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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) In order to facilitate the production of the B subunit of the heat-labile enterotoxin of <u>E. coli</u> (LT-B) for use as an immunologic carrier, we utilized recombinant DNA technology to construct a plasmid containing the genes for production of the B subunit only from a human isolate of enterotoxigenic <u>E. coli</u> . The 0.8kb gene fragment encoding synthesis of LT-B was cloned into plasmid pBR322 following sequential digestion of the enterotoxin plasmid with restriction endonucleases PstI and HindIII. Cloned		

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B subunit was isolated from cell lysates in its oligomeric form, was structurally identical to native B subunit when examined by SDS-PAGE, immunologically identical in ELISA, and contained no demonstrable A subunit in either assay. Despite deletion of the A subunit, cloned LT-B did induce morphologic alterations in cultured mouse Y1 adrenal cells at high concentrations, an indication of residual toxicity. The 0.8kb gene fragment was recloned into the plasmid vector pUC8 and this resulted in a construct which coded for synthesis of LT-B free of biologic activity but still capable of binding to the epithelial cell surface. Peptide fragmentation resulted in loss of binding activity, suggesting a conformational binding determinant. Preliminary studies utilizing the entire B subunit as a carrier for haptens have shown that LT-B is an effective carrier for mucosal priming for production of anti-hapten mucosal secretory IgA.

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Annual Report

Immunopotential of mucosal secretory IgA responses by use of a ganglioside binding probe.

ONR Contract No. N00014-83-K-0192

Introduction

This report presents the work carried out during the 12 months period from March 1983 to March 1984 on ONR Contract No. N00014-83-K-0192: Immunopotential of mucosal secretory IgA responses by use of a ganglioside binding probe. Diseases involving infection of mucosal surfaces are the single largest cause of morbidity and mortality among the world's populations. Diarrheal and respiratory illness together accounting for more than 15 million deaths annually and are among the most debilitating infectious diseases afflicting people of all ages around the globe. A major limitation to the development of effective immunoprophylaxis against mucosal pathogens is the inability to stimulate significant levels of secretory IgA antibody directed against specific virulence determinants. This project is designed to explore the potential for immunopotential of the secretory mucosal immune response against specific antigens by coupling those antigens to a ganglioside binding probe.

Background

Escherichia coli causes diarrhea by a number of mechanisms including invasion of the colonic epithelium and production of one or more plasmid mediated enterotoxins. One of the *E. coli* toxins, the heat-labile enterotoxin (LT) is immunologically related to cholera enterotoxin and appears to produce diarrhea by the same basic mechanism-- activation of adenylate cyclase followed by increase in intracellular levels of cAMP. LT is a 91,000 dalton polymeric protein composed of two major, non-covalently associated, immunologically distinct regions or domains ("LT-A" and "LT-B"). Of these, the B region is responsible for binding of the toxin to the host cell membrane receptor, the ubiquitous monosialoganglioside GM_1 . The B subunit is nontoxic and appears to be the immunodominant moiety of the holotoxin.

In addition to the large molecular weight heat-labile toxin, *E. coli* also produce a low molecular weight (c.a. 2,000 dalton) heat-stable enterotoxin which is non-immunogenic and which appears to produce diarrhea through stimulation of guanylate cyclase. We have recently

developed an effective vaccine against diarrheal disease caused by *E. coli* expressing either or both of these enterotoxins. This was accomplished by purifying both toxins and chemically coupling the poorly immunogenic ST to either LT (LT/ST) or to the B subunit of LT (LT-B/ST). The product so derived was not only immunogenic, but the toxicity of the ST molecule was ablated as a function of the cross linking. In rats immunized by a combined ip/po route, this immunogen evoked a 4- to 7-fold increase over control values of serum IgG and mucosal secretory IgA antitoxin levels to each of the component toxins thus providing significant protection against challenge with either toxin or heterologous serotypes of viable strains which produce these toxins.

The relative success of this vaccine is likely a function of the ability of the LT-B to bind to the epithelial surface where it remains localized for antigen processing by cells of the mucosal immune system. It should therefore be possible to immobilize any antigen on the epithelial surface of the gut by covalently coupling the antigen to the G_{M1} binding component of LT-B. This would be accomplished by first identifying the G_{M1} binding region on LT-B and then by using that fragment as a carrier for a variety of haptenic molecules.

Production of LT-B

Our first goal was to obtain sufficient quantities of pure LT-B to assure an adequate supply. This was accomplished by genetic engineering--constructing a clone of *E. coli* that produced only the B subunit, free of contaminating subunit A, and in sufficient quantities to be of practical value. Previous cloning efforts in this lab had resulted in strains that appeared to be free of subunit A, but the products of which, when tested at high concentrations, retained a residual toxicity of undetermined nature. Since any contamination would adversely effect the subsequent peptide fragmentation and immunological studies, a pure form of LT-B had to be obtained. The standard guanidine-HCl gel filtration procedure previously employed for separation of the two subunits from holotoxin was deemed unacceptable due to poor recovery and the presence of residual toxicity in B fragments obtained by this technique as well.

The 60 md $LT^+ ST^+$ enterotoxin plasmid of human *E. coli* isolate H10407 was purified and cleaved with the restriction enzyme PstI. A 5.2 kb DNA fragment that includes the LT gene was inserted into the PstI site in the plasmid pBR322, producing a plasmid 9.4 kb in size, designated pDF82

(Fig. 1.A and 1.B). This plasmid contains a functional gene for tetracycline resistance (Tc^r) which enables direct selection for transformants of *E. coli* K-12 harboring the plasmid. These transformants were then screened for ampicillin sensitivity (Ap^s) due to insertion of a DNA fragment into the PstI site of pBR322 (insertional inactivation of the ampicillin resistance gene). Tc^rAp^s transformants were then tested for production of LT using the Y1 adrenal cell system and an Enzyme Linked Immunosorbent Assay (ELISA). Subsequent analysis of pDF82 with PstI, EcoRI, HindIII, HinfI, and Avall confirmed the size of the DNA insert and the absence of internal PstI sites.

The cloned LT DNA region was recloned from pDF82 into the single HindIII site in the tetracycline resistance gene of pBR322. pDF82 and pBR322 were cut with HindIII, mixed, and joined by T4 DNA ligase. The ligation mixture was again transformed into an *E. coli* K-12 and Ap^rTc^s transformants were identified after cycloserine enrichment for tetracycline sensitivity. The transformants were assayed for loss of biological activity on Y1 adrenal cells and for production of LT-B antigen by ELISA. Plasmid DNA was isolated from transformants negative in the Y1 assay and positive in ELISA and analyzed. One clone, designated pDF87 (Fig. 1.C), was cut by HindIII into two fragments, one identified as pBR322 and a smaller (0.8 kb) fragment coding for production of LT-B (Fig. 1.D). Cloned LT-B was isolated from cell lysates in its oligomeric form, was structurally identical to native B subunit when examined by SDS-PAGE (Fig. 2), immunologically identical in ELISA (Fig. 3), and contained no demonstrable A subunit in either assay. As mentioned above, despite deletion of the A subunit, LT-B purified from such transformants did induce morphologic alterations in cultured mouse Y1 adrenal cells at high concentrations (Table 1), an indication of residual toxicity.

Table 1. Comparisons of adrenal cell activities of LT, LT-B (pDF87), and LT-B (pJC217)

<u>Preparation</u>	<u>Biologic Activity^a</u>
LT	.038 ng
LT-B (pDF87)	39 ng
LT-B (pJC217)	ND ^b

^aAmount of trypsin activated material required to produce significant cell rounding (50%).

^bND, None Detected when tested at 25,000 ng per well.

The cloned LT-B DNA was recloned from pDF87 into the single HindIII site of the M13 derived cloning vector pUC8. pDF87 was cut with HindIII and the 0.8 kb fragment separated by electrophoresis through low melting point agarose. The agarose gel surrounding the fragment was excised, melted, and the LT-B DNA fragment extracted with phenol and recovered by ethanol precipitation. Plasmid pUC8 was cleaved with HindIII, mixed with the purified LT-B gene fragment, ligated, and transformed into an *E. coli* K-12. Transformants were selected for resistance to ampicillin and screened for loss of β -galactosidase activity, an indication of insertional inactivation and therefore presence of foreign DNA in the cloning vector. Transformants were subsequently tested for the ability to make LT-B by ELISA.

One positive clone, designated JM83(pJC217) (Fig. 4.A), was selected for further study. Plasmid DNA was purified from this clone and, when recut with HindIII, contained only two fragments: one corresponding to the 2.7 kb cloning vector, pUC8, and one of 0.8 kb coding for production of LT-B (Fig. 4.B). LT-B was efficiently transcribed utilizing the lac promoter of pUC8 and expressed in clones containing the plasmid. LT-B was then purified from this clone by agarose affinity chromatography and 50-60 mg of pure LT-B obtained per liter of culture. LT-B isolated from this strain was found to be structurally and immunologically indistinguishable from LT-B isolated from pDF87 and, more importantly, was completely non-toxic when tested in Y1 adrenal cells (Table 1).

Cyanogen Bromide Cleavage of LT-B

A variety of techniques for the selective chemical and enzymatic cleavage of proteins are available. We chose to start with cyanogen bromide cleavage since, at acidic pH, CnBr cleaves at the carboxyl side of methionine residues and there are five such residues in each purified LT-B monomer. The solubility of LT-B following dialysis against several acids was first determined. LT-B was insoluble following dialysis against 0.1 N HCl, 1% HCOOH, 5% HCOOH, and 70% HCOOH when redialyzed against Tris buffer. There was also a substantial loss of antigenicity as determined by ELISA.

LT-B was then dialyzed against water, in which it precipitates. The precipitate was found to be insoluble in 5-50% acetonitrile or methanol, but soluble in 0.01% formic acid, 0.1% acetic acid, or 0.1% NaCl. Initial HPLC gradients were therefore run using 5-100% acetonitrile in water, with both

solvents containing 0.01% HCOOH to act as a buffer and to solubilize the LT-B or its fragments. Samples were run on both isocratic (5% CH₃CN) and gradient (5-80% CH₃CN) columns. In both cases, a single peak emerged at 3 minutes (5% CH₃CN = void). No other peaks were apparent.

CnBr digests were prepared and run on the HPLC again, no peaks other than the three minute elution peak were observed. Several explanations were possible for the apparent observations:

1. The CnBr may not be fragmenting the LT-B.
2. The fragments were not retained, even at 5% CH₃CN and were therefore coeluting at three minutes.
3. The fragments were there, but not visible at 254 nm.

A variety of parameters were investigated, including CnBr concentration, NaCl concentration, and time of reaction. Optimum conditions were determined and CnBr digested fragments examined by polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE). Gel analysis showed complete digestion of the LT-B preparation when reacted with a 500 fold molar excess of CnBr in 70% formic acid overnight after removal of all NaCl from the sample.

A scale up of this preparation was chromatographed on a 1.5 x 60 cm column of Biogel P2 with 5% HCOOH + 1% 2ME as a running buffer. Fractions were collected and aliquots assayed at 280 nm and at 570 nm following treatment with ninhydrin. Several peaks were resolved at 570 nm. Pools were collected, lyophilized, and resuspended in one ml of distilled water, then assayed for antigenicity in the ELISA. All activity was present in the first pool from the column (void), probably representing a small residual amount of unreacted LT-B. This is consistent with positioning of a CnBr reactive site in the G_{M1} binding sequence. This possibility had been addressed in the original proposal and several options were discussed, including alternate chemical cleavage (i.e., BNPS-skatol) and enzymatic digestion. Continued analysis of the CnBr fragments proved to be unfruitful, as did subsequent analysis with aminopeptidase, carboxypeptidase, and submaxillary protease. What did become apparent is that any treatment which disrupts the pentavalency of the oligomer also reduces its ability to bind to GM1. This may indicate that binding is conformational and requires the interaction of more than one subunit.

Consequently, we have initiated studies to determine the efficacy of the entire B subunit as an immunologic carrier for haptens as a preliminary means of defining systems for subsequent work. We have coupled bradykinin to LT-B by both carbodiimide reaction and with glutaraldehyde and used the hapten-carrier conjugate to immunize groups of rats and mice. Sera and mucosal washings were examined for the presence of antibodies to bradykinin and to LT-B. This conjugate was effective at inducing both serum IgG and mucosal sIgA to the carrier and to the hapten. Comparative studies are now underway to optimize route of delivery, composition of antigen, and dosage for production of specific mucosal secretory IgA.

Figure 1. Agarose gel electrophoresis of partially purified lysates of pDF82 (lane A), HindIII- cleaved pDF82 (lane B), pDF87 (lane C), and HindIII- cleaved pDF87 (lane D).

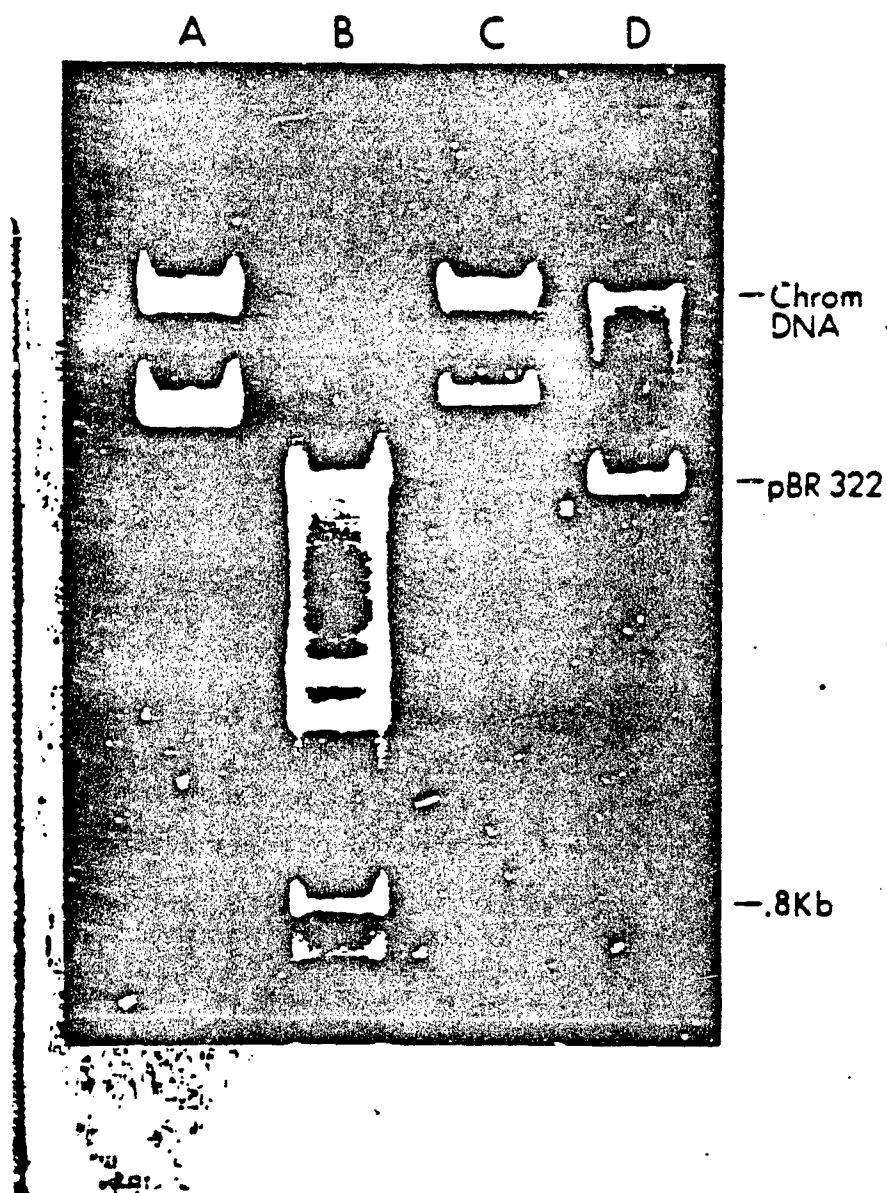


Figure 2. Analytical discontinuous electrophoresis in SDS-PAGE of agarose affinity purified preparations from pDF82 (lane A) and pDF87 (lane B). Both preparations contained a band corresponding to oligomeric B subunit (upper band). The band corresponding to the A subunit (lane A, lower band) was not present in pDF87.



Figure 3. ELISA comparison of agarose affinity purified preparations from pDF82 and pDF87. pDF87 did not produce antigen which was recognized by antiserum to cholera-A.

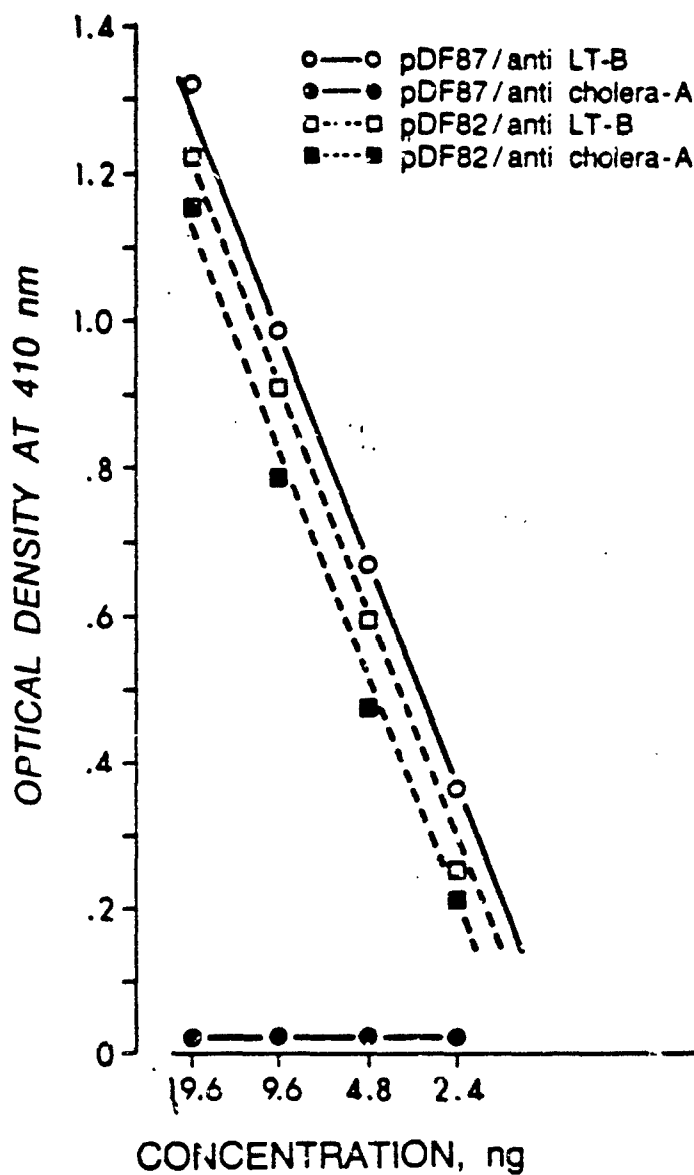
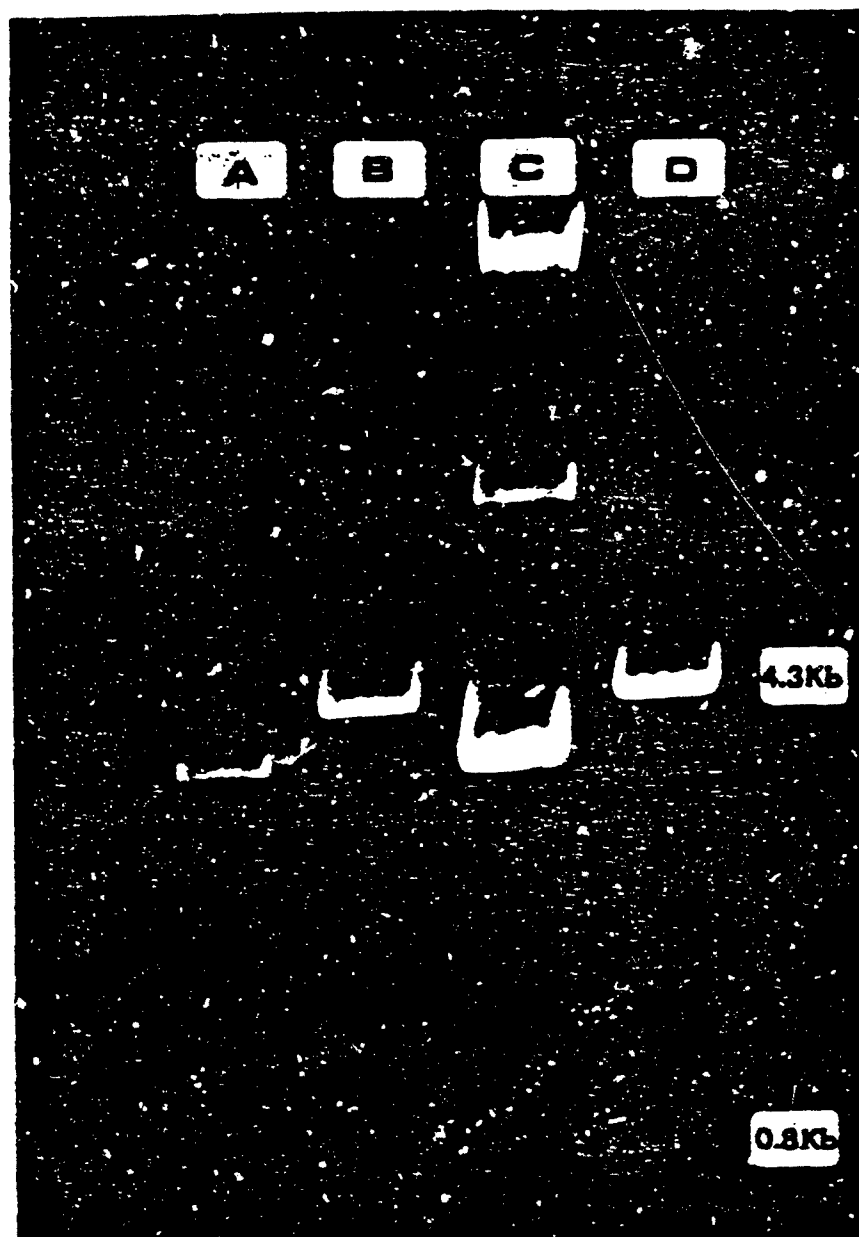


Figure 4. Agarose gel electrophoresis of partially purified lysates of A. *E. coli* JM83(pJC217); B. *E. coli* JM83(pJC217) cleaved with HindIII; C. *S. typhi*/SE12; and D. *S. typhi*/SE12 cleaved with HindIII.



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Fritz H. Bach, M.D.
Director, Immunology Research Center
University of Minnesota
Box 724, Mayo Memorial Bldg.
420 Delaware St., SE
Minneapolis, MN 55455

Francis A. Ennis, M.D.
Department of Medicine
University of Massachusetts
Medical School
55 Lake Avenue
Worcester, MA 01605

Fred D. Finkelman, M.D.
Department of Medicine
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4301 Jones Bridge Road
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University of Michigan Med. School
7620 Medical Science II Bldg.
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Borrison Laboratories, Inc.
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James J. Mond, M.D.
Department of Medicine
Uniformed Services University
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4301 Jones Bridge Road
Bethesda, MD 20814

Dr. Donna S. Sieckmann
Infectious Diseases Program Center
Naval Medical Research Inst.
National Naval Medical Center
Bethesda, MD 20814

Dr. John D. Clements
Department of Microbiology
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Tulane University Medical Ctr.
1430 Tulane Avenue
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Dr. Edward A. Havell
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P.O. Box 59
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Dr. Arthur G. Johnson
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Washington, DC 20007

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Laboratory, Dept. of Medicine
Naval Hospital
National Naval Medical Center
Bethesda, MD 20814

Dr. Robert I. Mishell
Dept. of Microbiology &
Immunology
Univ. of California, Berkeley
Berkeley, CA 94720

Dr. Page S. Morahan
Department of Microbiology
Medical College of Pennsylvania
3300 Henry Avenue
Philadelphia, PA 19129

Dr. Alan L. Schmaljohn
Department of Microbiology
University of Maryland
School of Medicine
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Dr. Jeannine A. Majde, Code 441CB
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University of Alabama
University, AL 35486

Dr. Phyllis R. Strauss
Department of Biology
Northeastern University
360 Huntington Avenue
Boston, MA 02115

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