Hawaii Biotechnology Group, Inc.

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10 February 94
Re: Contract Number N00014-93-C-0019

Commanding Officer
Naval Medical Research and Development Command
Scientific Officer: LT CDR P. Knechtges
National Naval Medical Center, Bldg. 1, 12th Floor
8901 Wisconsin Avenue
Bethesda, MD 20889-5606

Dear Lt. Commander Knechtges:

Enclosed is the progress report for the first year (1/1/93-12/31/93) of the contract period including all progress from the fourth quarter (10/1/93-12/31/93). The accompanying report describes our current progress for each portion of the contract. If you have any questions, please do not hesitate to contact me (ext. 380) or the project's lead scientist, Dr. Ogata (ext. 399).

Sincerely,

This document has been approved for public release and sale; its distribution is unlimited.

John M. Ivy, Ph.D.
Scientist, Group Leader
Molecular Genetics

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I. Overview

The following report covers progress made during the fourth quarter (10/1/93-12/31/93) as well as that for the entire first year (1/1/93-12/31/93) of contract number N00014-93-C-0019. Four topics are included in the contract; each topic's goal and current status are as follows:

1. The production of anti-ferret IgA antibodies. Status: **Development of purification protocol completed. Initial immunization studies in mice and production of ascites completed. Immunization of rabbits initiated.**

2. The purification of lipopolysaccharide from *Shigella* and *Campylobacter* species. Status: **Extraction and purification of LPS from *Shigella* species completed. Purification of *C. jejuni* LPS suspended due to current reductions in contract funding by the Navy.**

3. The development of an enzyme immunoassay for the detection of enteroaggregative *Escherichia coli* heat-stable toxin. Status: **Work suspended due to reductions in contract funding.**

4. The production of monoclonal antibodies against strain-specific antigenic epitopes on *Campylobacter coli* flagella. Status: **Tentative identification of a hybridoma which produces a strain-specific antibody. Second fusion performed for the production of additional hybridomas.**

II. Current Progress

1. Production of Anti-ferret IgA Antibodies

Immunoglobulin A (IgA) is the predominant immunoglobulin present in secretions such as milk and saliva and may be the first specific defense against natural infection. In serum, the molecule is present as a monomer; whereas, in secretions, IgA is present predominantly as dimers, although trimers, tetramers, and pentamers also exist. When in the dimeric form, the IgA monomers are covalently linked via intermolecular disulfide bonds between the heavy chain constant regions of the IgA monomers. Polymerization of IgA is initiated by a 14 kilodalton protein, designated "J-chain", which binds to the C-terminal cysteine of one of the monomers. In addition, a 70 kilodalton protein known as "secretory component" is linked to the macromolecule by either covalent or non-covalent forces; its function is to protect the molecule from

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proteolytic degradation.

Secretory IgA (sIgA) is thought to play a significant role in the prevention of diseases of the mucosal epithelia, such as bacterial diarrhea. Thus, studies on the production of sIgA by the host in response to the invading pathogen can provide valuable information regarding the disease process. For these studies, the use of an *in vivo* model which mimics the disease process in humans is of utmost importance. For the study of campylobacter infection, ferrets are currently used as a model. To facilitate study of the immune response to *Campylobacter jejuni* infections in ferrets, we will produce polyclonal anti-ferret IgA. The anti-ferret IgA antibodies will be a useful immunological reagent for the characterization of this *in vivo* model, thus providing insight into the disease process that occurs in man.

Methods & Materials

Preparation of clarified ferret milk. The initial step for the purification of ferret sIgA was the clarification of the whole ferret milk (supplied by Dr. Olgerts Pavlovskis of the Naval Medical Research Institute) for the removal of lipids and casein. To accomplish this, we utilized the method of Roque-Barreira and Campos-Neto (1985). Briefly, whole milk was diluted 1/2 with phosphate-buffered saline (4.3mM Na_2HPO_4, 2.7mM KCl, 137mM NaCl, pH 7.2) containing 0.02% sodium azide (PBS-azide). The diluted milk was defatted by centrifugation (12,000x*g*, 10 min, 4°C), and the aqueous layer between the floating fat layer and fatty cell pellet was collected. The aqueous layer was then treated with acetic acid until a final pH of 4.6 was achieved. Acidification of he solution precipitated casein (pI=4.6) which was then removed by centrifugation (12,000x*g*, 15 min, 10°C). The supernatant was collected, and the pH was adjusted to 7.2. The treated milk was aliquoted, then stored at -70°C. When clarified milk prepared by this method was loaded onto low-pressure chromatography columns, back-pressure and column compaction occurred. By sequentially passing the clarified ferret milk through 0.45μ and 0.2μ filters, we were able to alleviated these problems, thus indicating that filterable particles in the milk preparation were the source of the problems. For this reason, we further processed the clarified milk by centrifugation at 100,000x*g* for 90 minutes at 4°C; the resultant product was highly clarified and easily utilized in low pressure chromatography.

Identification of sIgA in ferret milk by SDS-PAGE and Western blot. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). Samples were run on 6, 8, 10, or 12% polyacrylamide gels overlaid with 3% stacking gels.

Samples were placed in sample buffer [30mM Tris buffer, pH 6.8, 3% (w/v) SDS, 10% (v/v) glycerol, and bromophenol blue], with or without 20mM dithiothreitol (DTT), then heated in a boiling water bath for 5 minutes. The samples were electrophoresed at 70V until the tracking dye neared the bottom of the gel. Proteins were visualized using a silver stain kit as described by the manufacturer (Sigma, St. Louis, Mo). Molecular weights of proteins were determined based upon their mobilities in the gel relative to those of protein standards (Bio-Rad, Hercules, CA). Murine immunoglobulins (Zymed, San Francisco, CA) were also included as standards. Western blot analysis of the milk components was performed as described by Towbin (1979). Alkaline phosphatase-labeled goat antibodies raised against murine IgA, murine IgM, and ferret IgG were used to probe the blots.

Affinity purification of sIgA. The method of Roque-Barreira and Campos-Neto (1985) was followed. Clarified ferret milk was loaded onto a jacalin agarose column (equilibrated against PBS-azide, pH 7.2) at a flow-rate of 0.1ml/minute. After loading was completed, the column was washed with fresh PBS-azide, pH 7.2, until the optical density at 280nm (OD_{280}) of the column effluent was ~0. Bound material was eluted by flushing the column with 0.5M d-galactose.

Size-exclusion and ion-exchange chromatography. The procedure is based upon the protocol of Cebra and Robbins (1966) in which IgA is separated from other immunoglobulins by size-exclusion and anion-exchange chromatography.

Clarified ferret milk was concentrated in Centriprep-100 ultrafiltration units (Amicon, Beverly, MA) by centrifugation at 330x*g* for 40-45 minutes. The filtrate was discarded, and the retentate was diluted with fresh PBS-azide then concentrated. This was repeated two times (total of 3 buffer exchanges). The buffer exchanges reduced the amounts of low molecular weight components that were present in the clarified milk preparation.

Following the final buffer exchange, the concentrate was loaded onto a Sephacryl S-400 column (Pharmacia, Uppsala, Sweden) that had been equilibrated with PBS-azide. The column flow-rate was maintained at 0.6ml/min using a peristaltic pump. The OD_{280} of the column effluent was monitored, and peak fractions were collected manually. The fraction containing the ferret sIgA was identified by SDS-PAGE and Western blot analyses.

The pooled sIgA fractions were treated with the zwitterionic detergent N-dodecyl-N,N-dimethyl-3-ammonio-1-propane sulfonate (DDAPS). DDAPS was added to a final concentration of 1% (w/v), and

the mixture was incubated at room temperature for 30 minutes. Following this, three buffer exchanges were performed in a Centriprep-100 using Tris-buffered saline (TBS; 10mM Tris, 100mM NaCl, 0.02% azide), pH 7.5, containing 1% (w/v) DDAPS as the exchange buffer. After the final buffer exchange, the concentrated sample was diluted 1/10 with fresh, cold (4°C) TBS, pH 8.5.

The detergent-treated, partially purified sIgA was further purified by anion exchange chromatography on diethylaminoethyl (DEAE) Bio-Gel A (Bio-Rad, Hercules, CA). The column was equilibrated with cold TBS, pH 8.5, and the sample was loaded at a flowrate of 0.6ml/min. Once loaded, the column was flushed with fresh TBS, pH 8.5, containing 0.1% (w/v) DDAPS until the OD_{280} of the eluent was constant. The column was then flushed with TBS, pH 8.5, without DDAPS until the baseline returned to 0. Bound material was eluted by flushing the column with TBS, pH 8.5, containing 0.15M NaCl then TBS, pH 8.5, containing 0.3M NaCl. The eluted material was concentrated in a Centriprep-30. The purity of the concentrated product was checked by SDS-PAGE and silver stain.

Immunization of Mice and Rabbits. Prior to injection into animals, the azide was removed from the immunoglobulin preparation by diafiltration against PBS using Centricon-30 devices. After the buffer-exchange was completed, the sIgA solution was sterile filtered, then stored frozen until used for inoculation of animals.

Female, 18-20 grams, BALB/c mice (Simonsens, Gilroy, CA) were immunized with purified ferret sIgA in order to examine their antibody response to the immunogen and to prepare immune sera for the development of the cross-adsorption protocol. The mice were given a primary, intraperitoneal (IP) injection of 25μg of sIgA diluted 1/3 in Freund's complete adjuvant. At two week intervals, they were boosted by IP injections of 12.5μg of sIgA diluted 1/3 in Freund's incomplete adjuvant. Test bleeds were taken prior to beginning the immunizations and approximately 1 week after each boost. Antibody responses were evaluated by Western blot using a Bio-Rad Mini-PROTEAN II multiscreen apparatus. Ascites production occurred either spontaneously or was induced by intraperitoneal injection of myeloma cells. After two collections of ascites fluid, the mice were sacrificed.

Two New Zealand White Rabbits (8-10lbs; Simonsens, Gilroy, CA) were immunized by subcutaneous and intradermal injections of sIgA in multiple sites on their backs. The primary inoculation contained 250μg of sIgA diluted 1/2 in Freund's complete adjuvant. Boosts contained 125μg of sIgA diluted 1/2 in Freund's incomplete adjuvant. Test bleeds were taken before the inception of the immunization scheme and after the first boost. Antibody responses

were evaluated by Western blot. Once the antibody titers were satisfactory, the blood was collected by cardiac puncture terminal bleeding.

Purification of Ferret IgG. Ferret IgG was purified by affinity chromatography using Staphylococcus Protein A sepharose (Sigma, St. Louis, Mo). Material from the IgG peak fractions obtained by size-exclusion chromatography was diluted 9/10 with 1M Tris-HCl, pH 8.0. The sample was pumped through a Staph Protein A column (equilibrated against 100mM Tris-HCl, pH 8.0) at a flow-rate of 0.6ml/minute. Once the sample was loaded, the column was washed with 100mM Tris-HCl, pH 8.0, until the OD_{280} of the effluent was constant, then subsequently washed with 10mM Tris-HCl, pH 8.0, until the OD_{280} was 0. The bound IgG was eluted with 100mM glycine-HCl, pH 3.0. Eluted material was immediately neutralized with 1M Tris-HCl, pH 8.0 (25μls of 1M Tris-HCl per 500μls eluate produces a final pH ~7.3).

Cross-adsorption of Immune Mouse Sera and Ascites. Cross-reactive antibodies were removed from immune mouse sera by adsorption with purified ferret IgG as follows. Immune serum was diluted in TBST [10mM Tris, 150mM NaCl, 0.05% (v/v) Tween 20, pH 8.0] containing 1% (w/v) skim milk then mixed with ferret IgG. The mixture was incubated at room temperature for 2 hours then used to probe blots of clarified ferret milk to visualize the effects of the cross-adsorption.

For the cross-adsorption of mouse ascites fluid, ferret IgG was covalently attached to an Affi-gel 10 (Bio-Rad, Hercules, CA) matrix as described by the manufacturer. Ascites fluid was diluted in 0.1M 3-[N-morpholino]propane-sulfonic acid (MOPS), pH 7.5, containing 0.02% (w/v) sodium azide then passed through a column containing the immobilized ferret IgG at a rate of ~0.5ml/minute. The column eluent was collected, and the efficiency of the adsorption was determined by Western blot. The column was regenerated using 40mM citric acid, pH 3, containing 20mM NaCl.

Results

Identification of sIgA in ferret milk. The electrophoretic pattern of the clarified ferret milk possessed a large number of protein bands. Comparison of the non-reduced milk proteins with murine IgA, IgG, and IgM standards (Zymed, San Francisco, CA) provided tentative identification of two bands as ferret sIgA (Fig 1). Both proteins were greater than 200kD in size. Their large molecular weights were consistent with the fact that secretory IgA exists as

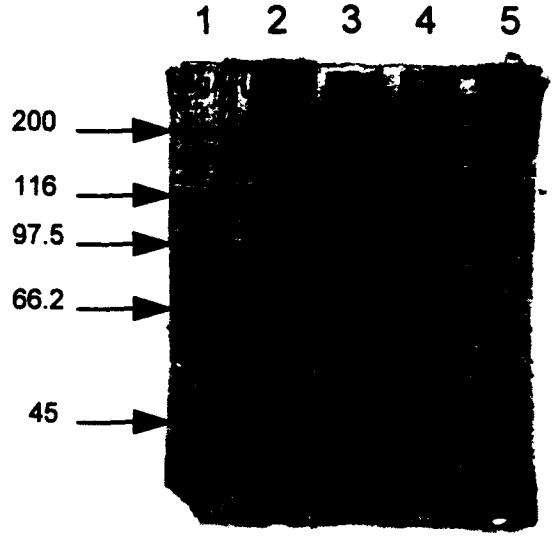


Figure 1. Silver-stained SDS-PAGE of clarified ferret milk. Lane 1) Broad MW standards (sizes indicated in kD); 2) Clarified ferret milk; 3) Murine IgA standard; 4) Murine IgG standard; 5) Murine IgM standard. Electrophoresis performed in 8% acrylamide gel. Ferret sIgA bands indicated on gel.

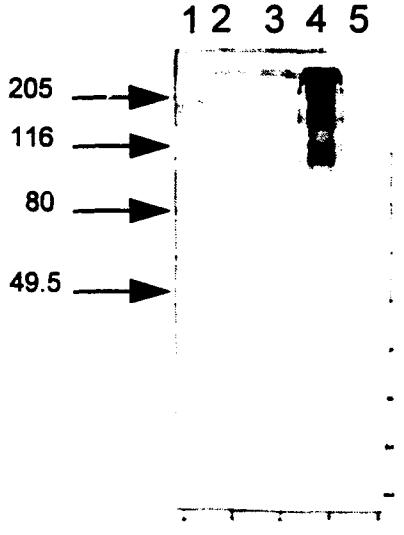


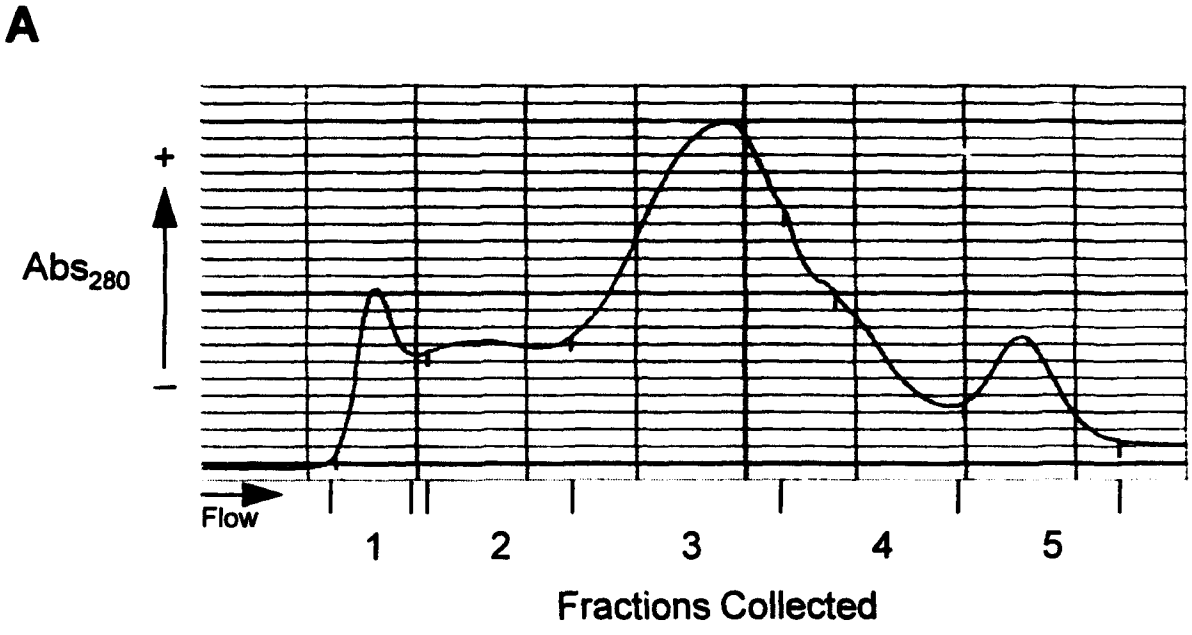
Figure 2. Western blot of clarified ferret milk and ferret "sIgA" purified by electroelution. Blots were probed with α-murine IgA. Lane 1) Pre-stained MW standards [approximate locations and sizes (kD) are indicated]; 2) Clarified ferret milk; 3) Electroeluted ferret "sIgA"; 4) Murine IgA standard; 5) Murine IgM standard. Material was blotted from a 10% acrylamide gel.

dimers and larger polymeric structures (Kerr, 1990). This identification was supported by Western blot analysis (Fig 2). Both bands were weakly cross-reactive with an α-murine IgA probe, but neither reacted with α-murine IgG nor α-murine IgM probes. Additionally, reduction of this material with DTT released a molecule which was reactive with α-murine IgA and possessed the same relative molecular weight as that of heavy chains from a murine IgA standard. These findings support and are consistent with our conclusion that these bands are ferret sIgA.

Purification of sIgA by affinity chromatography. Jacalin is a lectin that is extracted from the Jackfruit (Roque-Barreira and Campos-Neto, 1985) and selectively binds sIgA in human colostrum (Kondoh et al, 1987, Roque-Barreira and Campos-Net, 1985). Purification of human sIgA using jacalin provides a simple alternative to classical methods which involve numerous steps. For the purification of ferret sIgA, however, jacalin did not prove to be effective. Ferret sIgA did bind to the jacalin column; however, contaminant components were also bound. In addition, the efficiency of binding of the ferret sIgA to the jacalin was poor, thus allowing much of the immunoglobulin to pass through the column during loading. It has been reported that IgA from pig, goat, horse, cow, or dog sera have little or no affinity for jacalin (Wilkinson and Neville, 1988). Our findings indicate that sIgA from ferret milk is also poorly reactive with jacalin.

Purification of sIgA by size-exclusion and anion-exchange chromatography. Due to the poor performance of the jacalin, we switched to a more conventional method for the purification of ferret sIgA. The new approach was based upon the method of Cebra and Robbins (1966) which was developed for the purification of sIgA from rabbit colostrum.

The milk components were first separated by size exclusion chromatography in a Sephacryl S-400 column. Early experimentation produced chromatograms similar to that in figure 3a. Analysis of the collected fractions demonstrated separation of ferret sIgA from IgG (Fig 3b) despite the relatively poor peak separation that was seen in the chromatogram. Improved clarification of the ferret milk by ultracentrifugation as well as modifications in the column conditions led to improved peak separation. Although size exclusion chromatography was effective in separating the sIgA from the IgG, the resultant sIgA fraction contained a variety of contaminant proteins. Further purification of the sIgA fraction by anion-exchange chromatography did not remove any of these contaminant milk proteins. The contaminants exhibited a wide-range of molecular weights when examined by SDS-PAGE under non-reducing conditions, yet they co-chromatographed on the Sephacryl column.



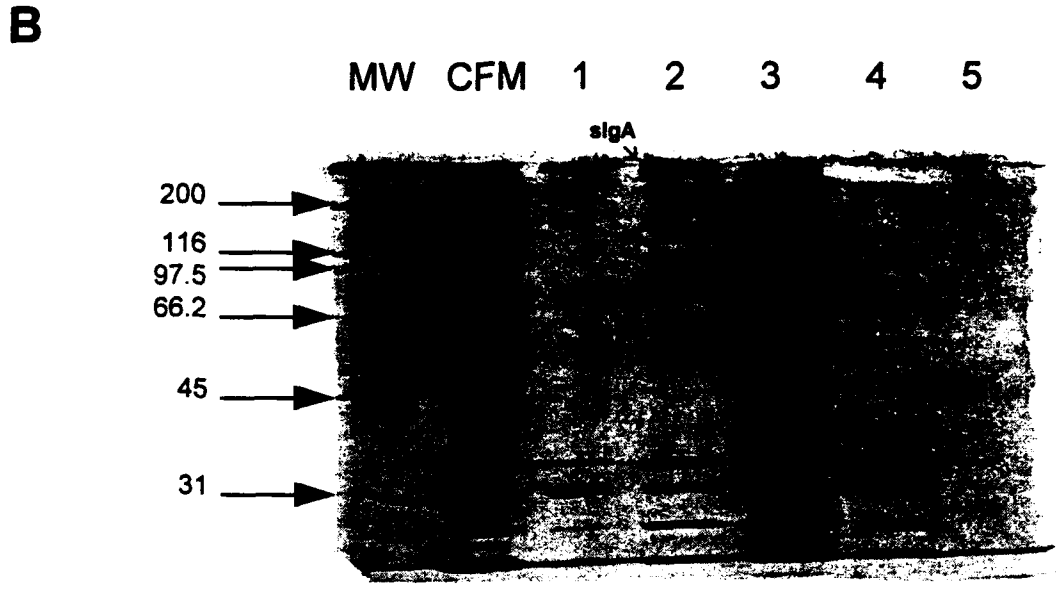
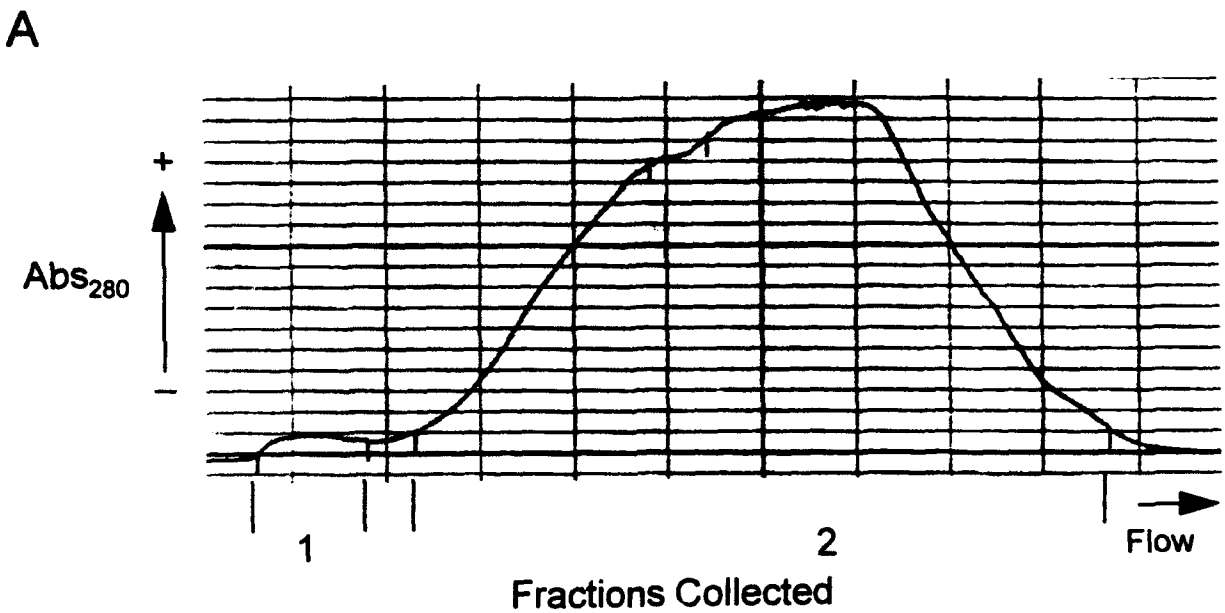


Figure 3. Separation of ferret milk components by size-exclusion chromatography. A) Chromatogram from Sephacryl S-400 column. Sample was ferret milk that had been clarified by 12,000x*g* centrifugation. Fractions that were collected are marked along X-axis as is the direction of column flow. B) Silver stained 10% SDS-PAGE of fractions 1-5 collected from the S-400 column. MW= molecular weight standards; CFM = clarified ferret milk preparation loaded onto column. Sizes of standards (kD) are indicated as are the sIgA and IgG bands.

The above findings suggested that the milk proteins were present as aggregates that were stabilized by non-covalent interactions. To test this theory, we treated clarified ferret milk with sodium dodecyl sulfate (SDS), then ran the mixture through the Sephacryl S-400 column. The resultant chromatogram (Fig 4a) was significantly different from that of untreated ferret milk. SDS-PAGE analysis revealed that the initial small peak contained sIgA with few low molecular weight contaminants (Fig 4b). This finding was consistent with the theory that the sIgA was present in non-covalently bound protein aggregates.

Size exclusion chromatography in the presence of SDS, however, was not a desirable method, since a significant amount of the sIgA co-chromatographed with the IgG (Fig 4b). In addition, SDS was not the ideal choice for the disruption of the milk protein aggregates, since it is highly denaturing and difficult to separate from proteins, as well as negatively charged, thus making it incompatible with anionic exchange chromatography. For these reasons, we experimented with other detergents in order to determine which could effectively dissociate aggregated proteins without interfering with the purification process. One of the detergents that was tested was N-dodecyl-N,N-dimethyl-3-ammonio-1-propane sulfonate (DDAPS) which is a zwitterionic detergent that has been reported to solubilize membrane proteins without destroying their biological activity (Gonenne and Ernst, 1978). We determined that a concentration of at least 1% (w/v) DDAPS was necessary for the dissociation of the protein aggregates, but inclusion of the detergent in the column buffer prevented efficient separation of the milk components by size-exclusion chromatography, probably as a result of micelle formation. Therefore, the sIgA was treated with the detergent prior to anion exchange chromatography. The dissociation of the protein aggregates led to the production of highly purified ferret sIgA (Fig 5).

During the development of the purification protocol, we exhausted our supply of clarified ferret milk which had been prepared from half of the milk that we received from Dr. Pavlovskis. We, therefore, prepared more material using the remaining whole ferret milk. We chose to modify the clarification method by centrifuging the whole milk at 100,000x*g* rather then 12,000x*g* prior to acid treatment in order to improve the clarification process. Unexpectedly, a larger casein pellet was produced by the subsequent acid precipitation, and we could no longer separate the sIgA from the IgG by size-exclusion chromatography on Sephacryl S-400. We believe that the improved clarification resulting from ultracentrifugation reduced the hydrophobicity of the solution, thus allowing improved precipitation of the casein which exists as micelles (McKenzie,



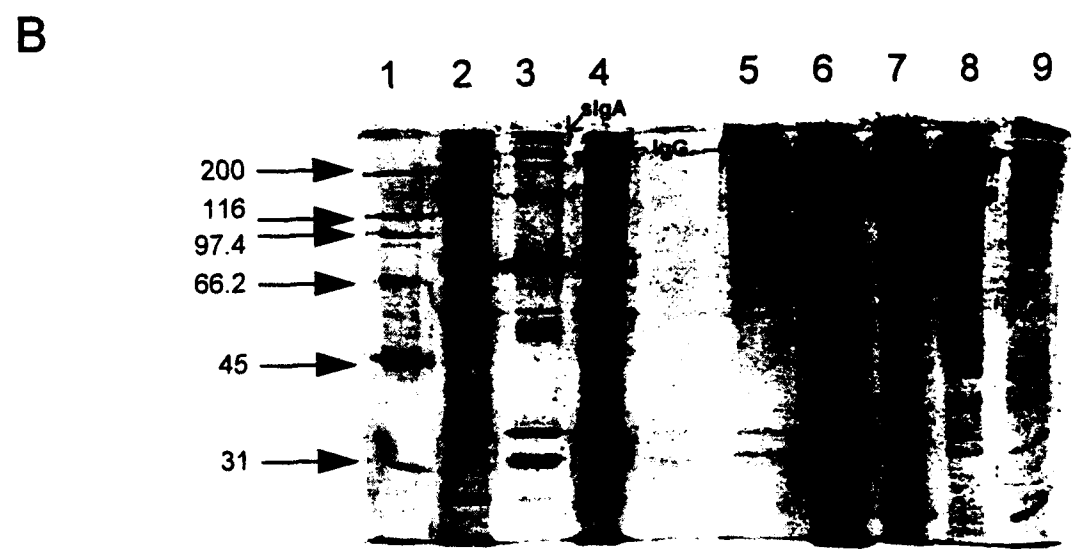


Figure 4. The effect of SDS upon the separation of milk components by size exclusion chromatography. A) Chromatogram from size-exclusion chromatography of SDS-treated ferret milk in Sephacryl S-400 column (same column as used for figure 4A). B) SDS-PAGE analysis of Sephacryl S-400 fractions. Lane: 1. Molecular weight standards (sizes indicated); 2. Whole clarified ferret milk; 3. sIgA fraction from S-400 without SDS-treatment; 4. IgG fraction from S-400 without SDS -treatment; 5. Fraction 1 from S-400 with SDS-treatment; 6. Fraction 2 from S-400 with SDS-treatment; 7. Murine IgA standard; 8. Murine IgG standard; 9. Murine IgM standard. Ferret sIgA and IgG bands are marked.

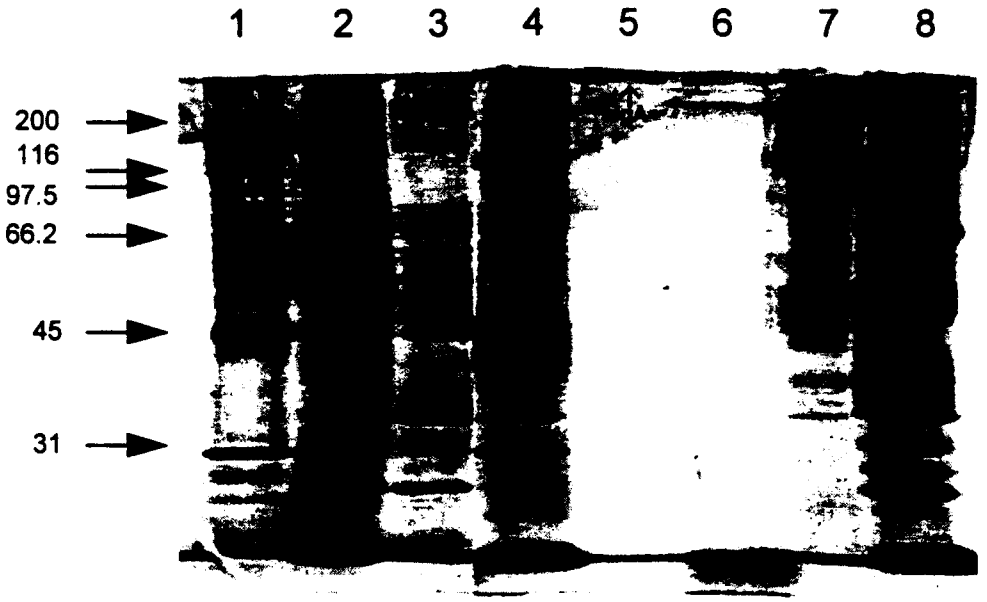


Figure 5. Effect of DDAPS treatment upon the purity of the sIgA eluted from the DEAE column. Lane: 1) Molecular weight standards (sizes indicated); 2) Clarified ferret milk; 3) DDAPS-treated sIgA fraction from Sephacryl S-400 column; 4) DEAE -bound material eluted with 0.2M NaCl; 5) pure sIgA eluted from DEAE column with 0.25M NaCl (location of band indicated); 6) DEAE-bound material eluted with 0.3M NaCl; 7) Murine igG standard; 8) sIgA fraction from Sephacryl S-400 column. Gel was 10% acrylamide. Proteins detected by silver staining.

1967). These micelles may have been responsible for the existence of protein aggregates in our initial clarified ferret milk preparation. Since our purification protocol was dependent upon the existence of large aggregates, it was necessary to modify our procedure to compensate for the reduction or removal of these structures.

We developed a modified protocol (below) in which ferret IgG was removed by affinity chromatography using a Staphylococcus Protein A Sepharose column prior to size-exclusion chromatography on Sephacryl S-300. By removing the IgG, we were able to adequately separate the sIgA from the other milk components. All other steps of the procedure remained unchanged with the exception of the ultrafiltration step which was performed prior to the addition of DDAPS to the sample. In the modified protocol, this was performed in a Centriprep-30 rather than a Centriprep-100. The modified method yielded ~40μg of sIgA per milliliter of milk.

The modified purification protocol is as follows: 1) The clarified ferret milk is centrifuged in a Centriprep 100 (Amicon) ultrafiltration device to reduce the amounts of IgG and other milk components in the starting material; 2) The Centriprep 100 retentate which contains the sIgA is passed through a Staphylococcus Protein A Sepharose column; 3) The components of the IgG-depleted mixture are separated by size-exclusion chromatography on a Sephacryl S-300 column, and the sIgA peak (first peak) is collected; 4) The sIgA fraction is concentrated and the buffer is exchanged by ultrafiltration in a Centriprep-30; 5) The concentrated sIgA solution is incubated with DDAPS to dissociate protein aggregates; 6) After incubation with DDAPS, the ferret sIgA is purified by anion-exchange chromatography on DEAE sepharose.

Immunization of Mice and Rabbits. We immunized two mice with purified ferret sIgA in order to examine their responses to the immunogen. Serum samples taken prior to immunization possessed no reactivity against ferret sIgA or other ferret milk components. Serum titers of α-sIgA in excess of 1:8000 were achieved after 3 boosts. Both mice possessed antibody which were reactive with ferret sIgA as well as many other milk components (Fig 6). Both sera recognized the same milk components although differences in the level of reactivity to certain milk components such as IgG can be seen (Fig 6). Adsorption of the immune sera with ferret IgG effectively removed cross-reactive antibodies without depleting those which were specific for sIgA as well as a milk component which appeared to migrate at the dye front (Fig 7). The low molecular weight component may be J-chain (MW=14-20kD) which is present in polymeric forms of IgA. J-chain is also present in IgM pentamers, and so the presence of α-J-chain antibodies may reduce

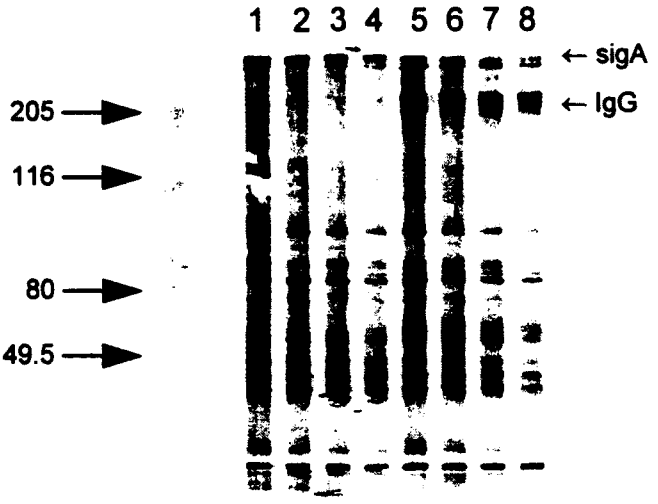


Figure 6. Murine serum antibody response against ferret milk components following a second boost with purified ferret sIgA. Lanes 1-4 were probed with dilutions of serum from mouse 1, while lanes 5-8 were probed with dilutions of serum from mouse 2. Dilutions of 1/500 (lanes 1 and 5), 1/1000 (lanes 2 and 6), 1/2000 (lanes 3 and 7), and 1/4000 (lanes 4 and 8) were used. IgG and sIgA bands are identified.

Figure 7. Cross-adsorption of cross-reactive serum antibodies from immune sera from mice immunized with ferret sIgA. Sera was cross-adsorbed by incubation with ferret IgG for 2 hours at room temperature. A 1/4000 dilution of immune serum obtained following a second boost was used. Lane 1) Immune sera; 2) Immune sera + 20 μg IgG; 3) Immune sera + 60 μg IgG. sIgA and the 14kD component that may be J-chain are identified.

the specificity of the sera. No immunoreactivity against ferret IgM, however, was detected, thus suggesting that the α-J-chain antibodies react with epitopes which are inaccessible when the molecule is associated with polymeric forms of immunoglobulins as is the case for epitopes on the secretory protein (Kerr, 1990). Therefore, the presence of α-J-chain antibodies should not adversely effect the specificity of the cross-adsorbed sera.

In order to obtain larger amounts of α-sIgA antibodies, we induced ascites production in both mice. One mouse produced ascites spontaneously following multiple boosts; whereas, the other mouse required intraperitoneal injection of myeloma cells. A total of ~8mls of ascites per mouse was collected. Both preparations possessed reactive antibody titers >1:8000 and recognized the same milk components as the immune sera. Cross-adsorption of the ascites fluid by passage through immobilized IgG effectively removed cross-reactive antibodies.

We began the immunization of the rabbits during November and recently collected their sera. The final α-sIgA serum titers for both rabbits were in excess of 1:64000. Sera taken prior to immunization were not reactive against ferret sIgA. The rabbit immune sera produced similar patterns to those of the murine immune sera when tested on Western blots. We collected approximately 130mls of immune rabbit sera.

2. Extraction and Purification of Lipopolysaccharide

Lipopolysaccharide (LPS) is present in the cell walls of all gram-negative bacteria. LPS is a heat-stable toxin as well as a major bacterial antigen. It has toxic, pyrogenic, and mitogenic properties. LPS induces the release of tumor necrosis factor by macrophages and acts as an immunoadjuvant. It is a virulence factor for pathogenic gram-negative bacteria and is responsible for the induction of fever and endotoxic shock during infection.

LPS is composed of three basic subunits: lipid A, the core, and the O-specific side chain. Lipid A is a phosphorylated glucosamine disaccharide esterified with fatty acids. The toxic properties of LPS are due to this portion of the molecule. The core is an oligosaccharide which commonly contains heptose and 3-deoxy-D-manooctulosonic acid (KDO). This portion, along with the O-specific side chain, define the antigenic portion of LPS. The O-specific side chain is a polysaccharide composed of repeating tri-, tetra-, or pentasaccharide subunits. The composition of the O-side chain is responsible for the antigenic specificity of LPS. Serotyping based upon this structure has been used extensively for the identification of different bacterial strains.

Despite being a major cell-surface antigen, the role of α-LPS antibodies in the development of immunity against infection by enterics such as *Shigella* is unknown. Some studies exploring this relationship in shigellosis have shown a direct relationship between the levels of α-LPS antibodies and protection from infection (Cohen et al, 1988, Cohen et al, 1991); whereas, other studies, such as that of Oberhelman et al (1991), have suggested that antibodies against invasion plasmid antigens, rather than those against LPS, are required for protection. Therefore, it is important to determine the levels of antibodies against both the invasion plasmid antigens and LPS for epidemiological analyses of infection rates or evaluation of vaccines. Thus, we will extract LPS from *Shigella sonnei* 53LB, *S. flexneri* 2457, and *Campylobacter jejuni* 81176 for the Navy's use in such studies.

Methods & Materials

Organisms and Culture Conditions. *Shigella sonnei* 53LB, *S. flexneri* 2a 2457, and *Campylobacter jejuni* 81176 were provided by Capt. L. Bourgeois and Dr. P. Guerry of the Naval Medical Research Institute. Starter cultures of *S. sonnei* 53LB or *S. flexneri* 2a 2457 were prepared by inoculating 50mls of brain heart infusion (BHI) broth (Difco, Detroit, MI) with one drop of a frozen stock. Cultures were incubated overnight at 37°C with shaking (150 rpm). The next day, 500ml aliquots of fresh BHI broth were inoculated with 0.5ml of starter culture and incubated overnight. Cells were collected by centrifugation (2900x*g*), washed three times with sterile deionized water, then killed by incubation in 50% (v/v) ethanol (EtOH) for 45 minutes at room temperature. The killed cells were washed once with 95% (v/v) EtOH, then stored at -70°C until extracted.

Campylobacter jejuni 81176 was cultured as described by Lee et al (1993). Frozen stocks were streaked onto Mueller-Hinton plates and incubated in Campy Pouches (BBL, Cockeysville, MD) at 37°C overnight. The next day, the plates were flooded with fresh BHI broth containing 1% yeast extract (BHI-YE), and the cells were suspended using a sterile loop. The optical density at 600nm (OD_{600}) of the cell suspension was adjusted to 0.05 by diluting the cells in fresh BHI-YE. The cell suspension was aliquoted into 25cm^2 tissue culture flasks containing 2mls of Mueller-Hinton agar (8mls of suspension per flask). The flasks were incubated at 37°C overnight under aerobic conditions. The resultant cell growth was used to inoculate 1 liter flasks containing a layer of Mueller-Hinton agar overlaid with 400mls of BHI-YE. Cells were added to produce a starting OD_{600} of 0.02. The biphasic cultures were incubated overnight at 37°C with shaking (100 rpm). Cells were collected, killed, and stored as previously described.

Extraction of LPS. Lipopolysaccharide was extracted using the method of Westphal and Jann (1965). Killed cells were suspended in sterile deionized water that had been heated to 68°C (5mls water per gram of cells). The cell suspension was added to an equal volume of 99% phenol. The mixture was heated at 68°C to melt the phenol then incubated for 15 minutes with agitation (110 rpm) at the same temperature. After incubation, the mixture was cooled in an ice water bath to facilitate phase separation and then centrifuged (2900x*g*) for one hour at 10°C. Centrifugation produced three distinct phases: an upper aqueous layer, a middle layer of insoluble material, and a bottom layer of phenol. The upper layer was collected, and the phenol layer was re-extracted with a second volume of fresh, sterile, deionized water. The aqueous phases from the two extractions were pooled, then dialyzed (dialysis tubing MWCO 6,000-8,000) against deionized water for 2 days at 4°C to remove residual phenol. After dialysis, the dialysate was centrifuged (8,150x*g*) at 4°C to remove particulate material. The supernatant was collected, then centrifuged at 80,000x*g* for 2 hours at 15°C. The supernatant was discarded and the pellet of LPS was resuspended in fresh deionized water, lyophilized, then stored at -70°C until analyzed. The purity of the LPS was determined by SDS-PAGE, protein assay (Bio-Rad, Hercules, CA), and UV absorbance. LPS was visualized in SDS-PAGE using the silver stain method of Tsai and Frasch (1982).

Results

Extraction of *Shigella* LPS. Using the phenol-water method, we extracted 25 grams of *S. sonnei* 53LB cell mass and obtained 300mgs of LPS. Assuming that the cells were still wet and that ~70% of the cell weight was due to water (Ingraham et al, 1983), the of LPS was 4% (wt LPS/dry cell wt). For *S. flexneri* 2a 2457, 30 grams of cell mass yielded 203mgs of LPS (2.2% yield). Both LPS preparations contained the O-specific side chain as demonstrated by SDS-PAGE analysis (Fig 8a). Coomassie staining did not visualize any material in the *S. sonnei* 53LB LPS; whereas, a single band was found in the *S. flexneri* 2a 2457 preparation (Fig 8b). The total protein content of each preparation was determined to be 2.8% (w/w), and the amount of nucleic acid contamination was negligible, as an absorbance peak at 260nm was not detected in either preparation. Two hundred milligrams of each preparation were aliquoted in 40mg amounts and sent to Captain Bourgeois prior to completion of this report.

Extraction of *Campylobacter jejuni* LPS. Due to the current reduction in funding for this project, work upon this task has been suspended indefinitely. Prior to the suspension of work, we performed a preliminary extraction using the phenol-water method of

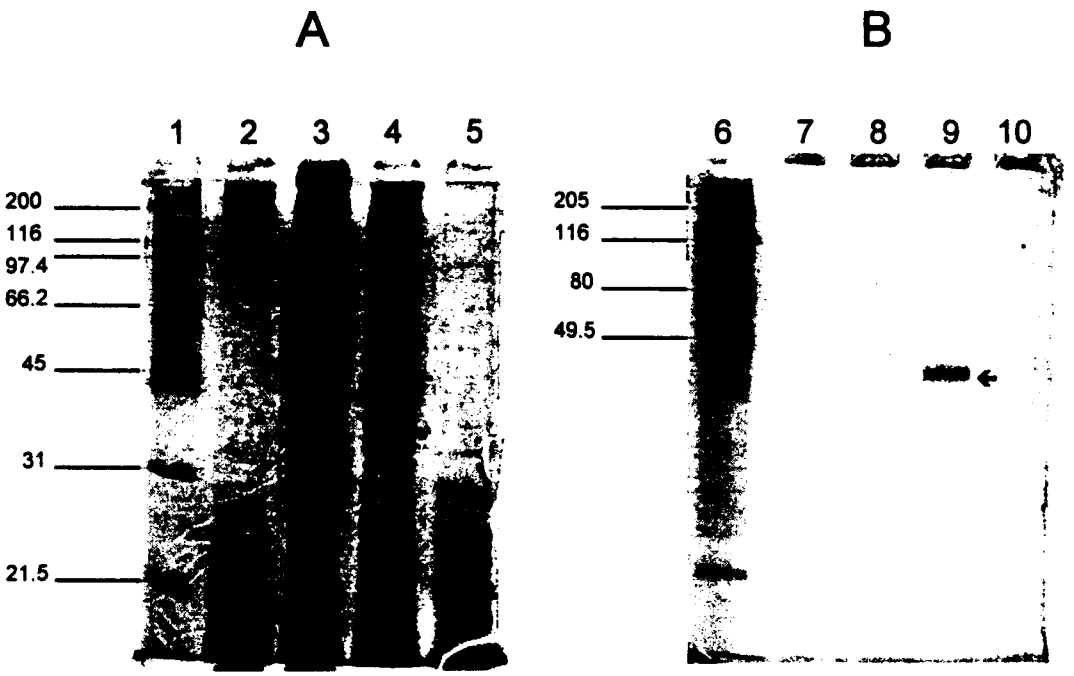


Figure 8. Results from SDS-PAGE analysis of purified LPS from *Shigella sonnei* 53LB and *S. flexneri* 2a strain 2457. Silver stained (A) and Coomassie stained (B) gels are presented. Lanes 1 and 6 contain protein molecular weight standards. The molecular weight of each standard is indicated. Lanes 2 and 7 contain LPS extracted from a rough phenotype strain isolated from the original stock of *S. sonnei* 53LB that was received from NMRI. Lanes 3 and 8 contain LPS from smooth type *S. sonnei* 53LB. Lanes 4 and 9 contain LPS from *S. flexneri* 2a strain 2457. Lanes 5 and 10 contain LPS from *Campylobacter jejuni* strain 81176. For the silver stained gel, 10 μg of LPS was loaded for each *Shigella* species, while 20 μg of each *Shigella* LPS was loaded onto the Coomassie stained gel. Approximately 5μg of *C. jejuni* 81176 LPS was loaded on each gel. Only the *S. flexneri* 2a strain 2457 LPS possessed material which stained with Coomassie; this material and its corresponding band on the silver stained gel are indicated by the arrows.

Westphal and Jann (1965) produced a yield of less than 0.05% (wt LPS/dry cell wt). This is not surprising since the campylobacter contain rough-type (R-type) LPS which is more hydrophobic than the smooth-type (S-type) LPS for which the phenol-water method works well. A method for the extraction of campylobacter LPS has been reported by Naess and Hofstad (1984). For this protocol, campylobacter cells are treated with pronase prior to extraction of their LPS by the phenol-water method. The pronase treatment removes a glycoprotein microcapsule which inhibits efficient extraction (Conrad and Galanos, 1990). If work upon this portion of the project is reinstated, we will experiment with this method first. If pronase treatment does not significantly improve our yields, we will then try the method of Galanos et al (1969) which was developed for the extraction of R-type LPS from *Salmonella* species.

3. Enteroaggregative *Escherichia coli* Heat-Stable Toxin 1 (EAST1)

Escherichia coli is the major facultative anaerobe inhabitant of the large intestine. Pathogenic strains of this bacterium are the most commonly isolated human pathogen responsible for urinary tract and wound infections, pneumonia, meningitis, and septicemia (Joklik et al, 1988). Additionally, certain strains of *E. coli* are important enteropathogens causing a wide variety of intestinal disorders. Several categories of these pathogenic *E. coli* have been established, including enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), and enterohemorrhagic *E. coli* (EHEC).

Recently, a fifth category, enteroaggregative *E. coli* (EAggEC), was identified. Epidemiological studies have associated members of this group with persistent diarrhea in young children. Animal studies with clinical isolates of EAggEC have produced histological results that are suggestive of the involvement of a toxin in the organisms' pathogenic mechanism (Vial et al, 1988). The existence of a toxin was confirmed by Savarino et al (1991), whose Ussing Chamber experiments demonstrated the presence of a heat-stable enterotoxin (EAST1) in the culture filtrates of EAggEC isolates. Further exploration has led to the identification of the EAST1 structural gene, *astA* (Savarino et al, 1993). The predicted amino acid sequence of EAST1 shares 50% homology with the enterotoxic region of the heat-stable toxin, STa, of enterotoxigenic *E. coli*. Based upon this finding, it has been suggested that EAST1 belongs to a new subfamily of heat-stable enterotoxins. Continued research upon EAST1 and other heat-stable enterotoxins will provide valuable information regarding the pathogenesis, diagnosis, and prevention of intestinal infections by pathogenic members of the Enterobacteriaceae. The purpose of this

portion of the contract is to produce polyclonal and monoclonal antibodies against EAST1 to aid in the purification of the toxin and for use in studies of its expression. In addition, we will develop an enzyme immunoassay for use in epidemiological and taxonomical studies.

Methods & Materials

Microorganisms. Dr. Stephen Savarino of the Naval Medical Research Institute provided us with two recombinant strains of *E.coli* as well as controls which possessed the vectors without *astA* inserts. The first recombinant, HMS174(DE3)pSS122, contained the *astA* gene in a pET11c vector (Novagen). Induction with IPTG has been reported to result in the secretion of active, recombinant EAST1 as demonstrated by Ussing chamber experiments (Savarino et al, 1993). The second recombinant, TB1pSS121, possessed the EAST1 gene, *astA*, in a pMALp2 vector (New England Biolabs, Inc.). When exposed to isopropyl β-D-thiogalactoside (IPTG), recombinants should produce a periplasmic fusion protein composed of EAST1 and maltose-binding protein (MBP). By ligating MBP to EAST1, purification of the fusion protein from other periplasmic components can be achieved by affinity purification on an amylose column. Additionally, a factor Xa cleavage site is included between MBP and EAST1, thus providing a means for the separation of the two components.

Production of EAST1 in HMS174(DE3)pSS122. The recombinant HMS174(DE3)pSS122 was grown in the presence of ampicillin (200μg/ml) in either NZCYM (NZ amine, casamino acids, yeast extract, $MgSO_4 \cdot 7H_2O$, NaCl) broth or Luria Bertani (LB) broth. Fifty ml starter cultures were grown overnight at 37°C with shaking (100 or 150rpm). The next day, fresh 50ml aliquots of culture media were inoculated with 0.5ml of starter culture and incubated for 4-6 hours at which time IPTG was added to a final concentration of 0.4mM. Three to five ml aliquots were removed from the induced culture at various times. The cells were pelleted by centrifugation (3200x*g*), and the supernatant was collected and sterile filtered with a 0.45μ syringe filter. Culture supernatants were stored at 4°C until all samples were collected. Collected supernatants were fractionated by sequential ultrafiltration in Centricon-10 and Centricon-3 (Amicon) devices. Retentates, filtrates, and whole cell lysates were examined by SDS-PAGE (Laemmli, 1970) and silver stained to visualize EAST1.

Production of MBP-EAST1 in TB1pSS121. The recombinant TB1pSS121 was cultivated in LB broth containing 200μg/ml ampicillin. Fifty ml starter cultures were grown overnight at 37°C with shaking (100-150 rpm). Fresh 50ml aliquots of LB containing ampicillin were inoculated with 0.5ml of the overnight starter, then incubated for

4 hours at 37°C with shaking. After 4 hours, the cells were induced by the addition of IPTG to a final concentration of 0.4mM. Cells were collected at various times by centrifugation (3200x*g*), then either solubilized in SDS-PAGE sample buffer for analysis of whole cell lysates or osmotically shocked to release periplasmic material for analysis.

Release of MBP-EAST1 by osmotic shock. The cytoplasmic components of TB1pSS121 were released by osmotic shock as described by the manufacturer (New England Biolabs, Beverly, MA). Briefly, induced cells were collected by centrifugation (3200x*g*), then resuspended in 30mM Tris-Cl, 20% sucrose, pH 8.0 (80mls per gram wet weight of cells). Ethylenediaminetetraacetic acid (EDTA) was added to a final concentration of 1mM, and the mixture was incubated at room temperature for 5-10 minutes with constant shaking. After incubation, the cells were pelleted by centrifugation (8000x*g*) for 20 minutes at 4°C, then resuspended in ice-cold 5mM $MgSO_4$. The suspension was incubated for 10 minutes in an ice bath with shaking. After incubation, the cells were removed by centrifugation, and the supernatant, which contained the crude cytoplasmic extract, was collected. The pH of the supernatant was adjusted by the addition of 1M Tris-Cl, pH 7.4 (8mls Tris-Cl per 400mls supernatant). The cytoplasmic material was either examined by SDS-PAGE or further purified by affinity chromatography.

Affinity purification of MBP-EAST1. The MBP-EAST1 fusion protein was purified from the crude cytoplasmic extract by amylose affinity chromatography as described by the manufacturer (New England Biolabs, Beverly, MA). The crude cytoplasmic extract was passed through an amylose resin column at a flow rate of 0.6ml/minute. After the sample was loaded, the column was washed with 8 column volumes of column buffer (10mM Tris-Cl, 200mM NaCl, 1mM EDTA, pH 7.4) or until the OD_{280} of the column effluent returned to 0. Bound material was eluted with column buffer containing 10mM maltose. Peak fractions were collected and analyzed by SDS-PAGE to determine the presence of the fusion protein.

Results

Purification of EAST1. We have completed our examinations of both *astA* recombinants and have been unable to establish the expression of EAST1 either as a fusion protein or as a free molecule. For the recombinant TB1pSS121, we examined whole cell lysates and periplasmic material from cells that had been collected at various times between 1 and 20 hours post-induction. We were able to visualize the MBP, but we could not detect a molecular weight shift relative to a MBP control. Proteins isolated by affinity chromatography of periplasmic material from both the recombinant

and a control containing the pMALp2 plasmid with no insert did not reveal any detectable differences between the sizes of the purified products Additionally, treatment of affinity purified periplasmic material from TB1pSS121 with Factor Xa did not release any detectable low molecular weight molecules as determined by silver staining a 10-20% SDS-PAGE gradient gel.

For the recombinant HMS174(DE3)pSS122, we examined lysates of cells that had been collected between 3 and 20 hours post-induction. No evidence for the presence of EAST1 in these preparations could be determined. Examination of culture filtrates revealed the presence of a small molecular weight molecule (~2kD) whose production appeared to be increased by induction with IPTG. Since the heat stable toxin STa is processed from 72 amino acids to 19 amino acids prior to secretion (Aitken and Hirst, 1992), we believed that it was possible that the EAST1 toxin was similarly processed, thus producing a molecule which was smaller than the predicted 4.1kD. The tentative EAST1 material, however, was present in comparable amounts in the culture filtrate of a IPTG-induced control containing the pET11c plasmid without an insert, thus negating this theory. No other potential EAST1 bands were found. In addition, examination of the two culture filtrates by reverse-phase high pressure liquid chromatography did not reveal any significant differences between the recombinant and the negative control.

During a discussion with Dr. Savarino in August, 1993, it was agreed that the recombinants were inadequate for our purposes and that the production of new recombinants was necessary. It was determined that Dr. Savarino would construct the new recombinants and deliver them to us in September or October. In October, however, we were notified by Capt. Bourgeois that anticipated cuts in the Navy's budget would require reductions in both the funding and scope of this contract. Since progress on this portion of the project was limited, all work on EAST1 was suspended indefinitely. In the event that funds become available, work will be reinitiated.

4. Type-Specific Campylobacter Flagellin Epitopes

Campylobacter coli and *Campylobacter jejuni* are significant enteric pathogens of humans (Blaser and Reller, 1981; Griffiths and Parks, 1990; Walker et al, 1986). Infections involving these motile, gram-negative bacteria most commonly manifest as gastrointestinal disorders characterized by fever, nausea, abdominal cramps, and diarrhea, although uncommon, extraintestinal disorders such as meningitis, cholecystitis, and urinary tract infections have been reported. In 1989, thirty thousand cases of enteritis were attributed to campylobacter infection in England and

Wales. The prevalence of campylobacter infections usually exceeds that of the better known enteric pathogens of the genus *Salmonella* (Griffiths and Parks, 1990).

The existence of various campylobacter virulence factors such as adhesins, enterotoxins, and cytotoxins have been investigated, yet the pathogenic mechanism of the *Campylobacter* remains poorly defined. The campylobacter flagellin has been the subject of great interest as a pathogenic determinant. These structures are the immunodominant protein antigens in human infection (Logan et al, 1987) and have been shown to be important to the organism's ability to colonize the mucosal epithelia. Additionally, antigenic variation of the flagellar epitopes has been reported (Harris et al, 1987); thus the organism may be able to avoid the host defenses. Harris et al (1987) isolated two such antigenic variants of *C. coli* VC167 (VC167T1 and VC167T2). Recently, it was reported that the antigenic variations seen between these two variants are due to undefined post-translational modifications (Alm et al, 1992). The purpose of this portion of the project is to develop monoclonal antibodies which recognize these post-translational modifications to aid in the elucidation of the nature of these antigenic variations.

Methods & Materials

Microorganisms and purified flagellin. *Campylobacter coli* strains VC167T1 and VC167T2 were provided by Dr. Trevor Trust of the University of Victoria (British Columbia, Canada). Purified *C. coli* VC167T2 flagellin was prepared by Dr. Mary Power, a post-doctoral fellow in Dr. Trust's laboratory. Bacteria were grown on Mueller-Hinton agar in Campy Pouches (BBL, Cockeysville, MD) at 37°C. Cells were scraped from the plate and suspended in sterile PBS (4.3mM Na_2HPO_4, 2.7mM KCl, 137mM NaCl, pH 7.2). The campylobacter were killed by the addition of formalin (37% formaldehyde) to a final concentration of 0.5% formaldehyde then left at room temperature for 1-2 hours. Killed cells were pelleted by centrifugation (3200x*g*), washed once with PBS-azide, then resuspended in a small volume of PBS-azide. The OD_{600} of the final cell suspension was determined using a Spectronic 20 spectrophotometer. Cell suspensions were stored at 4°C.

Immunization of mice. Mice were bled prior to injection, and their sera were tested by ELISA for reactivity against whole cells and purified flagellin. Female BALB/c mice (18-20 grams; National Cancer Institute) were given a primary intraperitoneal injection of 50μgs of purified T2 flagellin mixed with Freund's complete adjuvant (1 volume flagellin per two volumes adjuvant). Mice were boosted 3 times at two weeks intervals by intraperitoneal injection

of 25μgs of flagellin (mixed 1:2 in Freund's incomplete adjuvant). The mice were bled one week after each boost; α-campylobacter serum antibody titers were determined using a whole cell enzyme-linked immunosorbent assay (ELISA). The mice were given a final intravenous boost of 25μgs of flagellin three days prior to being sacrificed for production of hybridomas.

Production of α-flagellin MAbs. Mice were sacrificed by cervical dislocation, and their spleens were removed aseptically. The spleens were placed in a sterile petri dish with 7mls of RPMI media (Bio-Whittaker, Walkersville, MD). Scar tissue was removed, then the spleen cells were teased apart. Clumps of cells were allowed to settle out of the cell suspensions by gravity. The splenocytes were fused with P3X63-Ag8.653 myeloma cells using 50% (v/v) polyethylene glycol. Resultant hybridomas were selected using HAT medium. When adequate cell growth was achieved, culture supernatants were screened for IgG production by ELISA and strain-specific MAbs by ELISA and immunofluorescence.

Enzyme-linked immunosorbent assay. A whole cell ELISA was developed based upon the method of Van den Bosch et al (1993). Suspensions of formalin killed cells were diluted in fresh PBS-azide to an OD_{600} of 0.02. Fifty microliters of this cell suspension was added to the wells of a 96 well Immulon II microtiter plate (Dynatech, Chantilly, VA). The cells were dried onto the plates by incubation at 37°C overnight. The following day, the wells were washed twice with distilled water then each well was blocked by addition of 200μls of 3% bovine serum albumin (BSA) in TBS-azide (10mM Tris-HCl, 150mM NaCl, 0.02% sodium azide), pH 7.0, and incubated at room temperature for 1 hour. After the wells were blocked, they were washed twice with TBS-azide, then 50μls of the test antibodies (diluted in 3% BSA in TBS-azide if necessary) was added to the appropriate well. Plates were covered with parafilm and incubated at 37°C for 2 hours. After incubation, the wells were washed 4 times with TBS-azide. Fifty microliters of an appropriate alkaline phosphatase-labeled secondary antibody (diluted in 3% BSA in TBS-azide) was added, and the plate was incubated at 37°C for 2 hours. Once the incubation was completed, the wells were washed four times with TBS-azide, then 200μls of a 1mg/ml p-nitrophenyl phosphate solution in alkaline phophatase development buffer (100mM Tris, 100mM NaCl, 5mM $MgCl_2 \cdot 6H_20$, 0.02% NaN_3, pH 9.5) was added. The plates were developed at room temperature. Color development was measured by absorbance at 405nm using a Dynatech MR5000 plate reader.

For the determination of IgG production, the above procedure was used with the following changes: 1) Unlabelled goat α-mouse IgG was dried onto the wells; and 2) The incubation time for the

secondary antibodies was reduced to 1 hour.

Immunofluorescence assay. Ten microliters of formalin-killed cell suspensions (OD_{600}=0.02) were dried onto fluorescent microscope slides at 37°C. The dried cells were overlaid with 10μls of the test antibody, then incubated at 37°C in a humidified chamber for 30 minutes. After the incubation was complete, the slides were washed with TBS-azide, pH 7.0, containing 1% Tween-20, then rinsed with TBS-azide without detergent. Excess liquid was removed, and then the cells were overlaid with 10μls of an appropriate FITC-conjugated secondary antibody and incubated for 30 minutes at 37°C with humidity. After labeling was completed, the cells were washed with TBS-azide, then rinsed with deionized water. The slides were dried, and then coverslips were mounted with glycerol. Cells were examined for immunofluorescence using a Zeiss Axioskop 20 equipped for epi-fluorescence.

Results

Determination of endogenous α-campylobacter antibodies. Using the whole cell ELISA, we examined the sera of female BALB/c mice from two vendors for the presence of endogenous α-campylobacter antibodies. Mice from Simonsens (Gilroy, CA) possessed endogenous IgM which was strongly reactive with whole cells of both strains of *C. coli*. No IgG nor IgA reactivity was determined. When tested for reactivity to purified T2 flagellin, one of the three mice possessed reactive IgM. Mice that were purchased from the National Cancer Institute (NCI) also possessed IgM which reacted with whole cells of *C. coli* VC167T2; however, none possessed antibody (IgM, IgG, or IgA) which reacted with purified flagellin. It was evident that the presence of endogenous IgM which is reactive with T1 and T2 whole cells would be present regardless of the source of the animals. This, combined with the fact that purified flagellin would be used as an immunogen, influenced our decision to use the NCI mice.

Immunization of mice. Prior to immunization of the mice, we explored the efficacy of proteolytic cleavage of the flagellin as a method for the production of fragments possessing the strain specific epitopes. Treatment of flagellin with endoproteinase lysine-C produces a 30K fragment which retains the desired epitopes (M. Power, personal communication). This method, however, was not very efficient and would have required the expenditure of additional flagellin to determine the optimal conditions. Rather than using the protein for this, we chose to use whole flagellin for the immunizations.

Three female BALB/c mice were immunized with T2 flagellin.

After the third and final intraperitoneal boost, all three mice possessed IgG titers against both T1 and T2 that were >1:160,000 in the whole-cell ELISA. Unexpectedly, their sera responded to whole cells of *C. coli* VC167T1 as much as two-fold greater than against strain VC167T2 from which the purified flagellin had been extracted. We believe that this was due to different levels of expression of common flagellar epitopes, thus producing similar results as those reported for the reactivity of LIO 8 typing sera with purified flagellin from VC167T1 and VC167T2 (Harris et al, 1987) when tested in an ELISA format. In an attempt to demonstrate the presence of T2-specific antibodies, we cross-adsorbed the immune sera with VC167T1 cells. The cross adsorption reduced the response against VC167T1 cells to a greater extent than the response against VC167T2 cells, thus indicating the presence of antibodies against T2-specific epitopes.

Screening of hybridomas. Few monoclonal antibodies (MAbs) that were produced by hybridomas from the first fusion were reactive in the ELISA. This was unexpected since the mouse sera possessed a reactive titer of >1:160,000 in the ELISA prior to the fusion. On the assumption that the MAbs recognized antigenic epitopes that were being altered by the adherence of the cells to the surface of the microtiter plate wells, we modified the ELISA method by labelling the cells in suspension rather than adhering them to the surfaces of the wells. Using the suspension assay, we have identified several MAbs that have greater reactivities against *C. coli* VC167T2 cells than against VC167T1 cells; however, the differences in reactivities against the two cell types are not as great as that obtained with the polyclonal rabbit serum controls LAH-1 and LAH-2 (provided by Dr. Trust). Immunofluorescence microscopy has confirmed these results; however, whether these MAbs are truly strain specific must be established. We intend to analyze these MAbs by fluorescence activated cell sorting (FACS) in order to quantitate the differences in reactivity against the two *C. coli* strains. In addition, we are currently screening hybridomas from the second fusion. Unlike the first fusion, many of the MAbs from the second fusion were reactive in the ELISA, and we have identified additional MAbs that are potentially strain specific.

III. Plans for Present Quarter

1. Production of Anti-Ferret IgA Antibodies

We will complete the immunization of the rabbits then collect the immune sera. The IgG will be isolated from the immune sera by affinity purification using Staph Protein A, and the rabbit IgG will be further purified by adsorption with ferret IgG.

2. Extraction and Purification of Lipopolysaccharide

We will deliver the purified *Shigella* LPS to the Navy. All work upon the production of *C. jejuni* LPS has been suspended due to funding limitations.

3. EAST1

Due to reduced funding for this project and the lack of new starting material (i.e. additional recombinant forms of EAST1) for additional attempts, it was decided that this portion of the project would be suspended. In the event that the Navy attains additional funds, work upon EAST1 may be reinstated.

4. Type-Specific Campylobacter Flagellin Epitopes

We will continue screening the antibodies that are produced by the hybridomas from the first and second fusions. Additional fusions will be performed dependent upon need and available funds.

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