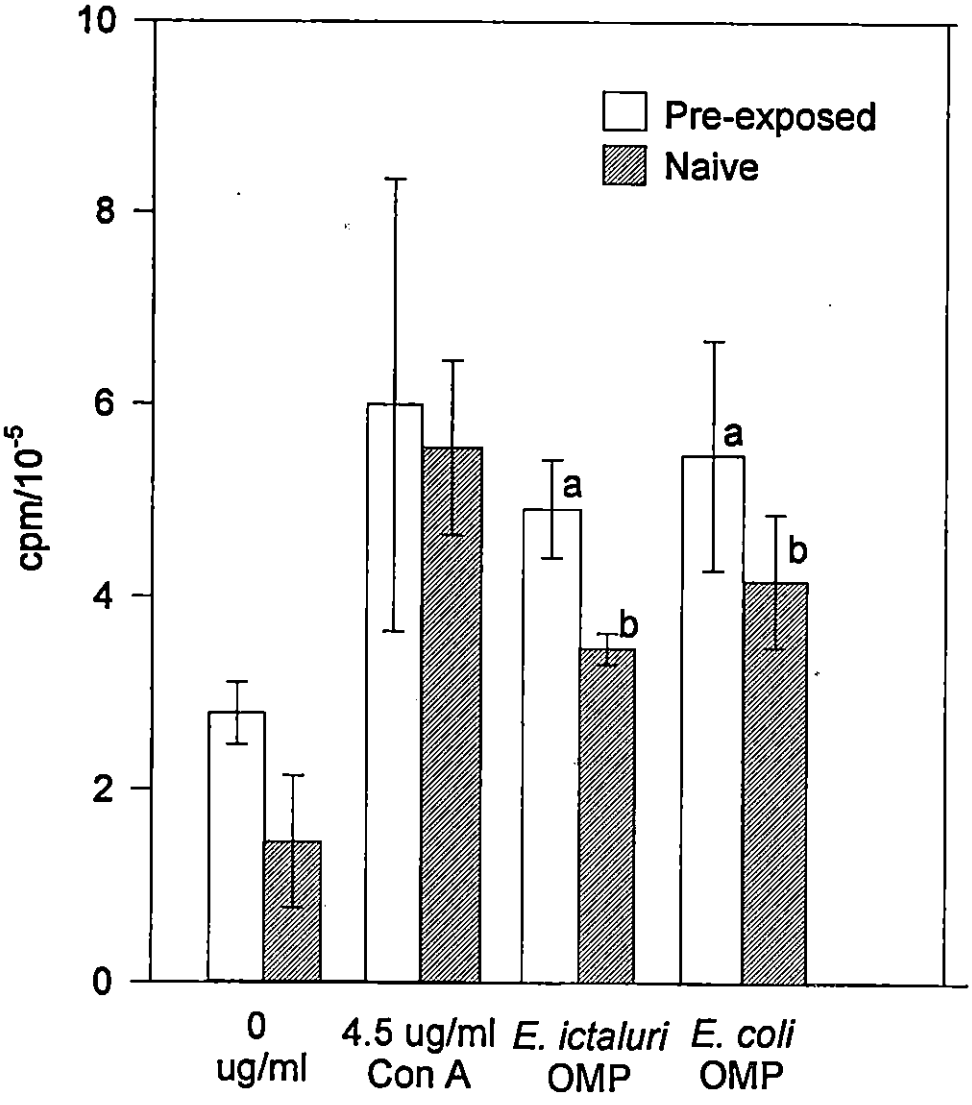
Control channel catfish T-cells respond to *E. coli* and *E. ictaluri* with similar intensities, but the response is smaller than pre-exposed channel catfish T-cells

The proliferative response of control channel catfish T-cells to *E. coli* outer membrane proteins are higher than responses of control channel catfish T-cells

stimulated with *E. ictaluri* outer membrane proteins. These data are also shown in Figure 9. Both *E. coli* and *E. ictaluri* outer membrane proteins stimulated control channel catfish T-cell cultures showed a significant increase in proliferation when compared to basal mitotic T-cell level with respective *p* values of p=0.0084 and p=0.0078. Although stimulated cultures were significantly higher than basal mitotic levels and *E. coli* induced a higher response than *E. ictaluri* there was not a statistically significant difference between *E. coli* and *E. ictaluri* stimulated control channel catfish T-cell cultures (p=0.1585). When comparing pre-exposed channel catfish T-cells to control channel catfish T-cells there is a statistically significant increase in basal levels of mitosis (p=0.0372). Pre-exposed channel catfish T-cells responded significantly higher than control channel catfish T-cells when stimulated with *E. ictaluri* outer membrane proteins (p=0.0095). On the other hand, pre-exposed channel catfish T-cells did not respond significantly higher than control channel catfish T-cell when stimulated with *E. coli* outer membrane proteins (p=0.1790).

Channel catfish T-cells do not respond well to lipopolysaccharide (LPS) of *E. ictaluri* or *E. coli*

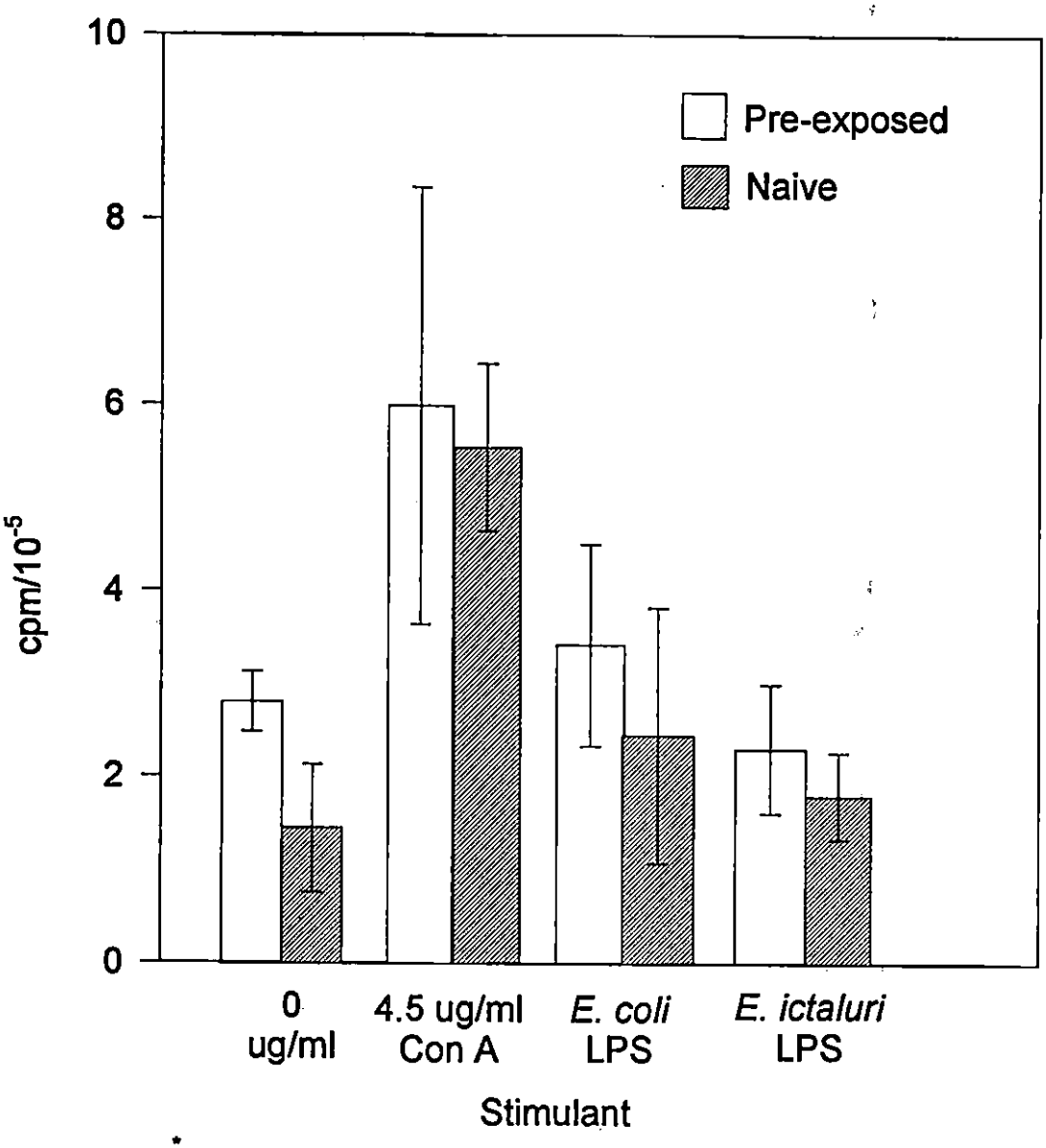
Pre-exposed and control channel catfish T-cells showed no significant increase in their proliferation response to either *E. ictaluri* LPS (p=0.3166 and p=0.1357) or *E. coli* LPS (p=0.4019 and p=0.0977) when compared to basal mitogenic levels. The results of these data are shown in Figure 10. Pre-exposed and control channel catfish T-cells did respond better to *E. coli* than to *E. ictaluri* but the increase was not statistically significant (p=0.2074 and p=0.4807).



[a] Pre-exposed channel catfish had significant increase compared to 0 ug/ml OMP.

[b] Naive channel catfish had significant increase compared to 0 ug/ml OMP.

Figure 9. Mitogenic responses of T-cells from channel catfish pre-exposed and never exposed (naive) to *E. ictaluri* to outer membrane proteins (OMP) from *E. ictaluri* and *E. coli* and concanavalin A (Con A).



*Neither pre-exposed nor naive channel catfish responded significantly to E. coli and E. ictaluri LPS in comparison to media alone.

Figure 10. Mitogenic responses of T-cells from channel catfish pre-exposed and never exposed (naive) to *E. ictaluri* to lipopolysaccharide (LPS) from *E. ictaluri* and *E. coli* and concanavalin A (Con A).

monocyte cell surface in association with major histocompatability complex molecules, much as it occurs in mammalian systems (Unanue, 1984; Chestnut and Grey, 1985; Chain et al., 1988). The T-cell can then bind this complex, be activated and initiate a mitogenic response.

All immune responses are effected by a number of factors which includes handling and transport stress (Ellsaesser and Clem, 1986). Most of this previous work involved the humoral response rather than the T-cell mediated response. Handling and transport stress, which was induced by transporting the fish from the hatchery to the laboratory in a confined tank, caused a marked reduction in the T-cell proliferative response. It also appears that basal mitogenic levels in transport stressed fish were less in comparison with acclimated channel catfish T-cells.

In order to save experimental materials, we also wanted to know what amount of *E. ictaluri* outer membrane protein would be sufficient stimuli for channel catfish T-cell cultures. When 5.0 μg of OMP was added to the culture wells, the response was comparable to basal level mitosis. When the amount of protein was increased to 10.0 μg we saw a dramatic increase in the T-cell proliferative response. At concentrations of 15.0 μg and 20.0 μg, the response was comparable to the response obtained by 10.0 μg of protein. Therefore, 10.0 μg was determined to be the optimal amount of protein to be added to each T-cell culture in order to obtain a significant stimulatory response.

In mammalian systems, the consequence of T-cell activation is a series of defined events which occur over a period of several hours. The earliest events, within seconds, appear to be a breakdown of membrane phospholipids and the activation of certain enzymes. The cell, which was in a resting state, then starts

to make new mRNAs, producing lymphokines and increasing cell surface lymphokine receptors. After about 48 hours, DNA was synthesized and the cell undergoes division (Berzofsky et al., 1987). The time required for monocytes of pre-exposed channel catfish to process antigen, present antigen and induce a T-cell mitogenic response occurred in the range of 54-66 hours. The time required for control channel catfish monocytes to process antigen, present antigen and induce a T-cell mitogenic response occurred in the range of 66-78 hours. This work supports the hypothesis that T-cells from channel catfish that have already been exposed to *E. ictaluri* are located in the G_1 phase (pre-DNA synthesis) of the cell cycle. Also, the slower and less extensive response of naive channel catfish T-cells in comparison to previously exposed channel catfish supports the hypothesis that resting T-cells are located in the G_0 phase (resting stage) of the cell cycle.

Channel catfish T-cell responses to *E. ictaluri* in comparison to other non-specific gram negative bacteria like *E. coli* were also investigated. The greater response obtained from T-cells by stimulation with *E. coli* suggest that *E. coli* and *E. ictaluri* outer membrane proteins share some cross-reactivity, the outer membrane protein preparations were contaminated with lipopolysaccharide, or *E. ictaluri* naive channel catfish T-cells are not naive of *E. coli*, but have been previously exposed to *E. coli*. In order to take away the possibility that outer membrane protein preparations were contaminated with lipopolysaccharide, channel catfish T-cell mitogenic responses to purified lipopolysaccharide were also investigated. The responses to lipopolysaccharide of *E. ictaluri* and *E. coli* showed no significant increase when compared to basal mitogenic levels. Therefore contamination by lipopolysaccharide was ruled out as an option.

The concept of cross-reactivity proposes that a single outer membrane protein may have more than one antigenic epitope found within the molecule. One of

these many antigenic epitopes found in an *E. ictaluri* and *E. coli* outer membrane protein may be exactly the same. After determining the antigenic epitopes cross-reactivity between *E. ictaluri* and *E. coli* could be investigated by producing monoclonal antibodies specific for each antigenic epitope and testing them to determine cross-reative binding.

Because *E. coli* is such an ambiguous organism, *E. ictaluri* naive channel catfish, may not be naive to *E. coli* therefore inducing a secondary response to *E. coli*. To rule out this third possibility T-cell responses of channel catfish naive to *E. coli* could be investigated and compared to *E. ictaluri* naive channel catfish.

Channel catfish T-cell mediated response investigation could drastically impact *E. ictaluri* vaccinlogy. A good vaccine should induce a sufficient immune response that results in the 100% destruction of the bacteria. In order to mount such an immune response both humoral antibody production and the T-cell mediated response must be activated. Channel catfish B-lymphocytes produce specific antibody in response to *E. ictaluri* (Miller et al., 1985) and proteins located in the outer membrane are the primary antigens that evoke an immune response (Klesius and Horst, 1991). Observations taken in these investigations show that *E. ictaluri* outer membrane proteins induce a sufficient T-cell mediated immune response and would make a good candidate for a 100% effective vaccine.

The results of this study may provide evidence which allows investigators to develop a more effective vaccine based on a better understanding of those mechanisms which induce protective immunity. By establishing and becoming familiar with a system designed to examine the *in vitro* interactions of channel catfish T-lymphocytes and specific antigens, other aspects of channel catfish T-lymphocyte biology can be investigated. Future investigations include the isolation and characterization of *E. ictaluri* specific T-cell receptors, the

interactions between channel catfish monocytes and T-lymphocytes, and the isolation and characterization of cytokines.

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Discussion

The immune system of a channel catfish is capable of eliciting both a humoral and cell-mediated response. Channel catfish B-lymphocytes specific antibodies against *E. ictaluri* which bind and target the pathogen for destruction by macrophages, neutrophils, and complement. Channel catfish neutrophils and macrophages are also capable of non-specific phagocytosis (Ainsworth, 1990). In mammalian systems, after macrophages phagocytose the pathogen, it is digested and proteins of the pathogen are presented on the surface of the macrophage where it is recognized by a T-cell. After recognition, a T-cell will respond a number of ways including proliferation of specific T-cell clones (Babbitt et al., 1985; Buus et al., 1986; Ashwell et al., 1988; Berzofsky et al., 1988). In the present work, we observed a marked increase in the T-lymphocyte mitogenic response when cultures were stimulated with crude preparations of *E. ictaluri* outer membrane proteins. We also confirmed the previously described findings that T-lymphocytes were mAb 13C10 reactive, and responded significantly to concanavalin A in the presence of monocytes as accessory cells (Ellsaesser et al., 1988). This appears to provide a compelling argument in favor of the necessity of the presence of monocytes in T-lymphoproliferative assays (Sizemore,1984).

Observation and analysis of the results show that T-cell cultures stimulated with concanavalin A responded to a greater extent than T-cell cultures stimulated with *E. ictaluri* outer membrane proteins. This may be because the concanavalin A stimulates T-cells directly by binding to a receptor located within the outer membrane of the channel catfish T-cell. In comparison, the response caused by outer membrane proteins was smaller than that of concanavalin A perhaps because outer membrane proteins must be processed by monocytes and presented on the