



Hawaii Biotechnology Group, Inc.

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April 27, 1995
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 ninth quarter (1/1/95-3/31/95) of the contract period. 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. 399).

Sincerely,

Steven A. Ogata, Ph.D.
Scientist

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distribution is unlimited.

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I. Overview

The following progress report covers the ninth quarter (1/1/95-3/31/95) for contract number N00014-93-C-0019. Four topics are included in the contract; each topic's goal and current status are as follows:

1. Production of anti-ferret sIgA antibodies. Status: Rabbit α -ferret sIgA antisera collected. Cross-adsorbed mouse ascites delivered to Naval researchers. Obtained and examined ferret colostrum. Purifying sIgA from colostrum
2. Purification of lipopolysaccharide from *Shigella* and *Campylobacter* species. Status: Lipopolysaccharide purified and sent to Navy. This portion of the project has been completed.
3. Development of an enzyme immunoassay for the detection of enteroaggregative *Escherichia coli* heat-stable toxin. Status: This portion of the project has been suspended.
4. Production of monoclonal antibodies against strain-specific antigenic epitopes on *Campylobacter coli* flagella. Status: Completed three fusions. Fourth fusion currently in progress. No strain-specific MAbs identified.

II. Current Progress

1. Production of Anti-ferret sIgA Antibodies

Purification of ferret sIgA for affinity purification of sIgA-specific antibodies. At the beginning of the ninth quarter, we received 10mls of ferret colostrum from Marshall Research Animals (North Rose, NY). Previously, we reported that Naval researchers had provided mature ferret milk as a source of ferret sIgA and that we had encountered difficulties obtaining satisfactory yields of sIgA from the milk. For these reasons, we examined colostrum as an alternative source, since this material should possess a higher concentration of sIgA than milk.

Clarified ferret colostrum (CFC) was prepared as previously described for ferret milk (see First Year Report). The CFC was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining to estimate the sIgA content. Based upon purified ferret sIgA standards, the concentration of sIgA in the CFC was ~2mg per milliliter; this was approximately 2-4 times more than the concentration in clarified ferret milk (CFM). Using our

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current purification protocol, which involves affinity purification, size-exclusion, and anion-exchange chromatographies, we purified sIgA from an aliquot of CFC and obtained a yield of ~10% (200 μ g sIgA per ml of CFC). Whereas this is greater than our sIgA yield from CFM (~40 μ g/ml), purification of adequate amounts of sIgA for the production of an immunoaffinity column with satisfactory binding capacity would still be difficult. Therefore, we modified the purification protocol in an attempt to improve our yield.

Originally, affinity chromatography using staphylococcus protein A sepharose had been incorporated into the protocol to separate milk sIgA from IgG, since anion-exchange chromatography poorly separated these components. Since we have not encountered the same difficulties separating colostrum sIgA and IgG, this step was omitted. Based upon our initial results, we concluded that omission of this step resulted in a 3-fold increase in yield, and therefore, we proceeded to purify sIgA from the remaining CFC. Analysis of the resultant sIgA preparations, however, produced conflicting results. We observed discrepancies between protein content [determined by absorbance at 280nm (OD₂₈₀)] and silver staining on SDS-polyacrylamide gels. Additionally, on Western blots, we found no correlation between protein content and sIgA immunoreactivity, thus supporting the SDS-PAGE results.

Upon further analysis, we determined that the increased yield was due to an increase in contaminants and not sIgA. These contaminants were initially overlooked, since they migrated at the dye front in the 8% gels that were used for our initial analyses of the purification product. Comparison of SDS-PAGE profiles of CFC and CFM revealed the contaminants to be unique to CFC, thus explaining why we had not encountered this problem during development of the purification protocol with CFM. Due to our concerns that the inclusion of additional purification steps would further reduce our yield, we were hesitant to modify the purification protocol. Rather, we chose to determine whether the contaminants would affect the quality of the affinity purification by binding antibodies that are not sIgA-specific. We probed Western blots of CFC with unadsorbed mouse ascites from animals injected with purified ferret sIgA and found that the contaminants were not immunoreactive; therefore, although the contaminants would reduce the coupling efficiency of ferret sIgA to the immunoaffinity column support, they should not compromise the quality of the affinity purified product.

2. Extraction and Purification of Lipopolysaccharide

At the beginning of the ninth quarter, we completed the purification and characterization of 200mg of *C. jejuni* 81176 lipopolysaccharide. The final product contained less than 2.2% protein and no detectable nucleic acid contamination. The material was sent to Captain Bourgeois of NMRI at the end of January, 1995, thus completing this portion of the contract.

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

Captain Bourgeois has informed us that Dr. Savarino has not been able to create recombinants that are satisfactory for our purposes. As financial funds are a major limitation, this task has been essentially terminated, thus further work was not performed during the past quarter.

4. Type-Specific *Campylobacter* Flagellin Epitopes

Production of strain-specific MAbs. At the end of December, 1994, we began immunizing mice with purified *C. coli* VC167T2 flagellin. Since our stock of purified flagellin was limited, we reduced the initial inoculum from 50 μ g per mouse to 25 μ g and the boosts from 25 μ g to 10 μ g. Four mice were purchased from Simonsen (Gilroy, CA) and tested for endogenous antibody that was reactive against either whole cells or flagellin. Test bleeds taken prior to the initiation of the immunization schedule possessed little reactive IgG or IgM when tested at 1/50 dilutions. This was in sharp contrast to our previous mice which possessed endogenous IgM that was reactive against whole cells but not flagellin. Based upon the results from the pre-bleeds, two mice were chosen for immunization.

During the ninth quarter, the immunization schedule was continued. Following the fourth boost (a total of five injections per mouse), both mice possessed reactive titers of less than 1/10,000 in the T2 whole cell ELISA. This was much lower than the titers from the previous mice which were approximately 1/160,000. The poorer response was probably a result of the lower inoculum, although it is possible that the previous mice possessed low levels of IgM that recognized shared or cross-reactive flagellar epitopes but were masked by the background in the ELISA. Therefore, it is possible that they were primed against flagellar epitopes, thus augmenting their immune response when inoculated with T2 flagellin.

We could not continue to boost the current mice, because our purified flagellin stock was almost exhausted; therefore, one mouse was sacrificed for the production of hybridomas in early March (fusion #4). We screened over 1,100 culture supernatants for the presence of reactive MAbs (IgG or IgM) in the whole cell ELISA. From the initial screenings, 27 hybridomas were chosen for further analysis. Eight of these potentially produced T2-specific MAbs, as their supernatants produced signal against T2 but not a T2 mutant that lacks the T2-specific, post-translational modification (T2316-7A). In addition, several hybridomas appeared to produce MAbs that were specific for the mutant. Inexplicably, none of the strain specific responses were reproducible even when supernatants that had originally produced specific responses were retested. Results from supernatants possessing non-specific reactivity were reproducible, thus indicating that the assay itself was not the source of the problem. We have investigated possible explanations for the

irreproducibility of the potentially strain specific results, but have not been able to determine one. At present, we possess approximately 10 hybridomas from the fourth fusion; none produce T2-specific MAbs.

Purification of flagellin. For possible use in future immunizations as well as further characterization of current MAbs, we prepared an immunoaffinity column for the purification of *C. coli* flagellin. As indicated in the second year report, ascites was induced in mice using three hybridomas from previous fusions (CCA 079, CCB 227, and CCC 212.1), and the MAbs were purified by affinity chromatography on staphylococcus protein A sepharose. We covalently linked MAbs CCA 079 and CCC 212.1 to an Affi-prep 10 matrix (Bio-Rad, Hercules, CA). The resultant column bound flagellin from glycine-HCl extracts of *C. coli* VC167T2. Although the elution product was enriched for flagellin, it still contained contaminants. To remove the contaminants which we believed were non-specifically adsorbed to the column support, we washed the column with a series of buffers containing increasing amounts of NaCl. Whereas the wash removed the contaminants, flagellin was co-eluted. Although the final product appeared to be free of contaminants, the yield was very low, thus requiring further optimization of the protocol.

As an alternative purification method, we developed a protocol that is based upon the ability of flagellin to self-assemble into flagella. The method involves concentration of glycine-HCl extracts in ultrafiltration devices to induce the production of large aggregates that can be removed by centrifugation at 16,000xg in a microfuge. Our initial attempt was successful and produced highly purified flagellin (based on SDS-PAGE and silver stain). A subsequent purification, however, produced products containing significant amounts of contaminants. Further experimentation led to the discovery that the protein concentration is critical to the preparation of contaminant-free flagellin. By the end of the quarter, we had determined a satisfactory concentration; however, further optimization of the purification conditions is necessary, since the recovery of flagellin is poor. In addition, while the approach appears to work for the purification of flagellin from the three *C. coli* strains that we possess, it has not been successful for the purification of flagellin from *C. jejuni* 81176, thus limiting the applicability of the method.

III. Plans for Present Quarter

At the beginning of the ninth quarter, we received notice from the Navy that our request for a no-cost extension had been approved. At present, the contract will run through the end of July, 1995.

1. Production of Anti-Ferret sIgA Antibodies

We have purchased and received an additional 20ml of ferret colostrum from Marshall Research Animals and will purify sIgA for preparation of a ferret sIgA column. We will perform limited experimentation with modifications of the purification protocol in order to improve our yield of sIgA. Regardless of the success of these modifications, we will prepare a ferret sIgA column and affinity purify antibodies from an aliquot of rabbit antisera.

4. Type-Specific Campylobacter Flagellin Epitopes

We will complete the characterization of MAbs from the fourth fusion and perform a fifth fusion. We will also continue to develop the flagellin purification protocols.