INCORPORATION OF THE BACTERIAL LIPOPOLYSACCHARIDE FROM Aeromonas salmonicida INTO LIPOSOMES AND THE EFFECTS ON ANTIBODY RESPONSES IN RAINBOW TROUT (Oncorhynchus mykiss)

ANTHONY N. NAKHLA

Incorporation of the Bacterial Lipopolysaccharide from *Aeromonas salmonicida* into Liposomes and the Effects on Antibody Responses in Rainbow Trout (*Oncorhynchus mykiss*).

by

ANTHONY N. NAKHLA

B.Sc. CONCORDIA UNIVERSITY, 1985

M.Sc. MEMORIAL UNIVERSITY, 1988

A Thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Biochemistry

Memorial University of Newfoundland

December, 1994

DEDICATED TO MY PARENTS

ABSTRACT

The Gram negative bacterial fish pathogen *Aeromonas salmonicida* is the causative agent of furunculosis in salmonid fishes. The lipopolysaccharide (LPS) from *A. salmonicida*, a major factor in determining bacterial virulence, was incorporated into liposomes to determine the ability of these formulations to function as delivery vehicles and immunological adjuvants in rainbow trout (*Oncorhynchus mykiss*). Several aspects were considered important in examining the potential of liposomal LPS formulations as vehicles and adjuvants.

^{1}H-Nuclear Magnetic Resonance (^{1}H-NMR), mass spectroscopic (MS), and polyacrylamide gel electrophoresis (SDS-PAGE) analyses indicated that the LPS from *A. salmonicida* was consistent with the structures previously proposed for the O-antigen and the core-oligosaccharide moieties using conventional chemical analyses. These studies also suggested that an unusual resonance at 0 ppm in the ^{1}H-NMR spectrum of the LPS may relate to both the LPS and a component of the antifoaming agent, Antifoam A™.

Incorporation studies demonstrated that LPS and PS from *A. salmonicida* can be incorporated into multi- and unilamellar vesicles composed of both positively and negatively charged lipid to form liposomes of varying incorporation ratios, sizes and lamellarities.

Biodistribution studies indicated that the uptake of LPS, liposomes, and liposomally-incorporated LPS administered via the IV, IP and IM routes was

primarily by the kidney and spleen in rainbow trout. Although oral administration of the antigens resulted in the lowest overall uptake, an enhanced uptake by the liver was observed as compared to the other tissues and organs examined. These results propose that liposomes may serve as efficient delivery vehicles for LPS to the primary hemopoietic organs of rainbow trout.

Immunization experiments demonstrated that multi- and unilamellar vesicles composed of both positively and negatively charged lipid are capable of prolonging the humoral immune response to LPS from *A. salmonicida* in rainbow trout when compared to the non-incorporated LPS.

ABx chromatography is a practical procedure for the purification of rainbow trout immunoglobulins from serum. It is likely that this protocol may be adapted for the isolation of IgM from other fish species.

Our data suggests that liposomally-incorporated LPS can safely be administered to rainbow trout and function as delivery vehicles and immunological adjuvants. These results propose that liposomal LPS formulations are worthy of further investigation in terms of potential and application in rainbow trout.

ACKNOWLEDGEMENTS

I would to like to express my sincere gratitude to my graduate faculty committee for their guidance: Dr. K. M. W. Keough, Dr. J. H. Banoub, and Dr. V. Skanes, who continuously provided valuable supervision throughout the course of this project. Their encouragement, interest, and advice both in and out of the laboratory were greatly appreciated. I would like to thank them for their kindness, careful attention to detail, and promptness.

I wish to thank Dr. A. J. Szalai, who willingly offered me valuable advice, constant motivation and friendship.

My thanks to Dr. P. J. Davis for his constant encouragement, periodical moral boosts and helpful comments.

My appreciation goes to Mr. Colin McGowan for his technical assistance in construction of the holding tanks in the Biotech building.

Special acknowledgement is due to the Department of Biochemistry of Memorial University of Newfoundland for permitting this Thesis to materialize and for their continued support.

I would also like to thank the Department of Fisheries and Oceans, Canada, for unconditional use of their facilities.

I am grateful to the Natural Sciences and Engineering Research Council of Canada for financial support. In addition, my extended gratitude to Dr. Keough, Dr. Banoub, and to the School of Graduate Studies for financial support.

Finally, I express extended gratitude to the numerous friends who have made this experience possible.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Fish diseases caused by a variety of bacterial species are systematically responsible for high mortalities of fish in situations commonly encountered in the aquaculture and fish hatchery industries. This research project has addressed a cell-surface antigen from the bacterium *Aeromonas salmonicida*, the causative agent of furunculosis in rainbow trout (*Oncorhynchus mykiss*). The aim of this investigation was to explore the potential of liposome-associated antigen formulations as a safe, economic and efficient delivery system for the aquaculture industry. This introduction is divided into sub-sections in order to identify the problem(s), explain the rationale behind the strategies employed and describe the objectives of this project. The chapters that follow will discuss particular questions addressed during the course of this investigation.

Overview of Bacterial Fish Diseases.

Bacteria are ıbiquitous in the aquatic environment where they are considered to play a major role in both synthetic and degradative processes. Consequently, the severity of bacterial fish diseases caused by a variety of pathogens has increased with the development and expansion of commercial aquaculture and studies on pathogenesis, ecology, epizooitology, and disease

management are well developed. Communicable diseases of fish are most likely to occur when susceptible host and virulent pathogen meet in the proper environmental conditions required for induction of disease (Snieszko, 1972) and it is believed that bacterial diseases of fish are almost inevitably stress related. In relation to fish, stress is defined as a stage produced by environmental or other factors which extend the adaptive responses of an individual beyond the normal range, such that its chances of survival are significantly reduced (Brett, 1958). Stressing factors such as crowding, poor water quality, fright, high temperature and trauma are important in precipitating disease, particularly in farmed populations.

A great deal of research has been concerned with the isolation, identification and classification of bacterial pathogens from the tissues of fish from farmed and wild stocks. Some virulent bacteria such as *Vibrio anguillarum* and *Aeromonas salmonicida* are considered important primary pathogens that are capable of inducing infection in fish which are subjected to only limited stress. Other pathogens such as *Aeromonas hydrophila* are more likely to infect compromised fish which are heavily stressed. Examples of some of the major bacterial pathogens of fish and the associated diseases induced are provided in Table 1. 1.

Table 1.1. Principal Bacterial Pathogens of Fish and Associated Diseases.*

CLASSIFICATION	PATHOGEN	DISEASE
Gram-negative facultatively anaerobic rods		
Enterobacteriaceae:	*Edwardsiella tarda*	edwardsiella septicaemia
	Edwardsiella ictaluri	enteric septicaemia of catfish
	Yersinia ruckeri	enteric redmouth, (ERM)
Vibrionaceae:	*Vibrio anguillarum*	vibriosis
	Vibrio ordali	vibriosis
	Vibrio salmonicida	Hitra disease
	Aeromonas salmonicida	furunculosis
Gram-negative aerobic rods		
Pseudomonadaceae:	*Pseudomonas fluorescens*	septicaemia
Gram-positive aerobic rods		
No family	*Renibacterium salmoninarum*	bacterial kidney disease
Gram-positive facultatively anaerobic cocci		
No family	*Streptococcus iniae*	septicaemia
	Streptococcus spp.	
Gram-positive anaerobic rods	Endospore-forming bacteria *Clostridium botulinum*	type E botulism
	Irregular non-sporing rods *Eubacterium tarantellus*	incriminated in neurological disease

*Reproduced with permission from Roberts, R. J. 1993. In "Bacterial Diseases of Fish" V. Inglis, R. J. Roberts and N. R. Bromage (ed's). Blackwell Scientific Publications, London. p. xvi-xvii.

Characteristics of *Aeromonas salmonicida* and Furunculosis Infections.

This investigation concerns the Gram-negative bacterium *A. salmonicida* which is the etiological agent of a fatal epizootic disease in salmonid fish called furunculosis. *A. salmonicida* is a facultatively anaerobic Gram-negative rod approximately 0.3-1.0 x 1-3.5 μm which is classified as a member of the Vibrionaceae family. *A. salmonicida* was the first disease causing bacterium to be characterized from a trout hatchery by Emmerich and Weibel in Germany in 1894 who had originally named the bacterium *Bacillus der Forellenseuche* or bacillus of contagious trout disease. In English it was known as *Bacillus salmonicida* until it was suggested (Griffin *et al.*, 1953) that it be reclassified in the genus *Aeromonas* of the family Vibrionaceae. *A. salmonicida* is presently divided into three subspecies as described by Belland and Trust (1988):

(1) Group 1 strains. *A. salmonicida* subspecies *salmonicida* which are strains typically derived from salmonid fish.

(2) Group 2 strains. *A. salmonicida* subspecies *achromogenes* which are atypical strains derived from salmonid fish and include the former subspecies, *achromogenes* and *masoucida*. Two Gram-negative microorganisms associated

with ulcer disease (Snieszko *et al*, 1950) and pasturellosis (Hasten and Bullock, 1976) are recognized as being such strains.

(3) Group 3 strains. *A. salmonicida* subspecies *nova* which are atypical strains associated with disease in non-salmonid fish.

The disease resulting from *A. salmonicida* infection was termed furunculosis because of the analogy with human furunculosis, which is a condition marked by the presence of furuncules or localized pyogenic infections (Stedmans Medical Dictionary, Illustrated 23rd Edition, 1977). Furunculosis may affect all species of salmonids and generally appears to develop as a septicaemia, a systemic disease caused by the multiplication of microorganisms in the circulating blood, and is often fatal. As seen in Figure 1.1, furunculosis results in 'boil-like' necrotic swellings of the musculature in fish, which may eventually ulcerate to develop into acute or chronic forms of the disease. Acute development of furunculosis displays few external signs whereas chronic cases may manifest darkening, lethargy, lightening of gills, ulceration and possibly the release of necrotic tissue debris and bacteria. The clinical pathology includes inflammation of the spleen, kidney, and intestine (principally in the

Figure 1.1. Furunculosis infection in brown trout (*Salmo trutta*) (top), and furuncule dissected to show the necrotic muscle containing large numbers of *Aeromonas salmonicida* bacteria (bottom). Reproduced with permission from Roberts, R. J. 1993. In.."Bacterial Diseases of Fish." V. Inglis, R. J. Roberts and N. R. Bromage (ed's). Blackwell Scientific Publications, London. p. 142.

pyloric and rectal regions), hyperemia in the swim bladder, hemmorrhages on the liver, eyes, and fins and severely depressed red blood cell numbers.

The specific changes that occur in fish which trigger the invasion and multiplication of the bacteria are likely due to suppression of non-specific immunological defences such as the reticuloendothelial system (RES) or alterations in the integrity of mucoid surfaces, which can provide a physical barrier to invasion, or both. *A. salmonicida* ssp. *salmonicida* may exhibit a cell-associated surface protein array known as the A-layer, whose presence or absence is considered to be critical in determining the virulence of a particular bacterial strain (Ishiguro *et al.* 1981). *A. salmonicida* is also known to produce extracellular products (ECP), which are considered to play an important role in pathogenesis and virulence (Ellis, 1991). Among the ECP that have been characterized are proteases (Rockey *et al.*, 1988; Price *et al.* 1989), membrane-damaging toxins which act as leucocytolysins (Fuller *et al.*, 1977), haemolysins (Titball and Munn, 1983; Titball and Munn, 1985), cytotoxins (Cipriano, 1982), and a glycerophospholipid: cholesterol acyltransferase (GCAT) (Lee and Ellis, 1990), in addition to other factors such as amylases and phospholipases (Campbell *et al.*, 1990) and lipopolysaccharide (LPS) (MacIntyre *et al.*, 1980). Collectively, bacterial ECP (summarized in Table 1.2) may permit the

Table 1.2. Extracellular Products (ECP) of *Aeromonas salmonicida* ssp. *salmonicida*.

Proteases

70 kDa protease (caseinase, serine protease)

Gelatinase (metalloenzyme)

Membrane-damaging toxins

Leucocytolysin

Cytotoxic glycoprotein

Haemolysins (T-lysin, H-lysin)

Glycerophospholipid: cholesterol acyltransferase (GCAT)

GCAT/lipopolysaccharide (LPS)

Other Factors

LPS

Esterases

Amylases

Ribonucleases

Glucosidases

Mannosidase

Alkaline phosphatase

Phospholipase

Lysophospholipase

*Modified with permission from Ellis, A. E. 1991. An appraisal of the extracellular toxins of *Aeromonas salmonicida* ssp. *salmonicida*. J. Fish Dis. 14:265-277.

bacterium to rapidly overcome the leucocytic defence systems of the blood resulting in the destruction of engulfing macrophages and monocytes, lysis of lymphocytes and digestion of muscle protein and glycogen. The bacteria may then be transported through the vasculature to localize in various organs.

Overview of the Mammalian Immune System.

The material contained in this section is a brief review of the mammalian immune system and has been written using the following immunology texts: Alberts *et al.,* 1989; Benjamini and Leskowitz, 1991; Coleman *et al.*, 1984; Kimball, 1986; andKuby, 1994. The material obtained from other sources are cited accordingly. The mammalian immune system consists of a network of lymphoid organs, tissues and cells and the products of those cells such as cytokines, cytotoxic substances and antibodies. The primary lymphatic organs of the mammalian immune system are the bone marrow and the thymus, where immature lymphocytes differentiate into antigen-
sensitive, mature B and T lymphocytes, respectively. The principal secondary lymphatic organs of the mammalian immune system are the spleen and the regional lymph nodes while other secondary lymphoid organs include the adenoids, tonsils, Peyer's patches of the small intestine and the appendix.

Bone marrow is found in the cavities of most bones in the human body and is the site of production of all major blood cell types, termed hemopoiesis. Hemopoietic marrow consists of sinusoids arranged peripherally around a central vein. The precursors of the different blood cell lines, or blast cells, and their more mature descendants are found within the sinusoids. Bone marrow contains intermediate and mature forms of erythrocytes, granulocytes, monocytes, lymphocytes and megakaryocytes. In addition to the progenitor cells for each blood type, the bone marrow contains stem cells, a generalized progenitor cell capable of yielding any of the blood cell lines. The bone marrow also provides a microenvironment for antigen-independent differentiation of B lymphocytes, which display an extensive repertoire of antigen receptors (immunoglobulin, Ig) at maturity. Mature B lymphocytes leave the bone marrow to circulate in the blood or lymph or to reside in other lymphoid organs.

The mammalian thymus is a flat, bilobed organ located high in the thoracic cavity which consists of a reticular network filled with a mass of lymphocytes and a small number of epithelial cells. The thymus, considered a critical organ of the immune system, consists of many lobules, each containing a cortex, or outer region, and a medulla, or central region. The thymus

provides an environment for antigen-independent maturation and development of T (thymus-derived) lymphocytes that prompt cell-mediated immunity and regulate most humoral and cell-mediated responses. Progenitor lymphocytes migrate through the bloodstream from the bone marrow to the thymus for the maturation process. Only about 5% of the cells produced survive to eventually leave the thymus as functional antigen-reactive T cells. The remainder of the T cells formed are eliminated by selection processes which allow only T cells responsive to foreign antigenic peptides associated with self MHC to be released from the thymus.

The spleen of mammals is a large, encapsulated, lymphoid organ which filters out antigens that enter the bloodstream. The spleen is largely responsible for the destruction of old and damaged red blood cells and is also a major site for mounting immune responses. The cortex of the spleen, which is composed of lymphoid tissue or white pulp, contains packed lymphoctes and is the area where immune responses are generated. Blood and (associated antigens) is deposited in the white pulp where the interaction between antigen, antigen-trapping phagocytes, and lymphocytes sets the stage for an immune response. The medulla consists of erythroid tissue or red pulp and is the area where old and defective red blood cells are destroyed and removed.

The lymph nodes are small bean-shaped organs that are located at the

major junctions in the lymphatic system and serve as filters for lymph. Like the thymus, lymph nodes consist of an outer cortex and an inner medula. When anitgens enter the lymph node via an afferent lymphatic vessel they may be trapped and phagocytosed by the macrophages. Antigen stimulation results in the differentiation of lymphocytes and plasma cells, producing an enrichment of lymph leaving the node through the efferent lymphatic vessel with antibodies and lymphocytes.

The ultimate target of all immune responses is an antigen, a foreign substance that can be specifically bound by antibody or T cell receptors. The immune response is a dual system, involving both antibody-mediated (humoral) and cell mediated responses.

Humoral immune responses involve immunoglobulins (Ig) that are expressed on the cell surface of B lymphocytes and function as antigen-binding receptors. When mature B lymphocytes specifically recognizes antigen, the B cell internalizes some of the antigen through receptor-mediated endocytosis. The antigen is processed by the B cell and is presented on the cell surface as a complex of peptide with major histocompatibility (MHC) proteins (see below), which is recognized by antigen specific helper T cells (T_H). The interaction between B cell and T cell triggers the secretion of a variety of lymphokines which activates the division and differentiation of the B cells. This process

generates a population of plasma cells and memory B cells and enhances antibody production. Plasma cells produce and secrete specific antibodies that circulate in the bloodstream and specifically bind to the antigen that induced their production. A proportion of the B cells transform back into resting, mature B cells known as memory cells, that are capable of being activated for a subsequent and more rapid response. Binding of antigens by antibodies inhibits the ability of antigens to bind to receptors on target cells. The antigen-antibody complex also enhances ingestion of the microorganism by phagocytic cells or activate a system of blood proteins known collectively as complement. Complement proteins undergo a cascade of proteolytic reactions that result in the assembly of membrane attack complexes that destroy microorganisms.

Following antigen recognition a biphasic antibody response occurs which is characterized by a lag period when antibody is not detectable, followed by a stage in which antibody levels rise markedly. A plateau with stabilized antibody levels is reached and this is followed by a period of decline. This primary response consists of IgM and IgG antibody isotypes although the production of IgM precedes the production of IgG. On second exposure to an antigen, memory cells are responsible for a more rapid, more pronounced, and longer lasting secondary response which is predominately IgG. The conversion from a response which is predominately IgM to one that is primarily IgG is

termed isotype switching. The anamnestic or memory response is the result of many more specific clones of memory B cells produced by the initial antigenic stimulation. The affinity of the antibodies produced in a secondary response is usually much greater (as much as 100- to 1000-fold) than those produced in a primary response. This process is known as affinity maturation and is believed to be a result of somatic mutation and antigen selection of high-affinity clones. Affinity maturation and isotype switching are known to lead to more effective elimination of pathogens.

Cell-mediated immune responses involve the production of specialized T lymphocytes that react with antigens on the surface of other cells. Cell-mediated immune responses may be involved in host protection against bacteria, viruses, fungi, parasites and cancer cells, as well as hypersensitivity reactions, rejection of histoincompatible tissues, and autoimmune disorders. The initial signal that cells have been infected is accomplished by molecules that transport peptides of the invading microbe to the surface of the infected cell. These transporters are proteins of the major histocompatibility complex (MHC) of genes. MHC molecules, synthesized in the endoplasmic reticulum, have deep grooves that conform to peptides that have been synthesized by an infected cell or produced by intracellular degradation of a foreign particle that has been ingested. After binding, the foreign peptide-MHC complexes migrate to the

cell surface. MHC molecules are divided into two classes designated class I and class II MHC molecules. Class I MHC molecules are found on almost all types of cells whereas class II MHC molecules appear only on cells involved in an immune response, such as macrophages and B lymphocytes. The foreign peptide-self MHC complexes displayed by an infected cell are recognized by receptors on the T cell. The structure of the T cell receptor is similar to the membrane-bound antibody molecule that acts as the receptor on B cells although T cell receptors recognize only foreign peptide-self MHC complexes. There are two subpopulations of T cells, each executing distinct functions and categorized by the cluster determinant (CD) differentiation antigens displayed on their surface. These T cells are designated as helper (T_H) and cytotoxic T (T_C) cells. T cells displaying the CD4 glycoprotein generally function as T_H cells whereas those displaying CD8 generally function as T_C cells. Class I MHC complexed to a foreign peptide on the surface of an infected cell is rsecognized by T cells which are phenotypically CD8. Complexes of class II MHC with a foreign peptide are recognized by T cells that are phenotypically CD4. Unlike CD8 T cells, CD4 T cells do not directly kill the cell but activate antigen presenting cells (APC) to destroy the cell (Janeway, 1993). Upon recognition of a peptide-MHC II complex, T_H cells are activated to secrete a variety of lymphokines thereby acting as effector cells that may activate B cells,

T_C cells and macrophages. Two mouse T_H cell subsets have been identified by the lymphokines which they secrete when activated and are categorized as T_H1 and T_H2 cells (Janeway, 1993). T_H1 cells are thought to be involved in delayed-type hypersensitivity (DTH) reactions and the activation of T_C cells (Janeway, 1993). In a DTH reaction, T_H1 recognizing certain peptide-MHC II complexes differentiate into T cells known as T_{DTH} following primary sensitization with antigen. This process takes 1-2 weeks and is followed by the secretion of a variety of lymphokines which function to attract and activate macrophages, induce hamatopoiesis of monocytes and neutrophils, and stimulate the extravasation of circulating monocytes and neutrophils. DTH is manifested by redness and swelling of the lesion at the site of antigen administration, takes longer to develop than antibody-mediated responses and plays an important role in host protection. The lymphokines secreted by activated T_H1 cells may also induce T_C cells that have recognized peptide-MHC I complexes to differentiate into cytotoxic T lymphocytes (CTL). CTL recognize and eliminate infected target cells by releasing chemicals that cause membrane damage resulting in the destruction of those cells. T_H2 cells are thought to be involved as helpers for B cell activation. T_H2 cells recognize peptide-MHC II complexes on the surface of antigen-presenting cells (APC) which include B lymphocytes, macrophages and dendritic cells. The contact between T_H2 and B cells results in the

secretion of lymphokines that activate the B cells, stimulate B cell proliferation and induce B cells to mature into antibody secreting cells. Other T cells, which have been identified as $CD8^+$ T cells, were originally referred to as supressor T cells (T_S). These T cells induce suppression of an immune response leading to a downward modulation of immune responses or diminish the reactivity of APC with other T cells. However, there has been no solid evidence to support the hypothesis that this supression is mediated by a distinct T_S cell population.

Antigens that require interactions with T cells in order to generate an immune response are known as thymus-dependent (TD) antigens. Some antigens, referred to as thymus-independent (TI) antigens, can induce B cell proliferation in the absence of T_H cells and APC. These antigens have repeating antigenic determinants and can stimulate B cells directly by crosslinking surface receptors for antigen. The resulting response produces low affinity antibodies of the IgM class. Most evidence suggests that LPS is a TI antigen which acts as a polyclonal activator or mitogen for B cells resulting in antibody production of a wide range of specificities (Morrison and Rudbach, 1981).

Comparison Between The Immune Systems of Mammals and Fish.

The immune system of fish has not been as extensively studied as the mammalian immune system, although in the last twenty years there has been increasing interest in the immune mechanisms of fish because of the expansion of the aquaculture industry. The immune system of fish is less complex than that of mammals although there has been a substantial amount of data generated that suggests that there are strong correspondences between the immune systems of fish and mammals.

The primary lymphoid organs of teleost fish are the kidney, spleen, thymus, the lymphoid tissue of the digestive tract, blood and lymph (Dorson, 1984). In fish, like mammals, cell lineages of immune and blood cells begin with the stem cells. Fish do not possess bone marrow but exhibit well developed bone marrow-like microenvironments in the kidney and spleen (Ellis, 1982) containing antibody producing cells and phagocytes, cells whose origins are in bone marrow in mammals (Rijkers, 1981). In teleosts, the spleen is capable of antigen trapping, a process concerned with the development of immunological memory (Ellis, 1988). The thymus of fish has many features of a mammalian thymus and is composed of differentiating lymphocytes, macrophages and epithelioid cells (Ellis, 1982).

Studies in rainbow trout (Kaastrup *et al.*, 1988; Hayden and Warr, 1985) and channel catfish (Ellsaesser *et al.*, 1988) have demonstrated that peripheral blood lymphocytes can be separated into two subpopulations. One of these expresses membrane bound immunoglobulins or antigen-binding receptors (mIg^+) and is analogous to B cells while the other is (mIg^-) and is analogous to T cells. Those studies additionally demonstrated the presence of accessory cells including macrophages and monocytes. Studies in channel catfish have demonstrated that immune responses to LPS require the presence of mIg^+ lymphocytes and monocytes whereas immune responses to known TD antigens such as concanavalin (ConA) require both mIg^+ and mIg^- lymphocytes as well as monocytes (Miller *et al.*, 1985). This study suggests that LPS is a TI antigen in fish as well as mammals.

In mammals, five isotypes of immunoglobulin (IgG, M, A, D, E) are known, whereas only one has been identified in teleost fish. This immunoglobulin is a tetramer of approximately 700 kDa and is classified as IgM because of its similarity to mammalian IgM with respect to subunit structure and molecular weight (Voss *et al.*, 1980; Lobb and Clem, 1983; Atanassov and Botev, 1988). Secondary immune responses (defined by higher antibody titres) have been reported in several fish species (Avtalion, 1969;

Trump and Hildemann, 1970; Ambrosius and Frenzel, 1972; Arkoosh and Kaattari, 1991) although the processes such as isotype switching and affinity maturation are apparently absent (Kaattarri, 1994). In addition, differences in serum antibody concentrations between primary and secondary exposures to antigen are not as pronounced as they appear to be in mammals (Dorson, 1984).

Cellular antigen processing events in fish are believed to be similar to those in mammals (Vallejo *et al.*, 1992). Studies in channel catfish have confirmed the presence of APC which take up and degrade antigen, and subsequently present fragments of the antigen to specific lymphocytes (mIg$^-$) by molecules which resemble MHC (Vallejo *et al.*, 1992). The existence of MHC molecules has not been confirmed in teleosts although putative class I and class II MHC genes have recently been identified in carp (Kaufman *et al.*, 1990). There are other resemblances between the immune systems of fish and those of higher vertebrates. Nonaka *et al.*, (1981) demonstrated that rainbow trout had a multicomponent complement system which can form a lytic membrane attack system similar to the one in mammals. In addition, phagocytic activity has been described in cell populations of salmonid fish including the monocyte-macrophage cell lineage and neutrophils (Griffin, 1983; Suzuki, 1984, O'Neil,

1985) as well as thymocytes (Suzuki, 1984). Recent evidence also suggests that fish leukocytes secrete a cytokine with macrophage activating factor (MAF) activity (Graham and Secombes, 1988) that is analogous to mammalian interferon-gamma (IFN) (Graham and Secombes, 1990).

Overview of Anti-Furunculosis Vaccine Development.

The first substantiated attempts to develop an anti-furunculosis vaccine were performed by Duff in 1942 (Duff, 1942). Subsequently, attempts to treat furunculosis outbreaks induced in salmonid fish by *A. salmonicida* infections involved the use of chemotherapeutic agents and antibiotics. These approaches were generally ineffective as they proved to be expensive and were of only short-term benefit. However, the most consequential drawback of chemical and antibiotic treatment against bacterial disease was the potential of the generation of drug-resistant strains of bacterial pathogens (Snieszko and Bullock, 1957; Aoki *et al.*, 1970). More recently, as a logical alternative to chemical treatment, vaccination programs have been initiated to induce protection against furunculosis in the aquaculture industry.

Many approaches have been explored for development of vaccines for fish. As a measure of prevention against various infectious organisms, these

strategies have included immunizations with O-antigen preparations (Anderson and Jenney, 1991), adjuvanted whole-cell bacterial formulations (Tatner, 1990; Bogwald *et al.*, 1991; Niki *et al.*, 1991), isolated ECP (Hastings and Ellis, 1988; Lund *et al.*, 1991), LPS (Velji *et al.*, 1990), polysaccharides (Robertsen *et al.*, 1990; Yano *et al.*, 1991), glycoconjugates (Banoub *et al.*, 1989) and oral enteric coated vaccines (Wong *et al.*, 1992). Vaccines developed against some bacterial diseases of fish (e.g. vibriosis) have been quite effective in inducing protection (Velji *et al.*, 1990; Bogwald *et al.*, 1991; Wong *et al.*, 1992). However, despite a considerable amount of research that has produced some encouraging results regarding immunity to *A. salmonicida* (Olivier *et al.*, 1985; Adams *et al.*, 1988) and the existence of commercially available anti-furunculosis vaccines, heavy losses of cultured salmonids due to furunculosis continue to occur (Ellis, 1988). Having the ability to induce immunosupression within its host (Porreau *et al.*, 1986), *A. salmonicida* is an efficient pathogen and accordingly, an inefficient antigen in terms of stimulating protective immunity in salmonid fishes.

LPS as a Candidate for Vaccine Development.

As described above, there are several components which can be isolated from the cell envelope of *A. salmonicida* that contribute to bacterial virulence and allow the organism to induce a diversity of pathophysiological effects (discussed briefly below). The ECP each have distinct functions and, logically, one component or a combination of several components could potentially be utilized in formulating an anti-furunculosis vaccine. As alluded to previously, vaccines composed of whole cell bacterial preparations (Bogwald *et al.*, 1991; Wong *et al.*, 1992) or extracellular components (Velji *et al.*, 1990) have been quite effective in inducing protection in salmonids against other pathogens but efforts to induce protection against *A. salmonicida* have been only moderately successful (Ellis, 1988; Kennedy-Stoskopf, 1993).

Throughout the course of this study, we have utilized LPS as the antigenic component in our formulations. LPS is a significant cell surface antigen from *A. salmonicida* and a prominent factor in determining virulence of Gram-negative bacteria (Luderitz *et al.*, 1966 a,b). Our attempts were directed at inducing enhanced serum anti-LPS antibody responses, which might consequently lead to the generation improved levels protection against *A. salmonicida* in fish. LPS, the outermost constituent of Gram-negative bacteria,

is a complex macromolecule composed of both toxic and immunogenic moieties (discussed in the following section). LPS, is also known to be essential to bacterial viability and mutants that are unable to produce LPS are not viable.

Because of its crucial role and its exposed position, LPS represents an ideal target of disease-producing bacteria for antibodies and appears to be a reasonable choice as the major component for a formulation designed to modulate the immunity against *A. salmonicida* infections in rainbow trout.

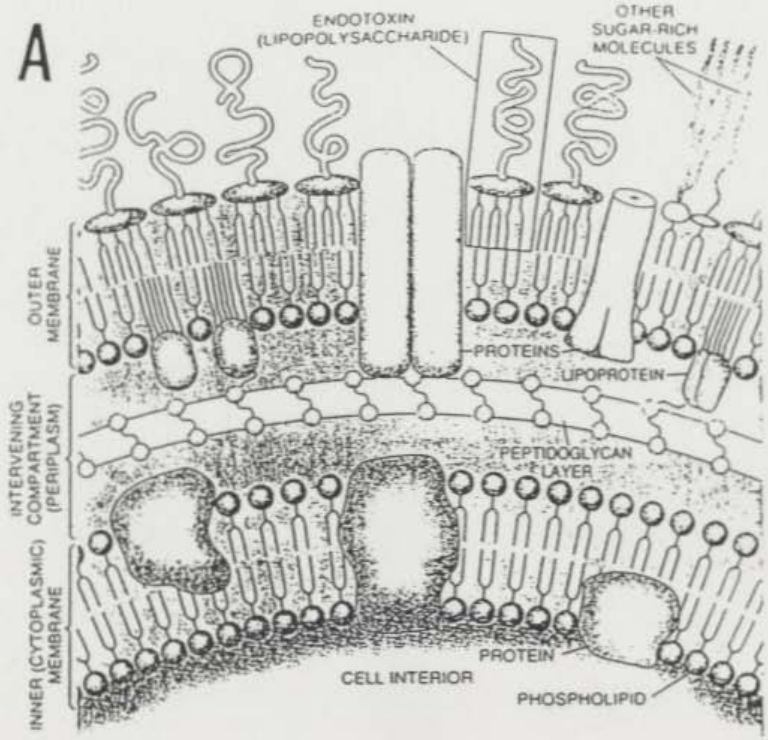
Structural Properties of LPS.

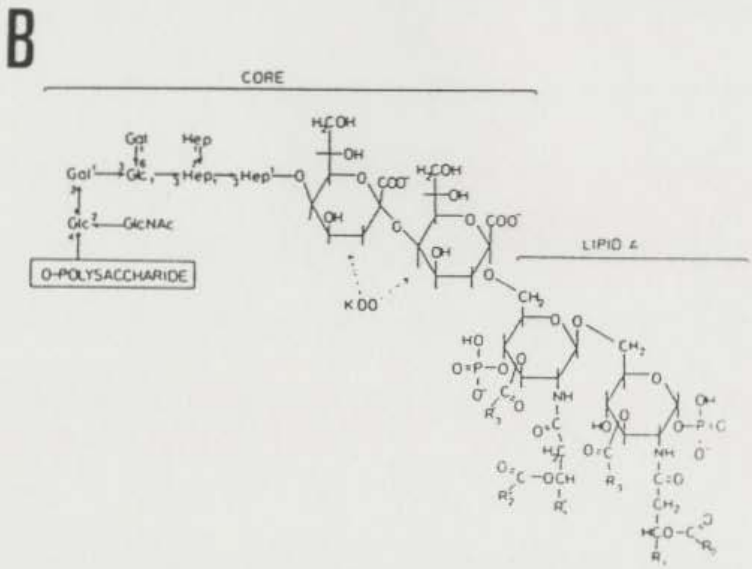
LPS is synthesised exclusively by Gram-negative bacteria and exists as a complex amphiphilic macromolecule firmly bound to the cell wall representing the outermost constituent of the outer membrane (Rietschel and Brade, 1992). LPS was first described as an endotoxin by Richard Pfeiffer (1892) while examining *Vibrio cholerae*. Pfeiffer used this term to distinguish this heat-resistant toxic substance from exotoxin, which was described earlier as the heat-labile toxic material released from live bacteria in growing cultures or acute infections. Endotoxin, in contrast to exotoxin, is released only during cell death or bacterial cell multiplication when bacteria undergo disintegration. In the 1940's, the term lipopolysaccharide was introduced based on the chemistry

of the macromolecule, and accordingly, the terms LPS and endotoxin are frequently used synonymously. It has been demonstrated that LPS can bind to mammalian erythrocytes without causing haemolysis (Neter, 1956) while it has been suggested that there is a relationship between the lethal effects of endotoxin *in vivo* and its affinity for erythrocytes (Hill and Weiss, 1964). Comparative studies on the toxicity of *E. coli* LPS indicated that this LPS was more toxic to mammals than to fish (Berzci *et al.*, 1966). Recently, it was reported that salmonid erythrocytes were susceptible to lysis by bacterial LPS from several species (Lee and Ellis, 1991).

It has been estimated that one bacterial cell contains approximately 3.5 x 10^6 LPS molecules occupying an area of 4.9 μm^2 (Rietschel *et al*; 1994) which can amount to three-quarters of the surface area in the case of an *E. coli* cell (total surface area, 6.7 μm^2) (Rietschel *et al*; 1994). Classically, LPS from smooth (wild-type) strains of bacteria (S-LPS) can be viewed as an amphiphilic molecule composed of three covalently linked segments: the O-antigen, the core oligosaccharide, and lipid A as originally proposed by Luderitz *et al.* (1966b) (Figure 1. 2.). Each moiety of LPS manifests a distinct composition, biosynthesis and biological function. The lipid A component is embedded in the outer membrane of bacterial cells whereas the polysaccharide portion

FIGURE 1.2. A) A schematic representation of the outer membrane of Gram-negative bacteria, B) The general architechture of Gram-negative bacterial lipopolysaccharide. R indicates the locations of the fatty acids. Reproduced with permission from Rietschel, E. T., and Brade, H. 1992. Bacterial endotoxins. Scientific American. 267:54-61.


A
ENDOTOXIN
(LIPOPOLYSACCHARIDE)
OTHER
SUGAR-RICH
MOLECULES
OUTER
MEMBRANE
PROTEINS
LIPOPROTEIN
INTERVENING
COMPARTMENT
(PERIPLASM)
PEPTIDOGLYCAN
LAYER
INNER (CYTOPLASMIC)
MEMBRANE
PROTEIN
CELL INTERIOR
PHOSPHOLIPID


B
CORE
LIPID A
O-POLYSACCHARIDE
GlcNAc

protrudes into the extracellular environment. The O-antigen (also referred to as the O-specific chain) is the most variable segment of the molecule and the part that can evoke specific immune reactions. Typically, the chain consists of 20 to 40 repeating units that include up to eight sugars per unit (Luderitz *et al.*, 1966b; Nowotny, 1984). The O-antigen differs from one bacterial species to the next and between different strains of the same species. The types and the sequence of sugars within the O-antigenic chain and the number of repeating units often differ and therefore may generate production of diverse antibodies.

The core oligosaccharide which is divided into two regions, is neither as variable nor as immunogenic as the O-antigen chain, although antibody production can be stimulated in response to mutant (from rough strains) LPS (R-LPS) which lack O-antigen (Luderitz *et al.*,1966b; Nowotny, 1984). The inner core is linked to lipid A via 3-deoxy-D-manno-2-octulosonic acid (KDO) and is mostly composed of heptoses whereas the outer core is linked to the O-antigen. One of the chief characteristics of the core oligosaccharide is its substitution by phospho, pyrophospho, and ethanolamino residues which express a net negative charge that may have important physiological implications.

The lipid A moiety of LPS is the least variable and the most examined of

the structural units. It is virtually always composed of a glucosamine disaccharide with attached phosphate(s) and fatty acids although the number of fatty acids and the sites of attachment are variable. It is generally accepted that lipid A is responsible for the toxic properties of LPS since endotoxins consisting of only lipid A and KDO are as toxic and pyrogenic as molecules that comprise a full polysaccharide (Brade *et al.*, 1986). In addition, it appears that structural derivatives of lipid A are not as potent as native lipid A, indicating that at least the entire lipid A component and not some particular fraction of it is needed for maximal endotoxic activity (Brade *et al.*, 1986).

Physiological Effects of LPS.

When bacterial cells multiply, or when they die or lyse, LPS is secreted from the cell surface. This liberated LPS is capable of recruiting host cells such as macrophages and monocytes that can be activated to secrete mediator molecules which elicit a diversity of responses. In mammals, it is believed that there exists a number of potential specific LPS membrane receptors (for a review, see Lynn and Golenbock, 1992). In one model, circulating endotoxins bind to a molecule known as LPS-binding protein (LBP) (Wright *et al.*, 1989) and the resulting LPS-LBP complex is subsequently anchored with a receptor

on the surface of macrophages and monocytes, referred to as CD14 (Wright *et al.*, 1991). Consequently, the activated host cells release a small protein called tumor necrosis factor alpha (cachectin) (TNF). When administered to mammals, purified TNF can induce some of the harmful effects originally attributed to LPS such as fever and shock and can also recruit various defensive cells to sites of infection and efficiently destroy tumor cells (Beutler *et al.*, 1986; Old, 1988). LPS-stimulated macrophages also release interleukin-1 (IL-1) (Dinarello, 1984), IL-6 and IL-8 (Rietschel and Brade, 1992) which exert many of the same effects as TNF. These cells also secrete a variety of lipids, such as prostaglandin E_2, thromboxane A_2 and platelet-activating factor (PAF), which may contribute to fever and regulate the activity of immune system cells (Rietschel *et al.*, 1980). Stimulation of macrophages by endotoxin may also produce oxygen free radicals which, when present on the cell surface or intracellularly, may contribute to microbial destruction (Dijkstra *et al.*, 1989). In mammals, the effects of moderate levels of the mediators mentioned above can be beneficial and may aid in the recovery process with mild fever, stimulation of the immune system and microbial killing. If infection is severe and large amounts of LPS accumulate in circulating blood, large quantities of those mediators are released and harmful physiological effects such as violent

fever, hypotension, disseminated blood clotting and lethal shock can result (Rietschel and Brade, 1992). These mediators have paracrine effects at the local cellular level which may be important in stimulating and regulating specific immunity.

It has been shown that fish display enhanced resistance to LPS from *E. coli* as compared to mammals (Berczi *et al.*, 1966). Similarly, Patterson and Fryer (1974) demonstrated that LPS from *A. salmonicida* was toxic to mice but not toxic to coho salmon at the same doses. It appears then, that LPS may be more toxic to mammals than fish, regardless of the origin of the LPS (isolated from human or fish pathogens).

The Weakness of LPS as an Antigen.

One disadvantage in developing LPS-based vaccines is that LPS is a TI antigen in both mammals (Morrison and Rudbach, 1981) and fish (Miller *et al.*, 1985). Certainly, humoral immunity plays a role in providing resistance to some of the harmful effects of LPS although the response appears to be limited (Elkins *et al.*, 1989). T-cell mediated immunity is also considered to be of prime importance to the enhancement of protective immunity against bacterial disease. It is believed that intracellular bacterial multiplication is controlled by

the presence of activated macrophages (generated as a result of T-cell mediated response) or cytotoxic T cells or both which can destroy infected cells (Hahn and Kaufman, 1981). Therefore, in efforts to enhance the immunogenicity of LPS from *A. salmonicida*, we have investigated the practicality of the incorporation of the LPS macromolecule into phospholipid bilayer vesicles (liposomes) as will be discussed below.

Applications of Liposomes.

Liposomes were originally described by Bangham and Horne (1964) who recognized that in aqueous environments, phospholipid films would spontaneously form closed structures that encapsulated part of the aqueous medium in their interior. Hydrophobic interactions between the phospholipids are the primary driving force for the formation of liposomes when they are confronted with water. Liposomes may differ in their size, composition, charge, and lamellarity. A wide range of compounds may be incorporated into either the lipid or trapped aqueous space, and such flexibility has presented several potential applications to scientific investigators.

Initially, liposomes were attractive to biophysicists as model systems for biological membranes. The lipid bilayer structures of liposomes mimic the

barrier properties of biomembranes, and therefore, they offered the potential of examining the behaviour of membranes of known composition. Liquid-crystalline bilayers are generally permeable to hydrophobic and small non-electrolyte, water-soluble molecules. The fluidity of the lipid bilayer, which is influenced by membrane lipid composition, is critical in determining the rate of diffusion across the membrane. For instance, decreasing acyl chain length and increasing acyl chain unsaturation increases fluidity whereas increasing acyl chain length and decreasing acyl chain unsaturation will reduce fluidity (Davis and Keough, 1984a; Davis and Keough, 1985). Introduction of cholesterol (Davis and Keough, 1984b; Davis and Keough, 1986) and α-tocopherol (Nakagawa *et al.*, 1980) into lipid bilayers also effectively reduces fluidity, thereby enhancing bilayer stability. Thus, by altering the lipid composition of the bilayer or the material incorporated, it was possible to establish differences in membrane properties. Model membranes have facilitated the study of the lipid-protein interactions occurring in biological membranes and have been used in a multitude of research projects concerning membrane structure and function.

Liposomes have been adapted to numerous other potential applications, including their use as vehicles to achieve specific delivery of therapeutic drugs to target organs, to reduce toxicity of antimicrobial, antiviral and

chemotherapeutic agents, or to potentiate the immunogenicity of antigenic substances, that is, to act as immunological adjuvants. Accordingly, there have been a myriad of drugs and antigens incorporated into liposomes to achieve those objectives and several examples of these formulations are provided in Table 1.3.

Because of their biodegradable and non-toxic nature, liposomes can be safely administered without serious side effects (Sculier *et al.*, 1989) and have been frequently used as drug delivery vehicles. One of the basic goals of chemical therapeutics is to deliver the drug efficiently and specifically to the site of disease. Some drugs may be delivered in their free form whereas others require a carrier such as a liposome in order to reach and enter their final destination because a) they are rapidly cleared from the area of introduction or the circulation or b) they are obstructed by biological barriers which they cannot permeate. Liposomes are regarded as suitable carriers because they can serve as a depot system for the sustained release of an associated compound. In addition, liposomes can alter the biodistribution of biologically active substances (Proffitt *et al.*, 1983; Liu and Huang, 1992), protect the enclosed materials from inactivation by the host defense mechanisms (Heath *et al.*,1980; Gabizon, 1992; Ahmad *et al.*, 1993; Vaage *et al.*, 1993) and decrease the

Table 1.3. Diversity of Materials Incorporated into Liposomes and their Associated Applications.

Site Specific Drug Delivery
Doxorubicin (Gabizon, 1992; Ahmad *et al.*, 1993; Vaage *et al.*, 1993)
Epirubicin (Gabizon, 1992)
Vincristine (Vaage *et al.*, 1993)
Methotrexate-8-aspartate (Heath *et al.*,1980)

Reduced Drug Toxicity
Actinomycin D (Kaye *et al.*, 1981)
Adriamycin (Gabizon *et al.*, 1982)
Aminoglycosides (Fountain *et al.*, 1981)
Ampicillin (Bakker-Woudenberg *et al.*, 1985)
3'-azido-3'-deoxythymidine (AZT) (Philips *et al.*, 1991)
Cytosine arabinoside (Mayhew *et al.*, 1978)
Methotrexate (Kaye *et al.*, 1981)
Oxytetracycline (Gruner *et al.*,1985)
Penicillin (Gregoriadis, 1973)

Immunological Modulation
Diptheria toxin (Allison and Gregoriadis, 1974)
Epstein-Barr virus glycoprotein (Epstein *et al.*, 1985)
Hepatitis B surface antigen (Manesis *et al.*, 1979)
Influenza virus envelopes (Gregoriadis *et al.*, 1992)
Neisseria gonorrhoeae proteins (Kersten *et al.*, 1988)
Salmonella typhimurium LPS (Desiderio and Campbell, 1985)

detrimental side effects of some drugs (Gregoriadis, 1973; Mayhew *et al.*, 1978; Fountain *et al.*, 1981; Kaye *et al.*, 1981; Gabizon *et al.*, 1982; Bakker-Woudenberg *et al.*, 1985; Gruner *et al.*,1985; Philips *et al.*, 1991).

Another significant prospect for liposomes, and the most relevant to this study, is their potential as immunological adjuvants for the enhancement or modulation of immune responses to various antigens. Adjuvants are a diverse class of compounds which can enhance immune responses to particular antigens. They include aluminum hydroxide (alum), Freunds incomplete adjuvant (FIA), Freunds complete adjuvant (FCA), saponins complexed to cholesterol (immune stimulating complexes, ISCOMS), and muramyl dipeptide (MDP). Lipid A is also a potent adjuvant and therefore, LPS is considered as an antigen that carries its own adjuvant (Chilier *et al.*, 1973). However, some of these adjuvants, including lipid A, are toxic in mammals and may cause a wide range of side effects such as granulomas, acute and chronic inflammation, cytolysis, pyrogenicity and arthritis in mammals (Gregoriadis, 1990). The toxicity of these adjuvant compounds is clearly a detriment, and since liposomes are non-toxic, those harmful side effects might be circumvented with the use of liposomally-incorporated LPS (Dijkstra *et al.*, 1989; Petrov *et al.*, 1992).

Allison and Gregoriadis (1974) demonstrated that antibody titres to

diptheria toxin were much higher when the protein was administered in the liposome-incorporated form than when the protein was administered in the free form. Subsequently, the adjuvant effect of liposomes has been demonstrated with a wide spectrum of biologically active substances, including LPS from a variety of bacterial species, to potentiate immune response and stimulate host defence mechanisms (see Table 1.3.). Because of the structural properties of LPS, it is possible that this macromolecule can either be trapped in the aqueous compartments between the lipid bilayers or embedded within the lipid bilayer via the lipid A moiety with the carbohydrate moiety protruding from the liposomal surface. Thus, the use of liposomal LPS as vaccines has potential because of the efficiency with which the LPS can be delivered to the macrophages of the RES by the liposomes (see below), and the capacity of liposomes to act as immunological adjuvants resulting in an intensified presentation of LPS molecules to the host immune cells. The latter may be the most attractive feature of liposomes. Evidence in mammalian models suggests that liposomes can influence the immune response to an associated protein antigen to promote the generation of specific T-cell mediated immune responses (Tom, 1981; Garcon and Six, 1991). This phenomenon is likely due to the increased internalization of liposome-incorporated antigens by macrophages

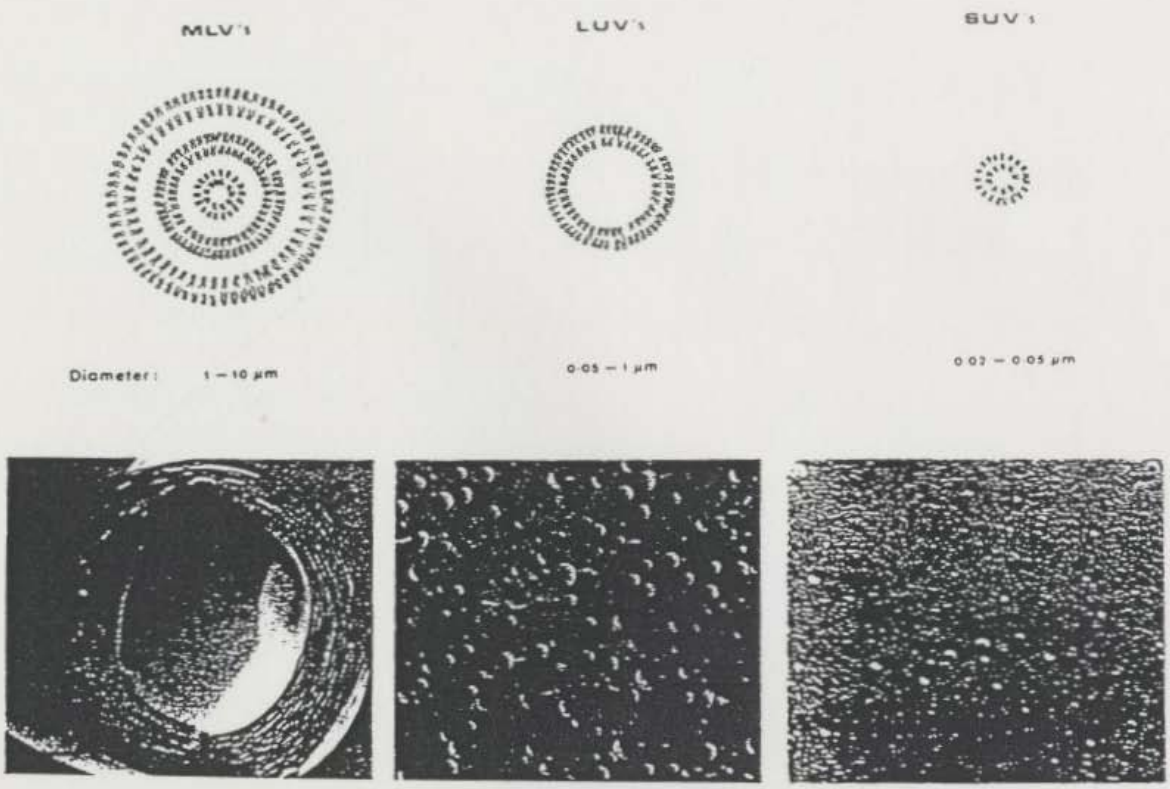
which allows for an increased antigen presentation to T cells (Harding *et al.*, 1991; Collins *et al.*, 1992). It has been suggested that adjuvants may stimulate accessory cells, such as macrophages or other APC, to produce soluble substances (cytokines) or membrane molecules that provide important co-stimulation of T cells (Therien *et al.*, 1991). As macrophages avidly and efficiently take up liposomes, it is postulated that macrophages serve as antigen-presenting cells for liposomal antigens (van Rooijen and Su, 1989; Alving, 1987). Further, Desiderio and Campbell (1985) reported that intraperitoneal administration of liposomal LPS from *Salmonella typhimurium* could modulate the host immune response in mice in favor of cell-mediated immunity, resulting in increased levels of protection against salmonellosis. Another study however, concluded that vesicles that contained LPS were not as efficient as vesicles coated with outer membrane proteins (containing residual quantities of LPS) in generating anti-LPS antibody responses when administered orally or intranasally to mice (Brownlie *et al.*, 1993).

Categories and Methods for Preparation of Liposomes.

With the many potential uses presented by these model membranes, the therapeutic applications of liposomes depend on the physical integrity and

stability of the lipid bilayer structure. There are three major categories of liposomes which can be prepared by numerous techniques and the resulting vesicles may be large, or small, and of unilamellar or multilamellar nature as illustrated in Figure 1.3. Multilamellar vesicles (MLV), composed of numerous concentric bilayers, are produced from mechanical agitation of a dispersion of dried lipid with an aqueous phase. Mechanical agitation is the simplest method for production of MLV that produces a suspension of large liposomes that are very heterogenous in size and exhibit a relatively low level of aqueous encapsulation. The aqueous encapsulation of materials into MLV can be improved by the dehydration-rehydration method (Kirby and Gregoriadis, 1984). This technique involves mixing liposomes with the material to be encapsulated, followed by lyophilization and rehydration. Another application of this procedure is to add the aqueous solution containing the material to be encapsulated with an ethanolic solution of lipid, which is then dried and gradually hydrated (Gruner *et al.*, 1985). In addition, the freeze-thaw procedure which involves the repetitive freezing of MLV followed by thawing at 40^{0}C can result in vesicles with enhanced trapping properties (Mayer *et al.*, 1985). Small unilamellar vesicles (SUV) can be produced by sonication of MLV (Abramson *et al.*, 1964) or by extrusion through a French press

FIGURE 1.3. Egg phosphatidylcholine multilamellar vesicles (MLV), large unilamellar vesicles (LUV) and small unilamellar vesicles (SUV). A) Schematic representation and B) as visualized by freeze-fracture electron microscopy. Reproduced with permission from Cullis *et al.* 1989. Advanced Drug Delivery Reviews. 3:267-282.


MLV's
LUV's
SUV's
Diameter: 1 – 10 µm
0·05 – 1 µm
0·02 – 0·05 µm

(Barenholz *et al.*, 1979). Large unilamellar vesicles (LUV) have been prepared via dilution from organic solvent (reverse-phase evaporation) (Szoka and Papahadjopoulos, 1978), detergent dialysis (Racker, 1973) or extrusion of MLV under pressure through membranes of known pore sizes (Hope *et al.*, 1985). The trapping properties of some of the methods of preparation and the corresponding classes of liposomes obtained are given in Table 1.4. These methods have been utilized to optimize the incorporation of a desired compound within liposomes, to limit the permeability of the membrane to the entrapped material and to alter half-life in circulation in attempts to enhance the therapeutic efficiency of a liposomal formulation. As mentioned previously, there are several factors to consider in liposomal formulation that can affect the rate of uptake of liposomes by the RES and their subsequent circulation time *in vivo*. These factors include lipid composition and overall surface charge (Senior and Gregoriadis, 1982; Gabizon and Papahadjopoulos, 1988; Allen *et al.*, 1991; Senior *et al.*, 1991; Huang *et al.*, 1992; Park *et al.*, 1992), vesicle diameter (Senior and Gregoriadis, 1982; Allen and Chon, 1987; Goren *et al.*, 1990; Allen *et al.*, 1991) and incorporated ligand (Bally *et al.*, 1990). The most prominent of these considerations appears to be liposomal surface charge which can be regulated by altering the lipid composition. For instance,

"conventional" liposomes which are composed of phosphatidylcholine, cholesterol, and some negatively-charged lipids (e.g phosphatidylglycerol or phosphatidylserine) manifest relatively short circulation half-lives in mammals and are cleared rapidly from sites of administration and are distributed within the RES, namely the Kupffercells of the liver and the macrophages of the spleen (Senior and Gregoriadis, 1982; Gabizon and Papahadjopoulos, 1988; Allen *et al.*, 1991; Huang *et al.*, 1992; Park *et al.*, 1992). There is also some evidence that positively-charged "conventional" liposomes containing stearylamine exhibit accelerated clearance *in vivo* compared to neutral liposomes (Senior *et al.*, 1993). Recently developed long-circulating liposomes depend on surface hydrophilicity and shielded surface charges imparted by the addition of modest molar equivalents of monosialoganglioside (GM_1) (Allen and Chon, 1987; Gabizon and Papahadjopoulos, 1988; Huang *et al.*, 1992), lipid derivatives of polyethylene glycol (PEG) (Allen *et al.*, 1991), hydrogenated phosphatidylinositol (HPI) (Gabizon and Papahadjopoulos, 1988), in addition to membrane rigidifying compounds such as cholesterol and/or sphingomyelin (Senior and Gregoriadis, 1982) in the formulations. Such long-circulating liposome formulations have been termed Stealth^R liposomes since the presence of such bulky, hydrophilic components may produce a steric hinderance of the

Table 1.4. Trapping Properties of some Liposomal Formulations.*

Vesicle Type	Preparation Procedure	Vesicle Diameter (μm)	Advantages	Disadvantages
SUV	sonication	0.025-0.040	· no detergents used · fast procedures	· dilute liposome suspension · low trapping efficiency
SUV	French press	0.020-0.050	· homogenous population · stable formulations · excellent reproducibility	
SUV	detergent removal	0.036-0.050	· reconstitution of proteins possible	· detergents difficult to remove completely
LUV	detergent removal	0.1-10.0	· high trapped volumes	· lengthy procedures · low trapping efficiency
LUV	reverse-phase evaporation	0.2-1.0	· high trapping efficiency	· exposure of entrapped materials to organic solvent and sonication
LUV	ether evaporation	0.1-0.5		· heterogenous population
LUV	extrusion	0.056-0.2	· high trapping efficiency · no detergents or solvents used	· trapped volumes relatively low unless freeze-thaw protocol is employed
MLV	mechanical agitation	0.4-3.5	· fast procedure · suitable for a variety of compounds · no detergent used	· low trapped volume and trapping efficiency · heterogenous population
MLV	freeze-thaw	0.5-5.0	· fast procedure · high trapped volumes and trapping efficiency	· solute dependent
MLV	sonicate-dehydrate-rehydrate	0.3-2.0	· high trapping efficiency	· limited by lipid solubility in organic phase · residual organic solvent
MLV	solvent-evaporation-sonication	0.3-2.0		

*Reproduced from Hope *et al.*, 1986, and Cullis *et al.*, 1989.

liposomal surface determinants thereby reducing recognition by the macrophages of the RES. Sterically stabilized Stealth^{R} liposomes demonstrate decreased uptake by the cells of the RES and have resulted in several promising applications that are currently being tested as chemotherapeutic agents in animal (Allen *et al.*, 1991; Huang *et al.*, 1992) and in human (Gabizon *et al.*, 1986; Treat *et al.*, 1987; Cowens *et al.*, 1989; Gabizon *et al.*, 1992) trials.

Research Objectives

This study was undertaken with the objectives directed at incorporating the LPS from *A. salmonicida* ssp. *salmonicida* into liposomes and examining the effects of these formulations on humoral immunity in rainbow trout (*Oncorhynchus mykiss*). LPS, a bacterial cell surface antigen, and likely a distinct target of disease-producing bacteria by antibodies, appeared to be a reasonable choice as the antigen component for a formulation to modulate immunity against *A. salmonicida* infections. Although LPS is regarded as being an antigen that carries its own adjuvant (Chillier, 1973), LPS induces a limited immune response (Elkins *et al.*, 1989) and is unable to induce T-cell mediated immunological memory (Morrison and Rudbach, 1981). Because of the potential ability of liposomes to act as both carriers and immunological

adjuvants, free and liposomally incorporated LPS were compared as immunogens. Accordingly, the "conventional" liposomes described above appeared to be most appropriate of the vesicle types to serve as a carrier because of their enhanced uptake by the RES compared to long-circulating liposomes.

In examining the potential of liposomes as plausible delivery vehicles for LPS, we have examined the following aspects;

1. The structural elucidation of LPS from *A. salmonicida* and the individual carbohydrate and lipid components by ^{1}H-Nuclear Magnetic Resonance (^{1}H-NMR) and coupled gas chromatography-electron-impact mass spectroscopy (GC-EIMS) in order to determine the nature of the preparations to which antibodies will subsequently be raised.

2. The incorporation of *A. salmonicida* LPS and polysaccharide devoid of the lipid A moiety (PS) at varying concentrations into "conventional" liposomes of differing lipid compositions, overall surface charge and lamellarity in order to determine the optimal liposomal formulation(s) to be employed for immunization experiments.

3. The effect of route of administration of free versus liposomal-LPS of *A. salmonicida* on the uptake and distribution of LPS among the organs and tissues of rainbow trout, particularly the kidney and spleen which are the principal hemopoietic organs in teleost fish, to establish the efficiency of liposomes as delivery vehicles.

4. The ability of liposomes to function as immunomodulators for the LPS from *A. salmonicida* in rainbow trout.

5. The development of an isolation procedure for rainbow trout immunoglobulin (Ig) that is based on the specific adsorption of Ig to a mixed mode, ion-exchange chromatographic matrix. The Ig isotype generated by immunizations with free and liposomal LPS from *A. salmonicida* was isolated by this method and partially characterized.

CHAPTER 2

STRUCTURAL ANALYSIS OF *Aeromonas salmonicida* LIPOPOLYSACCHARIDE BY NUCLEAR MAGNETIC RESONANCE AND MASS SPECTROSCOPY.

INTRODUCTION

In our attempts to enhance the immunity against *A. salmonicida*, one consideration was the nature and chemical structure of the domains of the LPS from this bacterium. Chemical structures of the various cell-surface antigens obtained from the LPS of the fish pathogens *Vibrio anguillarum* (Banoub *et al.*, 1987), *Vibrio ordali* (Banoub and Hodder, 1985) and *Yersinia ruckeri* (Nakhla, 1988) have been defined using conventional chemical analyses such as methylation, periodate oxidation and selective degradations. For *A. salmonicida*, these analyses have been used to elucidate the structure of the O-antigen and core oligosaccharide moieties, which are thought to contribute to the pathology of furunculosis (Shaw *et al.*, 1983, 1992). The O-antigen moiety was proposed to be a tetrasaccharide repeating unit of rhamnose, glucose and N-acetylmannosamine in a molar ratio of 1.0:1.58:0.83 with 75% of the N-acetylmannosamine substituted at position 4 by O-acetyl groups (Shaw *et al.*, 1983). The core oligosaccharide was proposed to consist of D-galactose, D-glucose, L-*glycero*-D-*manno*-Heptose and 2-acetamido-2-deoxy-D-glucose in a molar ratio of 0.96:1.0:4.02:0.91 (Shaw *et al.*, 1992). The original purpose of this study was to investigate the chemical structure of *A. salmonicida* LPS and of the carbohydrate and lipid moieties using ^{1}H-nuclear magnetic resonance

spectroscopy (^{1}H-NMR) and electron-impact mass spectrometry (GC-EIMS).

Another objective of the spectroscopic studies was to estimate how much of the LPS in liposomally-incorporated LPS formulations was exposed to the extracellular environment as opposed to that inaccessible to external agents (outside/inside ratios). Possibly, establishing differences in outside/inside ratios of LPS within those formulations would be helpful in evaluating the potential of the preparations to induce anti-LPS antibody production. The approach was to identify and assign the ^{1}H-NMR resonances to the functional groups of the LPS and to observe which, if any, of these resonances would be shifted in the presence of paramagnetic metal ions. Preliminary experiments did not however, lead to any substantial shifting or broadening of signals in a manner that could be qualified in the complex structure of LPS and so this avenue of study was discontinued.

While examining intact LPS from *A. salmonicida* using ^{1}H-NMR, an atypical resonance at -0.1 ppm was observed which was not anticipated from the functional groups attributed to the O-antigen (Shaw *et al.*, 1983) or the core oligosaccharide moieties (Shaw *et al.*, 1983). Signals resonating upfield of 1.0 ppm, which may be attributable to -CH_2- groups in a ring, are rare, although an unassigned, high-field signal was observed in the spectrum of LPS from

Escherichia coli at approximately 0.6 ppm (Qureshi *et al.* 1988). Cyclopropane fatty acids are characteristic of some lipids in Gram-negative bacteria (Gunstone *et al.*, 1994) that provide signals resonating upfield from 1 ppm in a ^{1}H-NMR spectrum; the protons from those functional groups resonate at approximately 0.6 ppm (Hopkins, 1961). Resonances near 0 ppm generally correspond with the resonance frequencies of silicon derivatives (Pouchert 1981).

Antifoaming agents are routinely used in the large-scale fermentation of bacterial cells in order to prevent bubble formation leading to the loss of media (Blenkharn and Wood 1987) and silicon-based emulsions are among those compounds. Throughout this project, Antifoam A™ was used in the large-scale fermentation of *A. salmonicida*. The Antifoam A™ emulsion used contains 30% of Antifoam A™ concentrate (a cyclic polysiloxane compound) in aqueous dispersion with non-ionic emulsifiers. Consequently, it was a possible source of the mysterious peak appearing in the ^{1}H-NMR spectra. Following assignment of the resonances observed in the ^{1}H-NMR spectra to the functional groups of the carbohydrate and lipid components of the LPS from *A. salmonicida*, an attempt was made to characterize the origin of the peculiar resonance observed in the ^{1}H-NMR spectrum using spectroscopic techniques, polyacrylamide gel electrophoresis (SDS-PAGE) and enzyme-linked

immunosorbent assay (ELISA).

MATERIALS AND METHODS

Bacterial Cultures and Isolation of LPS.

A virulent, autoagglutinating strain of *Aeromonas salmonicida* was provided by Dr. T. P. T. Evelyn (Department of Fisheries and Oceans, Nanaimo Biological Station, Nanaimo, British Columbia, Canada). Bacterial cultures were kindly grown by Howard J. Hodder of the Department of Fisheries and Oceans, St. John's, Newfoundland, Canada. Briefly, cultures were grown in Trypticase Soy Broth without dextrose (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) with continuous agitation to late stationary phase (approximately 26 h) at 25°C in 24 L batches in a New Brunswick Model MF-128S fermentor (see appendix I). Antifoam A™ (Sigma Chemical Co., St. Louis, MO, USA) emulsion was included in the culture broth at a final concentration of 0.1% (v/v). Alternatively, bacterial cells were grown in the absence of Antifoam A™ (in Trypticase Soy Broth) on an orbital shaker overnight at 24°C. Prior to harvesting, bacteria were killed by exposure to formalin at a final concentration of 0.3 % by volume, and the broth was stirred for an additional 18 h. Formaldehyde-fixed cells were collected by low-

speed centrifugation, washed once with 0.15 M NaCl, lyophilized and stored at -15°C until required.

Lipopolysaccharide was extracted from lyophilized bacteria by the aqueous phenol method (Westphal and Jann 1965) (see appendix I) and further purified by adherence to polymyxin coated Affi-prep beads (Bio-Rad Laboratories, Richmond, CA, USA). A slurry containing polymyxin beads (3 mL) was washed twice with 0.1 M NaOH and once in distilled H_2O. LPS (50 mg in 15 mL phosphate-buffered saline, PBS, pH 7.4, H_2O) was mixed with the washed polymyxin beads and the suspension was shaken overnight at room temperature. The suspension was centrifuged at 2500 x g_{max} and the supernatant containing non-adherent material was decanted. Adherent LPS was recovered from polymyxin beads by resuspension in 15 mL of 0.1 M NaOH, vortexing, incubation at room temperature for 10 min and centrifugation at 2500 x g_{max}. The supernatant was collected and the pellet was washed once more with 5 mL of 0.1 M NaOH and centrifuged as before. The supernatants were combined and the polymyxin-purified LPS (35 mg) was dialysed against 6 changes of distilled H_2O (3.5 L per change) and lyophilized. Phenol-extracted, polymyxin-adherent LPS from bacteria grown in the presence of Antifoam A™ is referred to as LPS^{+AFA} throughout the text. LPS extracted from cells not

exposed to Antifoam A™ is referred to as LPS^{-AFA}.

LPS was hydrolyzed with 1% acetic acid at 100°C for 90 min to liberate the lipid A component (Banoub *et al.* 1989, see appendix I). After centrifugation (5000 x g_{max}) to separate the lipid A and carbohydrate moieties, the supernatant was freeze-dried and resuspended in 47 mM pyridinium acetate buffer, pH 4.26. The sample was applied to a column of Sephadex G-50, eluted with 47 Mm pyridinium acetate buffer, pH 4.26 and the O-antigen and core-oligosaccharide units were fractionated and detected with a differential refractive index monitor (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Antifoam A™ was subjected to aqueous-phenol extraction and a sample (50 mL) from the combined aqueous layers was concentrated, resuspended in 15 mL pyrogen-free PBS and adhered to polymyxin beads. The extracted material was dialyzed extensively against distilled H_2O as described above, and lyophilized. The phenol-extractable, polymyxin adherent material (6 mg) recovered from Antifoam A™ is referred to throughout the text as AFAE (Antifoam A extract).

Polyacrylamide Gel Electrophoresis (PAGE).

Samples of O-antigen, lipid A, LPS^{+AFA}, LPS^{-AFA} and AFAE were boiled

for 2 minutes in the presence of 2% (w/v) sodium dodecyl sulphate (SDS). SDS-PAGE was performed in vertical slab gels (Laemmli 1970) containing 12.5 (w/v) polyacrylamide and 0.1% SDS (see appendix I). Polyacrylamide contained bis-acrylamide (0.8%) as a cross-linking agent. Gels were stained with Coomassie blue (proteins, see appendix I) and ammoniacal silver (proteins and LPS) (Tsai and Frasch, 1982, see appendix I) and carbohydrate-containing constituents were detected with periodic acid-Schiff (PAS) reagent (Van-Scuningen and Davril 1992, see appendix I).

^{1}H-Nuclear Magnetic Resonance (^{1}H-NMR).

^{1}H-NMR spectrum were recorded by Dr. J. H. Banoub using a Varian Gemini-300 spectrometer in the pulsed Fourier Transform mode at 300.1 MHz and 25°C (see appendix I). Solvents used for sample preparation were D_2O and perdeuterated dimethylsulfoxide (d_6-DMSO, 99.9% perdeuterated) and were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Chemical shifts are reported relative to that of external tetramethylsilane (TMS).

Coupled Gas Chromatography-Electron Impact Mass Spectrometry (GC-EIMS).

Samples of LPS^{+AFA}, LPS^{-AFA} and AFAE were subjected to methylation (Hakomori 1964, appendix I) and the methylated products were isolated by elution from a column of Sephadex LH-20 (Pharmacia LKB Biotechnology, Uppsala, Sweden) with chloroform. Alditol acetates of the methylated products were synthesized as previously described (Banoub *et al.* 1987, appendix I). Coupled GC-EIMS of the methylated alditol acetates was performed by H. J. Hodder using a Hewlett-Packard 5970 mass selective detector coupled to a 5890A gas chromatograph equipped with a packed column of CP-SIL-5CD at a source temperature of 200°C and an ionization voltage of 70 ev.

Enzyme-linked Immunosorbent Assay (ELISA).

ELISA was performed as described in appendix I. Briefly, LPS^{+AFA}, LPS^{-AFA}, and AFAE were brought to 100 μg/mL in 0.05M carbonate buffer (pH 9.6) and 100 μL was added to each well in duplicate rows of a 96 well polystyrene microtitre plate (Linbro Titertek, Horsham, Pa, USA). The plates were incubated in the appropriate coating buffer at 4°C overnight. Excess solution was discarded and 100 μL per well of 0.5 % gelatin in phosphate

buffered saline (PBS) was added as blocker for 1 hour at room temperature. The plates were washed five times with PBS containing 0.05% Tween-20 (PBST). During testing, positive controls were added to each microtitre plate (see below). Results of these controls indicated that when the same positive control serum was run on different microtitre plates with the same amount of LPS coated, variability in optical density was 4-21%. This suggests that the amount of LPS coated was relatively consistent from well to well and plate to plate. In the case of AFAE, we could not determine the exact amount of antigen bound to the plates.

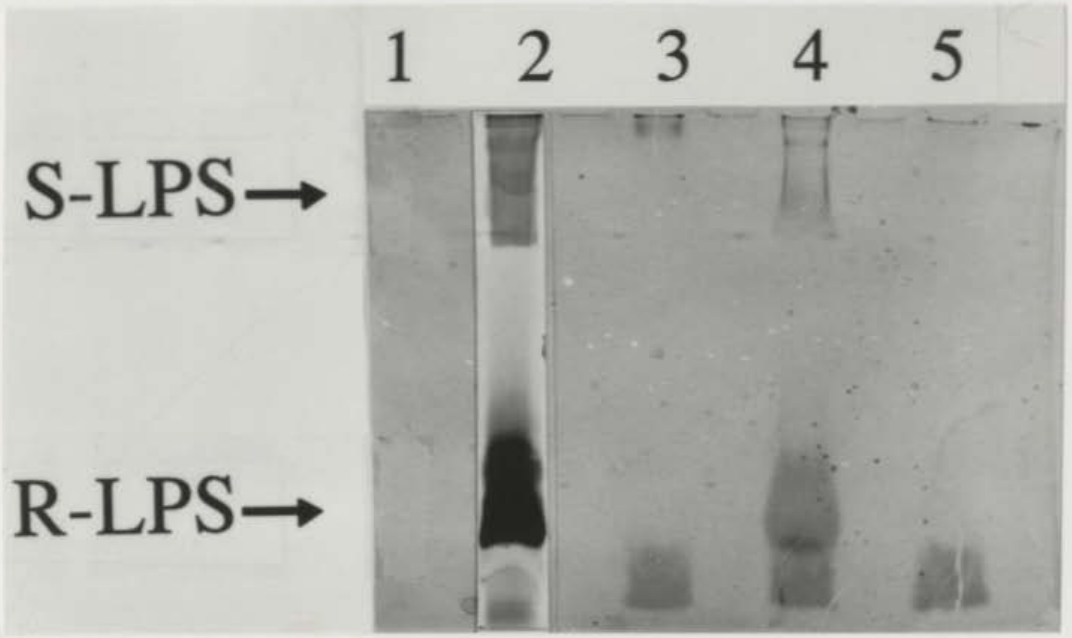
Test serum (rainbow trout serum generated by IP injections of LPS^{+AFA}) or normal trout serum (NTS, used as negative control) was added to the plates as serial doubling dilutions (from 1:40 to 1:40,960) in 0.25% gelatin in PBS containing 0.05% Tween-20 (diluent). Previously tested trout anti-LPS^{+AFA} serum and diluent were also added to each plate as positive and background controls, respectively, and they were incubated at 4°C overnight. The plates were washed as before and 100 μL of a 1% (v/v, in diluent) solution of anti-trout immunoglobulin murine monoclonal antibody was added to each well. The hybridoma secreting monoclonal antibodies (mAb 1.14 is a IgG_1, κ-class antibody) was kindly donated by Dr. Gregory Warr, University of South Carolina, and the hybridoma cells were cultured and the supernatants harvested

by Dr. A. J. Szalai, who was in our laboratory at the time and is now at the University of Alabama. Plates were incubated for 2 h at room temperature and washed as before and 100 μL of a 1:2000 dilution (in diluent) of rabbit anti-mouse immunoglobulin conjugated with horseradish peroxidase was added to each well. Following incubation at room temperature for 1.5 h and washing, 100 μL of hydrogen peroxide substrate solution (10 μL 30% H_2O_2 and 200 μL ABTS [2,2'-azino-*bis*-(3-ethylbenzthiazoline-6-sulfonic acid, 15 mg/mL in H_2O] per 10 mL of 0.05 M citrate buffer, pH 4.0) was added to each well. Finally, the plates were incubated at room temperature for 30 minutes and the optical density was read at 405 nm. On each plate, the absorbance values corresponding to the background controls were averaged and subtracted from the absorbance values of the standard and test sera. After the absorbance values had been corrected, positive reactions were arbitrarily determined to be those which gave an optical density greater than 0.02 Absorbance units. Anti-LPS antibody titres were defined as being the highest fold dilution of antiserum resulting in a positive reaction after correction of absorbance values.

RESULTS

LPS^{+AFA} subjected to SDS-PAGE was not visualized by reaction with Coomassie blue, suggesting that this LPS preparation was relatively protein-free (Fig. 2.1, lane 1). When visualized by ammoniacal silver, LPS^{+AFA} exhibited some bands in the middle to upper regions of the gel and other bands in the lower region of the gel (Fig. 2.1, lane 2). In order of decreasing electrophoretic mobility, these bands can be attributed to the core-lipid A (R-LPS) moiety and the complete LPS (S-LPS) as previously described for other LPS molecules (Tsai and Frasch, 1982; Darveau and Handcock, 1983). The absence of the molecular weight ladders (Goldman and Leive, 1980; Palva and Makela, 1980) suggests that the LPS from *A. salmonicida* is relatively homogenous, having a smaller variation in repeating units of the O-antigen moiety than LPS from other bacterial species (Goldman and Leive, 1980; Palva and Makela, 1980). Shaw *et al.* (1983) observed a similar banding pattern with the LPS from *A. salmonicida*. When the O-polysaccharide (i.e. O-antigen-core oligosaccharide devoid of lipid A), LPS and lipid A from *A. salmonicida* were analyzed by SDS-PAGE and the bands were visualized using the PAS staining procedure (Fig. 2.1, lanes 3, 4 and 5, respectively), the O-polysaccharide did not migrate from the wells (Fig. 2.1., lane 3). This observation suggests that

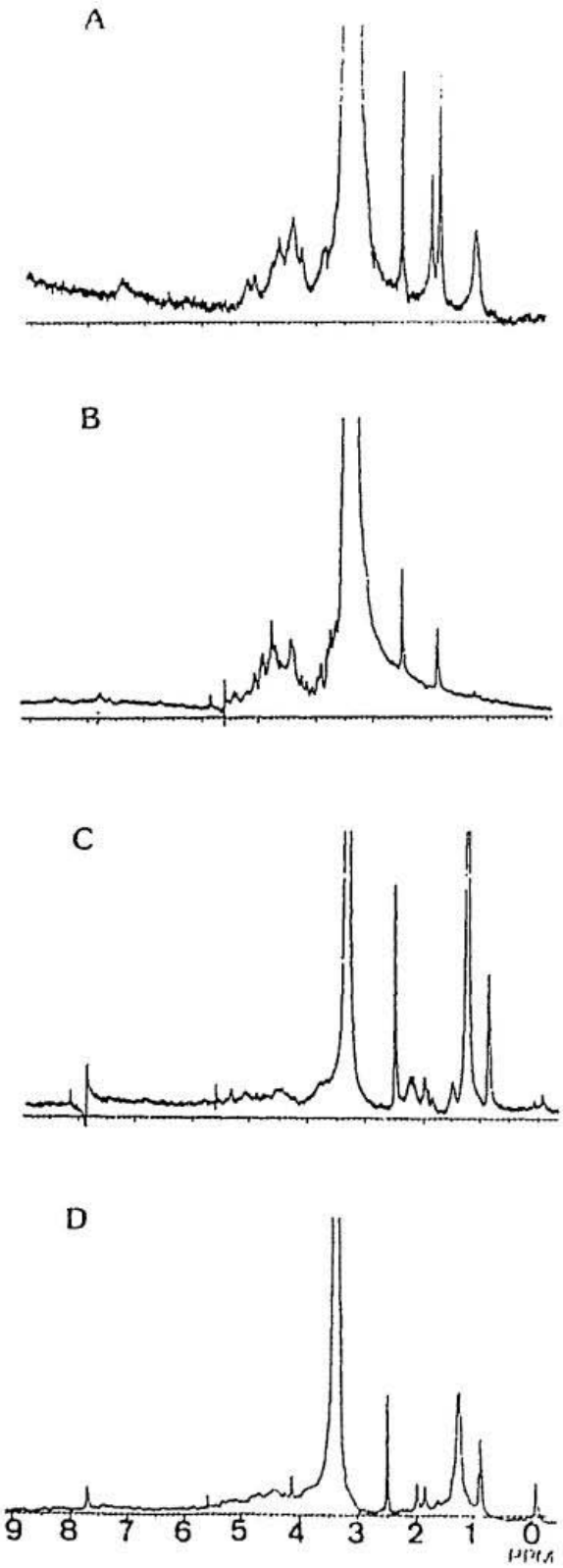
FIGURE. 2. 1. SDS-PAGE (12.5 % acrylamide) analysis of the LPS from *A. salmonicida* and its individual constituents: lane 1, LPS stained with Coomassie blue; lane 2, LPS stained with ammoniacal silver; lanes 3-5, O-polysaccharide, LPS and lipid A from *A. salmonicida*, respectively, as visualized with PAS staining procedure. S-LPS represent smooth (wild type) LPS while R-LPS denotes the LPS from rough (mutant) strains which lack the O-antigen.


1
2
3
4
5
S-LPS→
R-LPS→

the bands observed with LPS (Fig. 2.1, lane 4) are not solely carbohydrate. There was a fast-migrating, PAS positive band observed at the dye front in Fig. 2.1, lanes 3-5. PAS, a staining procedure routinely used to detect the carbohydrate portion of glycoproteins, is based on the oxidation of hexose vicinal 1,2-diols to aldehydes using periodate with subsequent staining by Schiff base (Zacharius *et al.*, 1969; Jay *et al.*, 1990; Van-Seuningen and Davril, 1992). The staining at the dye front may be due to the presence of small, charged carbohydrate fragments that may have resulted from the 1% acetic acid hydrolysis of the LPS. If this was the case, it would appear that the lipid A moiety was not detected using PAS stain (Fig. 2.1, lane 5). Another possibility would be that lipid A was detected by the PAS stain (Fig. 2.1. lane 5) and the presence of residual lipid A in the O-polysaccharide preparation could have caused the staining at the dye front in lane 3.

The ^{1}H-NMR spectrum (obtained in d_6-DMSO) of *A. salmonicida* LPS and of its structural components is represented in Fig. 2.2. The spectrum of the O-antigen of LPS shows one peak at 1.20 ppm and another at 1.85 ppm, corresponding to the methyl protons of the rhamnose and the protons of the O-acetyl groups which partially substitute the N-acetylmannosamine, respectively (Fig. 2.2, a). In addition, a signal at 2.00 ppm is present and is

FIGURE. 2.2. The ^{1}H-NMR spectrum of the LPS from *A. salmonicida* and its structural components obtained in d_6-DMSO: a) O-antigen ; b)core-oligosaccharide; c) lipid A and d) complete LPS. Chemical shifts are reported relative to that of external TMS.


A
B
C
D
9 8 7 6 5 4 3 2 1 0
PPM

attributable to the N-acetyl group of the N-acetylmannosamine. Two peaks characteristic of d_6-DMSO are observed at 2.5 and 3.4 ppm with the latter partially obscuring the ring protons of the O-antigen (Fig. 2.2, a). The signals for the anomeric protons can be observed between 4.3 and 5.3 ppm while a broad peak resonating at 7.70 ppm is likely due to NH protons from unacetylated N-acetylmannosamine (Fig. 2.2, a). The spectrum of the core oligosaccharide of LPS exhibits a signal at 1.85 ppm representing the protons of the N-acetyl group of N-acetylgalactosamine (Fig. 2.2, b). The ring protons are partially visible in the region between 3.6 and 4.2 ppm which are presumably representative of the D-galactose, D-glucose and L-glycero-D-manno-heptose residues. The anomeric protons can be seen resonating between 4.3 and 5.4 ppm although they have not been assigned. Fig. 2.2, c illustrates the spectrum of the lipid A moiety of LPS. Resonances at 0.85 and 1.25 represent the methyl and methylene functional groups of the acyl chains attached to the lipid A backbone, respectively, whereas two signals at 7.85 and 8.15 ppm are likely attributable to the NH protons from the N-acetylglucosamine backbone. A signal at -0.1 ppm was observed in the spectrum of the lipid A moiety (Fig. 2.2, c) but not in the spectra of O-antigen and core-oligosaccharide. In the spectrum of the complete LPS, the unusual signal at -0.1 ppm also appeared, in addition to the resonances which are

characteristic of the functional groups of LPS as assigned above (Fig. 2.2, d).

Silicon derivatives are commonly recognized sources of ^{1}H-NMR signals near 0 ppm. Since the antifoaming agent used in the bacterial fermentation (Antifoam A™) contains silicon, it may have been carried through in the preparation of the LPS and we attempted to see if this agent might be the source of the unusual high-field resonance. LPS was isolated from bacterial cells grown in the absence of Antifoam A™ (LPS^{-AFA}) and utmost caution was employed to prevent introduction of any exogenous silicon contaminants when cultivating the bacterial cells. LPS^{-AFA} and material extracted from Antifoam A™ (AFAE) were subjected to the same spectroscopic analyses as LPS^{+AFA} to determine the extent of the contribution, if any, of the antifoaming agent to LPS preparations. The ^{1}H-NMR spectra of LPS^{+AFA} and LPS^{-AFA} can be seen in Fig. 2.3, a and b, respectively. The spectra of the two LPS samples are very similar with both being comprised of the resonances assigned above in Fig. 2.2, including the peak at -0.1 ppm. The presence of the latter signal in LPS that was not exposed to Antifoam A™ suggests that this signal is from some other source associated with the LPS. The ^{1}H-NMR spectra of a commercial preparation of LPS extracted from *E. coli* using trichloroacetic acid did not exhibit a signal near 0 ppm (Fig. 2.4). However, other LPS preparations,

FIGURE. 2.3. The ^{1}H-NMR spectrum, obtained in d_6-DMSO of a) LPS grown in the presence of Antifoam A™, b) LPS grown in the absence of Antifoam A™ and c) material extracted from Antifoam A™. Chemical shifts are reported relative to that of external TMS.

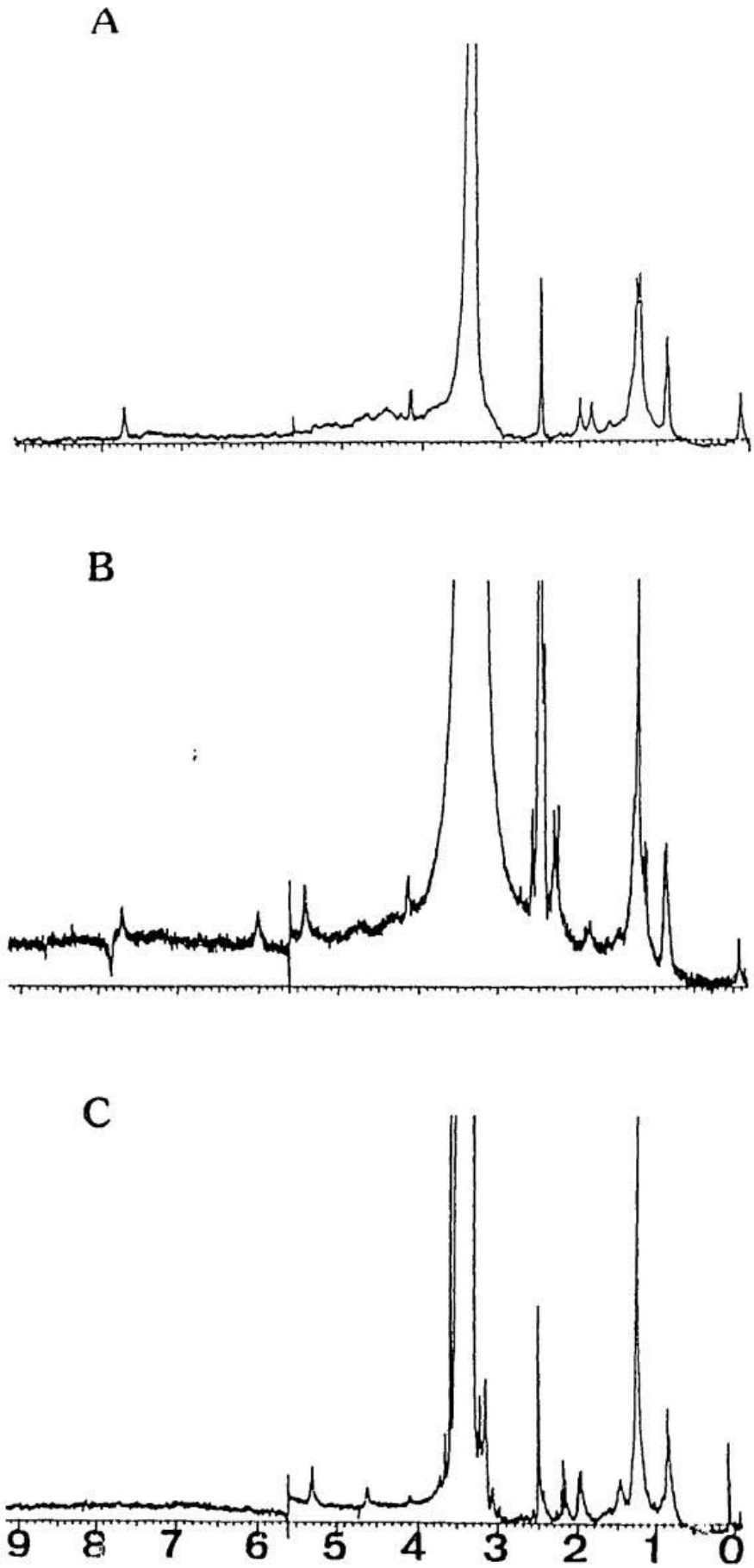
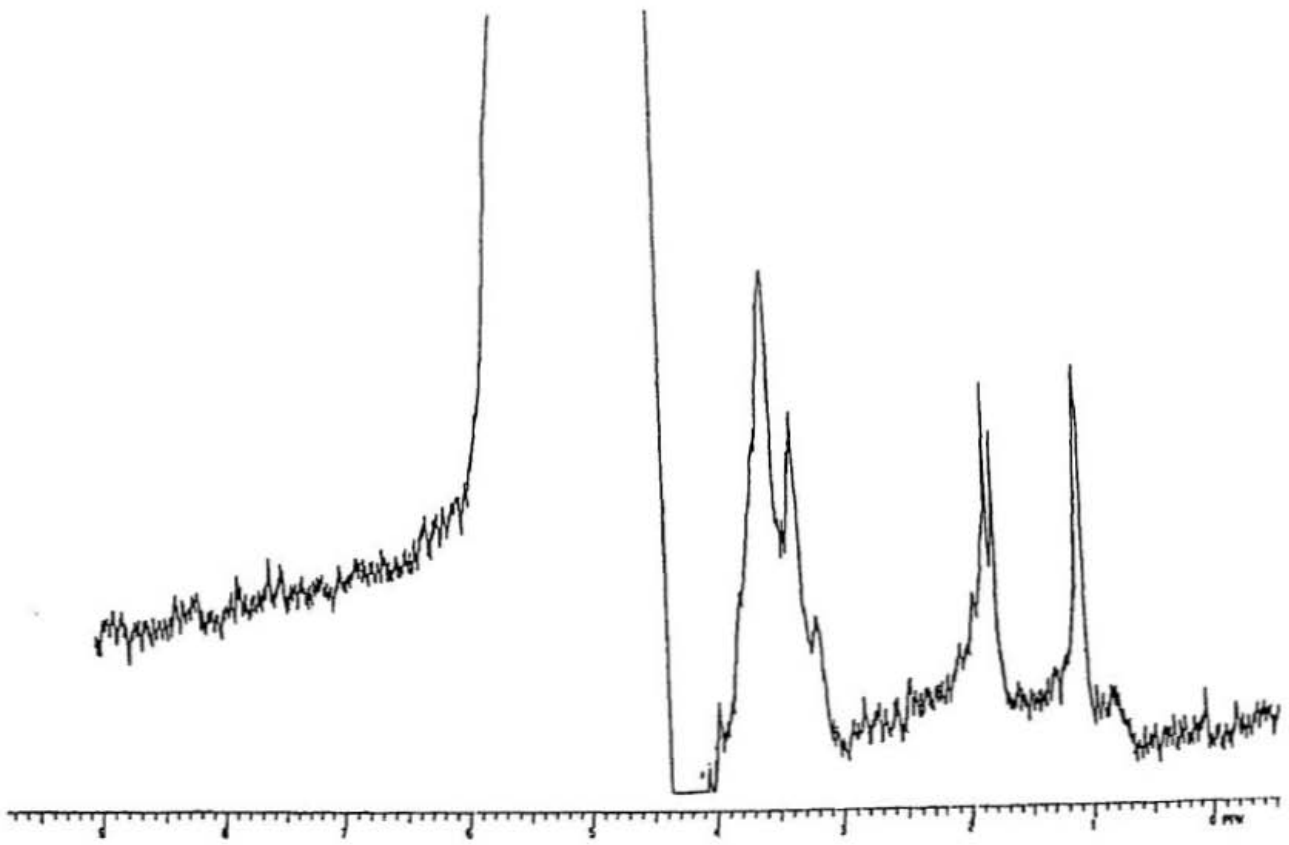

A
B
C
9
8
7
6
5
4
3
2
1
0

FIGURE 2.4. The ^{1}H-NMR spectrum of the LPS from *E. coli* obtained in D_2O. The LPS was prepared by trichloroacetic acid extraction and was purchased from Sigma Chemical Co.



including some obtained commercially, extracted from different bacteria by the aqueous-phenol method exhibited a resonance at approximately 0 ppm (Fig. 2.5, left side). In most cases, the strength of the high field signal of LPS was diminished when material was subjected to additional extraction with trichloroacetic acid (Fig. 2.5, right side). The material extracted from Antifoam A™ also exhibited the resonances observed in the ^{1}H-NMR spectrum of LPS, including the signal at -0.1 ppm (Fig. 2.3, c). Samples from three different lots of Antifoam A™ also yielded analogous ^{1}H-NMR spectrum, each consisting of a signal at roughly 0 ppm (Fig. 2.6).

LPS^{+AFA} and LPS^{-AFA} had similar monosaccharide compositions consisting of L-rhamnose, D-glucose, D-galactose, N-acetylglucosamine, N-acetylmannosamine, N-acetylgalactosamine and L-glycero-D-manno-heptose in approximate molar ratios of 0.4:1.0:0.3:0.3:0.5:0.1:1.0 (Table 2.1). In contrast, AFAE samples were devoid of monosaccharide as determined by GC-EIMS after treatment to produce alditol acetates (Table 2.1). This evidence, combined with the ^{1}H-NMR data, demonstrated that LPS^{+AFA} and LPS^{-AFA} were very similar. LPS^{+AFA} and LPS^{-AFA} also showed similar patterns when subjected to SDS-PAGE (Fig. 2.7, lanes 1 and 2, respectively). The material extracted from Antifoam A™ emulsion was similar to the lipid A moiety of LPS when analyzed by SDS-PAGE, where both the lipid A and the

FIGURE 2.5. The ^{1}H-NMR spectra of LPS prepared by the aqueous-phenol method (top) or preparations additionally extracted with trichloroacetic acid (bottom) from A) *Salmonella typhimurium* B) *Shigella flexneri* and C) *Vibrio ordali* and D) *Aeromonas salmonicida*. The LPS samples analyzed in A) and B) were obtained from commercial suppliers while the LPS analyzed in C) and D) were prepared in our laboratories.

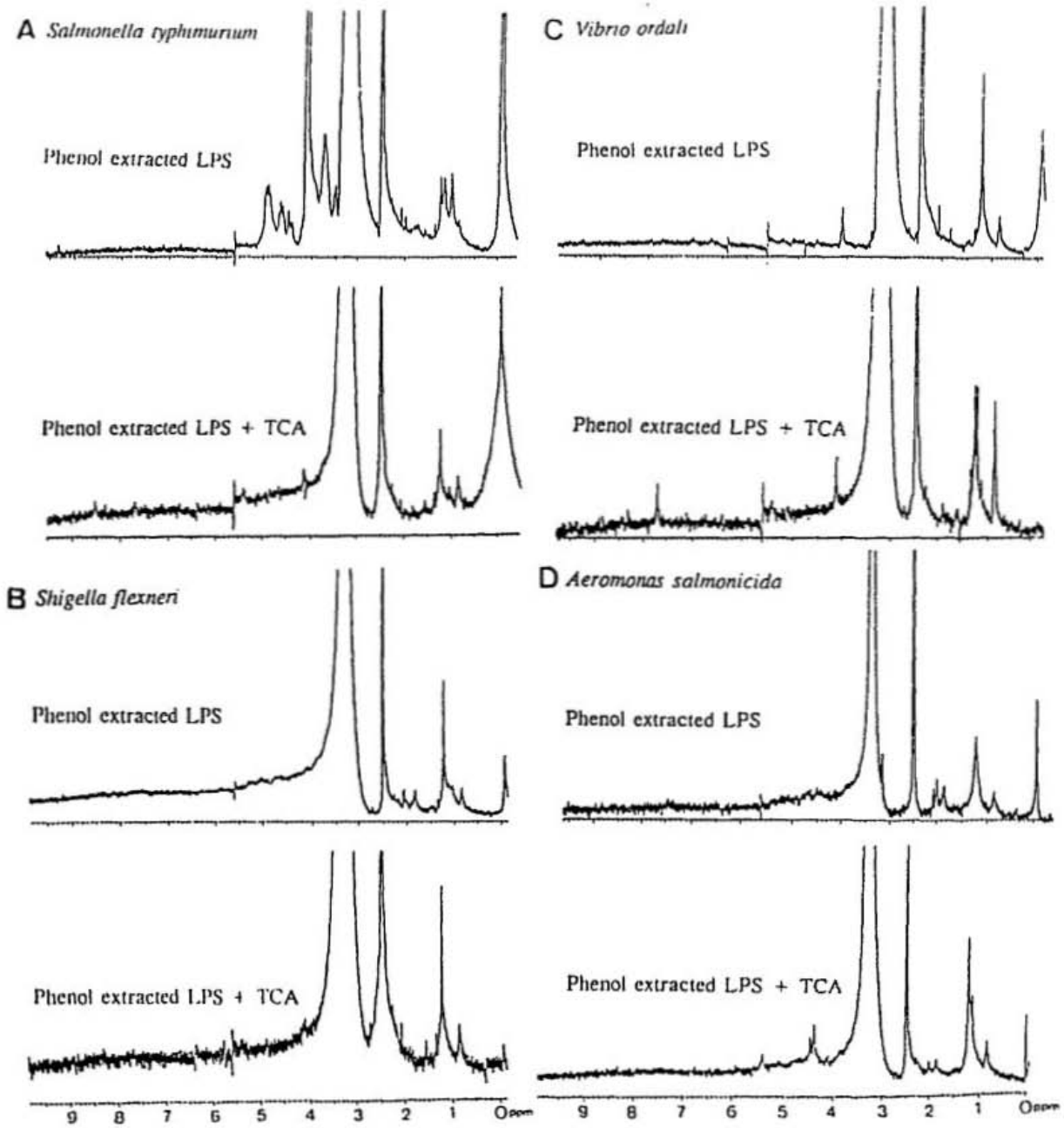

A Salmonella typhimurium
Phenol extracted LPS
Phenol extracted LPS + TCA
C Vibrio ordali
Phenol extracted LPS
Phenol extracted LPS + TCA
B Shigella flexneri
Phenol extracted LPS
Phenol extracted LPS + TCA
D Aeromonas salmonicida
Phenol extracted LPS
Phenol extracted LPS + TCA
9 8 7 6 5 4 3 2 1 0ppm
9 8 7 6 5 4 3 2 1 0ppm

FIGURE 2.6. The ^{1}H-NMR spectra of samples from three different lots of Antifoam A™. A) lot # 88F0052, B) lot # 23H0153, and C) lot # 112H0786.

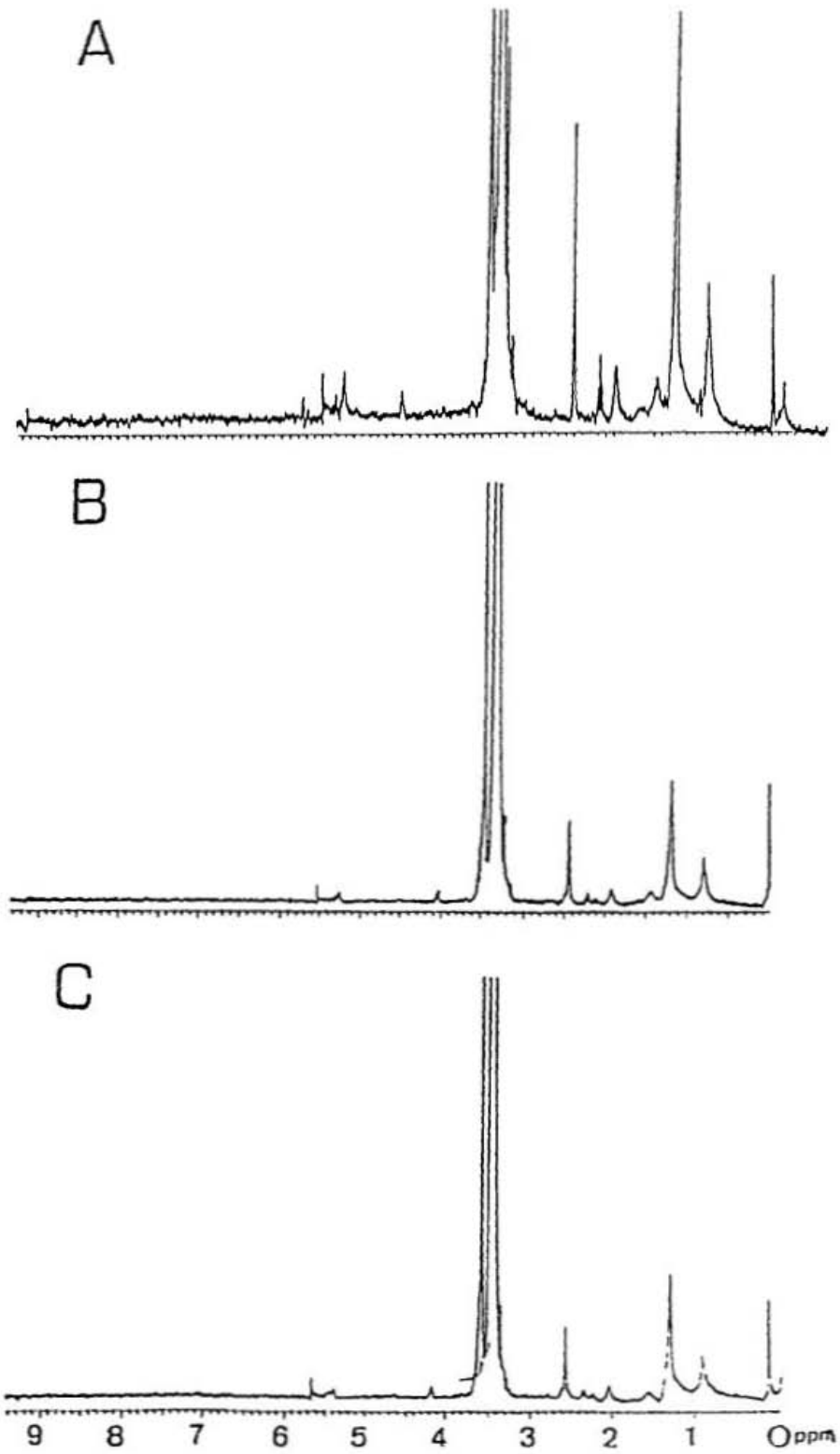
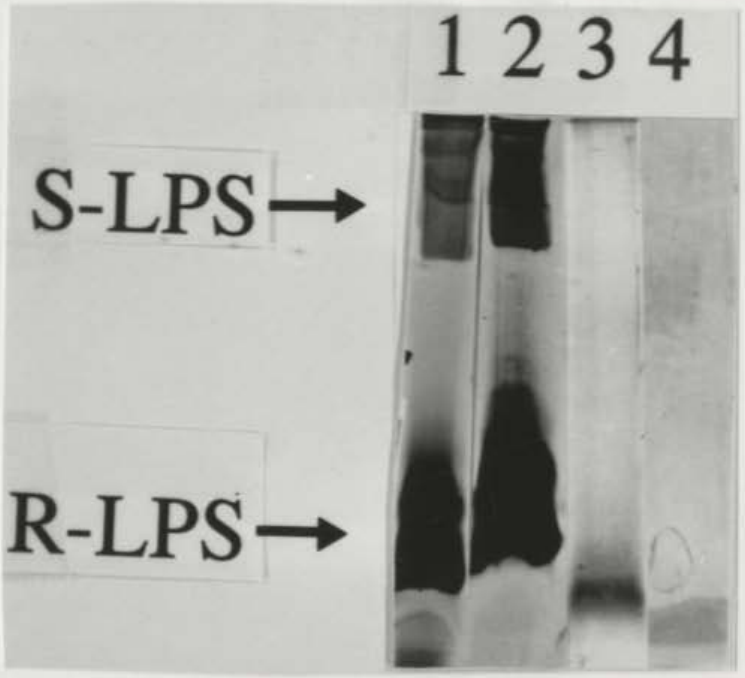

A
B
C
9
8
7
6
5
4
3
2
1
0ppm

TABLE 2.1. Monosaccharide Compositions of LPS^{+AFA}, LPS^{-AFA}, and AFAE as Determined by Coupled GC-EIMS.

	Molar Ratios		
Monosaccharide	LPS^{+AFA}	LPS^{-AFA}	AF
L-rhamnose	0.38	0.48	-
D-glucose	1.00	1.00	-
D-galactose	0.28	0.24	-
N-acetylglucosamine	0.29	0.26	-
N-acetylmannosamine	0.54	0.38	-
N-acetylgalactosamine	0.13	0.10	-
L-glycero-D-manno-heptose	1.00	0.74	-

FIGURE. 2.7. SDS-PAGE (12.5 % acrylamide) analysis of:lane 1, LPS^{+AFA} (same as lane 2 in Fig 2.1); lane 2, LPS^{-AFA}; lane 3, lipid A; lane 4, AFAE. Bands were visualized with ammoniacal silver. S-LPS represent smooth (wild type) LPS while R-LPS denotes the LPS from rough (mutant) strains which lack the O-antigen.


1 2 3 4
S-LPS
R-LPS

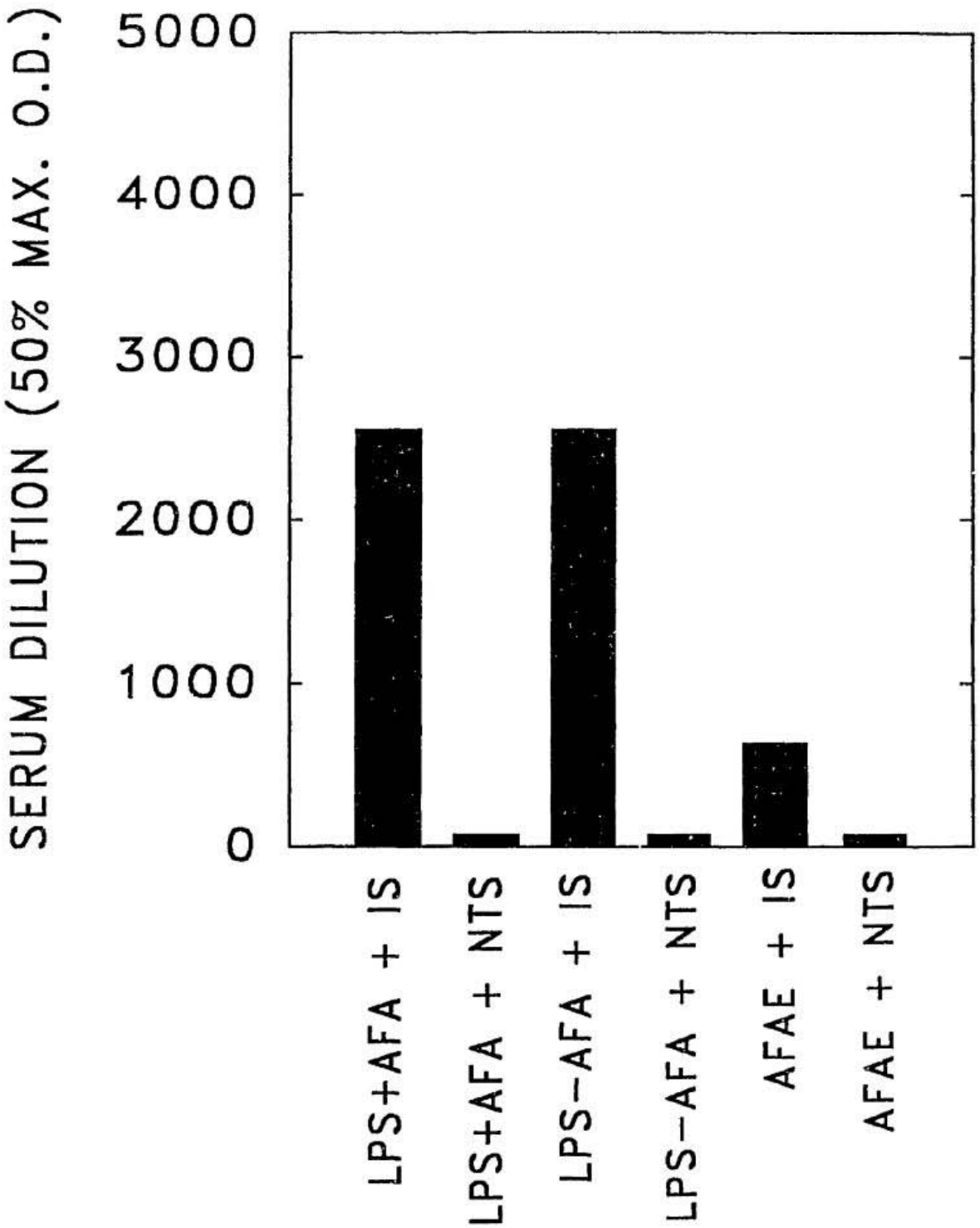
AFAE display only a single band which migrated close to the dye front (Fig. 2.7, lanes 3 and 4, respectively).

Fig. 2.8 demonstrates the reactivity of rainbow trout sera raised against LPS^{+AFA} to LPS^{+AFA}, LPS^{-AFA} and AFAE as well as corresponding negative controls with normal trout serum. The anti-LPS titres of rainbow trout sera raised against LPS^{+AFA} were comparable on ELISA using LPS^{+AFA} and LPS^{-AFA} as the solid phases. Trout immune serum demonstrated a weaker reactivity (titre of 1:640 as compared to titre of 1:2560) against purified AFAE than against LPS^{+AFA}.

DISCUSSION

As described earlier, one of the objectives of this investigation was to calculate outside/inside ratios for the functional groups of LPS incorporated into liposomes using ^{1}H-NMR in order to determine the most efficient formulations. To accomplish this task, it was necessary to assign the resonances from the ^{1}H-NMR spectrum of LPS from *A. salmonicida* to its functional groups and to observe which, if any, of these resonances could be shifted in the presence of paramagnetic metal ions. It was not possible to calculate outside/inside ratios

FIGURE. 2.8. Reactivity of LPS^{+AFA}, LPS^{-AFA} and AFAE with sera from rainbow trout immunized with LPS^{+AFA} (immune sera, IS) or with normal trout serum (NTS) as determined by ELISA. 1) LPS^{+AFA} + IS; 2) LPS^{+AFA} + NTS ; 3) LPS^{-AFA} + IS; 4) LPS^{-AFA} + NTS ; 5) AFAE + IS; 6) AFAE + NTS. Data are presented as antibody titres as defined in the Materials and Methods.


SERUM DILUTION (50% MAX. O.D.)
5000
4000
3000
2000
1000
0
LPS+AFA + IS
LPS+AFA + NTS
LPS−AFA + IS
LPS−AFA + NTS
AFAE + IS
AFAE + NTS

based on this technique because the only signal that appeared to be shifted significantly was the unusual resonance at approximately 0 ppm and there was uncertainty as to the origin of this signal. In addition, it proved difficult to shift resonances on a reproducible basis, and at times wide broadening of resonances was observed. It was not possible to deal with these effects in a qualitative manner given the complexity of the spectra.

Shaw *et al.*(1983) reported that the O-antigen from *A. salmonicida* was a tetrasaccharide repeating unit composed of rhamnose, glucose and N-acetylmannosamine in a molar ratio of 1.0:1.58:0.83 with 75% of the N-acetylmannosamine substituted at position 4 by O-acetyl groups. Our ^{1}H-NMR spectrum for the O-antigen from *A. salmonicida* exhibited resonances which could be attributed to the functional groups of these monosaccharide constituents. Similarly, for the core oligosaccharide from *A. salmonicida*, the resonances in the ^{1}H-NMR spectrum were consistent with the functional groups of the D-galactose, D-glucose, L-*glycero*-D-*manno*-Heptose and 2-acetamido-2-deoxy-D-glucose residues which constitute the oligosaccharide moiety as described by Shaw *et al.* 1992. Lipid A displayed resonances which were consistent with the functional groups of the N-acetylglucosamine backbone and the acyl chains which are attached to it. The ^{1}H-NMR spectrum of the intact

LPS displayed the characteristic resonances expected from the individual carbohydrate and lipid components.

Intact LPS and lipid A from *A. salmonicida* produced an unusual signal at -0.1 ppm in the ^{1}H-NMR spectrum, a region where silicon derivatives are known to resonate. Resonances of this nature have not been described for LPS although an upfield signal has been observed at about 0.6 ppm in the ^{1}H-NMR spectrum of LPS *Escherichia coli* (Qureshi *et al.*, 1988). However, the authors did not assign this unusual upfield signal to a specific functional group. Since the LPS had been extracted from cells grown in the presence of Antifoam A™, a silicon-containing compound, appearance of this atypical resonance led to the investigation of Antifoam A™ as perhaps being responsible for this uncommon signal at -0.1 ppm.

Lipopolysaccharide from *A. salmonicida* was extracted from cells grown either in the presence or absence of Antifoam A™ and material was extracted from the Antifoam A™ emulsion via the hot aqueous-phenol procedure, a method used for the extraction of LPS from bacterial cells. This extracted material was further purified by adherence to polymyxin, an antibiotic with a high affinity for LPS (Morrison and Jacobs, 1976). The ^{1}H-NMR spectrum of LPS from *A. salmonicida* (grown either in the presence or absence of Antifoam

A™) and that of the Antifoam A™ extract were similar, exhibiting resonances with coinciding chemical shifts, including the resonance at -0.1 ppm. These data suggest that the resonance at -0.1 ppm may relate to a functional group in LPS and to a component of Antifoam A™. When analyzed by GC-EIMS, LPS extracted from cells grown with and without Antifoam A™ contained similar monosaccharide compositions. LPS^{+AFA} was composed of L-rhamnose, D-glucose, D-galactose, N-acetylglucosamine, N-acetylmannosamine, N-acetylgalactosamine, and L-*glycero*-D-*manno*-Heptose in molar ratios of 0.38:1.00:0.28:0.29:0.54:0.13:1.00 whereas LPS^{-AFA} contained the same monosaccharides in molar ratios of 0.48:1.00:0.24:0.26:0.38:0.10:0.74. Carbohydrate was not detected in the material extracted from Antifoam A™. Although this material appeared similar to LPS in an ^{1}H-NMR spectrum, the GC-EIMS data suggests that perhaps this Antifoam A™-derived material is only partially structurally similar to *A. salmonicida* LPS.

SDS-PAGE demonstrated that LPS extracted from cells grown with and without Antifoam A™ were similar. Both types of LPS exhibited similar patterns and contained the bands which were previously attributed to the core-lipid A moiety (R-LPS) and to the intact LPS (S-LPS) molecule. The LPS from *A. salmonicida* appears to have a smaller variation in repeating units of

the O-antigen in comparison to the LPS from *S. typhimurium* and *E. coli*, which displayed several more bands when analyzed by SDS-PAGE (Tsai and Frasch, 1982). The material extracted from Antifoam A™ and lipid A both exhibited one major band which migrated close to the dye front. These data suggest that the material extracted from the Antifoam A™ emulsion is structurally more similar to the lipid A moiety than to intact LPS.

Antiserum against LPS^{+AFA}, raised in rainbow trout, exhibited similar reactivity to LPS from cells grown in the presence or in the absence of Antifoam A™. The same immune serum demonstrated a lower reactivity to AFAE suggesting that trout antiserum raised against LPS^{+AFA} either cross-reacts with LPS-like epitopes of AFAE or that more than one population of antibodies was generated when LPS^{+AFA} was administered to rainbow trout, some reactive with LPS and others reactive with AFAE. The reactivity of the immune serum against AFAE was substantially less than against LPS, suggesting that the antibodies induced were primarily against LPS as opposed to a component of Antifoam A™. Since AFAE contained some material which had lipid A-like characteristics, it is possible that antibodies against both LPS and the lipid A portion were generated by our procedure. In separate experiments, it was determined that antibodies generated in trout by administration of liposomally-

incorporated LPS^{+AFA} were reactive with both intact LPS and its lipid A moiety (see Chapter 5).

The ^{1}H-NMR spectrum and sugar analysis by GC-EIMS of LPS from *A. salmonicida* indicates that the LPS extracted from cells grown in the presence or absence of Antifoam A™ were very similar and that both contained a signal resonating at approximately 0 ppm. The ^{1}H-NMR spectra presented here corresponds with that which would be expected from the structures previously suggested for the O-antigen (Shaw *et al.* 1983) and core-oligosaccharide (Shaw *et al.* 1992) moieties of the LPS from *A. salmonicida* with the exception of the appearance of this unusual signal at -0.1 ppm, which could not be readily assigned to any of the well-recognized components of LPS. We also found that some commercially-obtained phenol-extracted LPS (*S. typhimurium* and *S. flexneri*) preparations showed the unusual resonance at approximately 0 ppm. In contrast, a commercially-available preparation of LPS form *E.coli* that had been extracted with trichloroacetic acid did not exhibit a resonance near 0 ppm. The material obtained from Gram-negative bacteria by phenol extraction contains lipid as well as polysaccharide whereas that obtained by trichloroacetic acid extraction is more complex and contains protein (Staub, 1965). It was questioned whether the high field signal at -0.1 ppm could be associated with

the method of extraction of LPS from Gram-negative bacterial cells. When phenol extracted material was subjected to additional extraction of the LPS with trichloroacetic acid, it was observed that, for certain preparations, the strength of the high field signal observed in the ^{1}H-NMR spectrum was diminished. This observation suggests that extraction of LPS from bacterial cells with aqueous phenol produces preparations that include a component which may not be as prominent in LPS preparations extracted with trichloroacetic acid.

We found that Antifoam A™ contains a constituent which is reminiscent of the lipid A moiety of *A. salmonicida* LPS when analyzed by ^{1}H-NMR and SDS-PAGE. We have been unable as yet to assign the resonance at -0.1 ppm observed in the spectra of both LPS and AFAE. Although maximum efforts were made to avoid the introduction of contaminants to the culture media, the possibility that there were exogenous silicon-containing contaminants cannot be completely disregarded. Consequently, LPS isolated by the aqueous phenol method from bacterial cells cultivated in the presence of Antifoam A™, may contain additional material which have some properties coinciding with those of native LPS. The possibility that the high field signal could have come from some part of the LPS molecule cannot be completely eliminated, but the fact that the second extraction with trichloroacetic acid reduced the intensity of that

signal tends to argue against such an interpretation. It was not possible to easily distinguish LPS from different bacterial species simply by ^{1}H-NMR due to structural similarities and hence, functional groups with comparable chemical shifts.

CHAPTER 3

INCORPORATION OF LIPOPOLYSACCHARIDE AND POLYSACCHARIDE FROM *Aeromonas salmonicida* INTO LIPOSOMES.

INTRODUCTION

LPS extracted from the outer membrane of *A. salmonicida*, is an amphiphilic macromolecule containing constituents that are potentially immunogenic and toxic (Luderitz *et al.*, 1966) whereas polysaccharide (PS), the water-soluble derivative of LPS produced by mild acidic cleavage of the lipid A moiety of LPS, is non-toxic. It is postulated that LPS and PS-based formulations may be useful as vaccines against bacterial diseases of fish. However, a disadvantage of using LPS and PS for immunization is that, based on studies in mammals, both macromolecules are regarded as being T-independent antigens that are incapable of inducing cell-mediated immunity (Morrison and Rudbach, 1981).

Multilamellar liposomes are formed spontaneously during hydration of dried lipid films, with the lipid self-associating to form concentric bilayers enclosing a series of aqueous compartments (Bangham and Horne, 1964). It is possible to trap water-soluble antigens derived from bacteria in the aqueous compartments of liposomes, or to incorporate amphiphilic compounds such as bacterial LPS into lipid membranes. Liposomes can be used as carriers for vaccines because of the diverse nature of antigen molecules which can be entrapped (Gregoriadis, 1990), the efficiency with which these antigens can be

delivered to various organs of the RES by liposomes (Lasic, 1992), and the ability of liposomes to act as immunological adjuvants for a variety of substances *in vitro* and *in vivo* (Poznansky and Juliano, 1984; Gregoriadis, 1990; Buiting *et al.*, 1992; Fries *et al.*, 1992). It is well documented that there are numerous considerations in formulating liposomes, particularly lipid composition, that can influence the rate of uptake of liposomes by the macrophages of the RES (Allen *et al.*, 1989; Allen *et al.*, 1991; Gabizon and Papahadjopoulos, 1992). For instance, drugs which are potentially targeted for non-RES organs and tissues can be incorporated into long-circulating or Stealth^{R} liposomes which depend on surface hydrophilicity and shielded surface charges imparted by the addition of monosialoganglioside (GM_1) (Allen and Chon, 1989; Gabizon and Papahadjopoulos; 1988 Huang *et al.*, 1992), lipid derivatives of polyethylene glycol (PEG) (Allen *et al.*, 1991), hydrogenated phosphatidylinositol (PI) (Gabizon and Papahadjopoulos, 1988) or membrane rigidifying compounds such as cholesterol or sphingomyelin or both (Senior and Gregoriadis, 1982) to avoid uptake by the cells of the RES. For vaccines, enhanced uptake by the macrophages of the RES is desired. Conventional liposomes composed of phosphatidylcholine, cholesterol, and a small molar equivalent of charged lipid (e.g. phosphatidylglycerol for negative charge and

stearylamine for positive charge) manifest relatively short half-lives in circulation, and are cleared rapidly from sites of administration. These liposomes are distributed within the Kupffer cells of the liver and the macrophages of the spleen (Senior and Gregoriadis, 1982; Gabizon and Papahadjopoulos, 1988; Allen *et al.*, 1991; Huang *et al.*, 1992; Park *et al.*, 1992). In mammals, immune responses to liposomally-encapsulated protein antigens are predominantly T-cell mediated (Tom, 1981; Garcon and Six, 1991; Alving, 1992), which may be a critical property for inducing protection against a virulent infectious agent. Incorporating LPS or PS into liposomes has potential for the production of formulations that may be capable of enhancing the immunogenicity of antigens, thereby improving host protection against the causative agents of disease in mammals or fish.

The incorporation of LPS or PS from *A. salmonicida* into liposomes of varying lipid composition and lamellarity as a function of the LPS and PS concentration was investigated. The mean vesicle diameters and size distributions of the liposomal formulations were estimated using quasielastic light scattering (QELS). In this chapter, some of the properties of liposomal LPS and PS are discussed that were considered to be important when devising formulations to be employed for immunization experiments in rainbow trout.

MATERIALS AND METHODS

Materials.

Egg phosphatidylcholine (PC), "egg" phosphatidylglycerol (PG) prepared by transphosphatidylation of egg PC, cholesterol (CH), stearylamine (SA) and metrizamide were purchased from Sigma Chemical Co., St.Louis, Mo., USA. NaB^3H_4 (specific activity 490 mCi/mmol) used for radiolabelling LPS was obtained from New England Nuclear, Mississauga, Ont., Canada. All other materials were of reagent grade or better, and were purchased from various suppliers.

Bacterial Cultures and Isolation of A.salmonicida LPS.

Bacterial cultures of *A.salmonicida* were grown to late stationary phase as briefly described in chapter 2 and in more detail in appendix I.

LPS was extracted from lyophilized bacterial cells by the aqueous phenol method (Westphal and Jann, 1965, appendix I) followed by dialysis against 6 changes of distilled H_2O (3.5 L per change) and lyophilization. Polysaccharide (PS) devoid of lipid A moiety was obtained as previously described (Banoub *et al.*, 1989). Briefly, LPS (100 mg) was hydrolyzed in 10 mL of 1% aqueous acetic acid for 2 h at 100°C after which the solution was cooled and centrifuged

at 5000 x g_{max} for 30 min at room temperature. The supernatant was dialyzed against 6 changes of distilled H_2O (3.5 L per change) and lyophilized to yield 71 mg PS.

Radiolabelling of LPS.

Radiolabelling of LPS *A.salmonicida* was performed using the method of Laude-Sharp *et al* (1990) with minor modifications (appendix II). Briefly, 50 mg of LPS were oxidized in the presence of 0.2 M $NaIO_4$ (10 mL) for 20 minutes with stirring at room temperature. Ethylene glycol (2 mL) was added to the reaction mixture and the solution was dialysed against 6 changes of distilled H_2O (3.5 L per change). The oxidized LPS was subsequently reduced at 4°C overnight in the presence of 5mCi (0.01 mmole) NaB^3H_4 in 0.5 M borate buffer, pH 9.0. Following destruction of the excess NaB^3H_4 with 1% acetic acid, the reaction mixture was centrifuged at 290,000 x g_{max} and the pellet was resuspended in distilled H_2O and dialysed against 6 changes of H_2O (3.5 L per change) to remove any non-LPS material. Any free residual radiolabel was removed by passage of dialysed ^{3}H-LPS through a PD-10 desalting column (1.5 x 8 cm, Pharmacia LKB Biotechnology, Uppsala, Sweden). Purified ^{3}H-LPS was assayed for carbohydrate by the phenol-

sulphuric acid procedure (Dubois *et al.*, 1956, see appendix II) and its radioactivity was determined using a Beckman LS 1801 liquid scintillation counter. Purified ^{3}H-LPS had a final specific activity of 0.16 μCi/μg LPS (5920 Bq/μg LPS).

Preparation of Liposomally-Associated LPS and PS.

^{3}H-LPS and PS from *A. salmonicida* were incorporated into multilamellar vesicles (MLV) composed of either PC:CH:PG or PC:CH:SA in a 6:3:1 molar ratio according to a modification (Dijkstra *et al*, 1988) of the method of Bangham and Horne (1964) for liposome preparation as described in appendix II. Briefly, the lipids (ranging from 10-100 μmoles in Fig. 3.1 and 50 μmoles thereafter) were suspended in chloroform-methanol (2:1, v:v) in 15 mL glass vials and the solvent was evaporated under a continuous stream of nitrogen. Resultant lipid films were held *in vacuo* overnight and subsequently stored at -15°C. The dried lipid films were warmed to room temperature and hydrated at room temperature with 1 mL of a solution of ^{3}H-LPS (1, 2.5 or 5 mg LPS/mL in 0.15 M NaCl containing 1.8 x 10^{5} dpm) or PS (1, 2.5 or 5 mg PS/mL, no radioactive label) with mild agitation on an orbital shaker at moderate speed (1500 rpm) at room temperature for 1 hour. The dispersions

were then vigorously vortexed for 3 one-minute intervals resulting in the formation of MLV. Large unilamellar vesicles were prepared from MLV at room temperature by extrusion through two stacked polycarbonate filters of 200 nm pore size (Nucleopore Corp., Pleasanton, Cal., USA) using an "Extruder" (Lipex Biomembranes, Vancouver, B.C., Canada) under pressures ranging from 250-650 lb/in^2 (see appendix II). The material was successively extruded ten times and the resulting vesicles referred to as $LUVET_{200}$ (Hope *et al.*, 1985). Sonicated vesicles (SV) were obtained by placing MLV in a bath sonicator (Papahadjopoulos and Walkins, 1967; Liu and Huang, 1992) for two 12-minute periods with intermittent vortexing for 1 minute (Dijkstra *et al.*, 1988a). Liposomal formulations with initial lipid compositions of either PC:CH:SA or PC:CH:PG are symbolized by + or - respectively, throughout the text.

Separation of Unincorporated LPS and PS from Liposomes.

Free PS was separated from liposomal PS by resuspending the liposomes in 0.15 M NaCl followed by centrifugation of the formulation for 1 h at 15,000 x g_{max} at 4°C in a Beckman 80 Ti rotor. Resulting pellets were resuspended in 0.15 M NaCl and the centrifugation process was repeated twice more. The

liposomal formulations were assayed for carbohydrate by the method of Dubois (appendix II) using PS as a standard. Total lipid phosphorous was determined by a modification (Keough and Kariel, 1987, see appendix II) of the method of Bartlett (1959). Total lipid was estimated by multiplying total lipid phosphorous by a correction factor. The correction factor was determined by dividing the total weight of lipid by the total weight of phosphorous in a preparation. Incorporation ratios are given as μg PS/μg lipid in the liposomal formulations following separation of unincorporated PS.

^{3}H-LPS was separated from liposomal ^{3}H-LPS by density gradient centrifugation using a discontinuous metrizamide gradient (Heath, 1987, see appendix II) with minor modifications. For MLV, 0.5 mL of dispersion in 0.15 M NaCl was mixed with 1 mL 10% (w/v) metrizamide (in 0.077 M NaCl to achieve an osmotic strength of 280 mosm//L) in 5 mL polyallomer centrifuge tubes. The suspension was carefully layered with 3 mL of 5 % metrizamide (w/v) in 0.077 M NaCl and finally with 0.5 mL of 0.077 M NaCl. The gradients were centrifuged at 4°C for 2h at 150,000 x g_{max} in a Beckman SW 50.1 rotor (150,000 rpm). For extruded and sonicated vesicles, preparation of gradients was similar with the exception that 30% and 15% metrizamide were used for mixing and layering, respectively, and ultracentrifugation was for 16 hours. Following centrifugation, the buoyant pellicles (liposomes) were

collected from the gradients whereas unincorporated LPS was recovered from the infranatant layers, and both materials were dialysed extensively and stored at 4°C. The liposomes were diluted in 0.15 M NaCl and residual metrizamide was removed from the liposomes by centrifugation at 50,000 x g_{max} for 1 hour. This was followed by extensive dialysis of the resuspended pellets against 0.15 M NaCl. Radioactivity present in liposomes was estimated using a Beckman LS 1801 liquid scintillation counter and specific activity of the original LPS solution and the activity of the final liposome preparations were used to calculate the concentration of LPS in the preparations. Lipid content in the buoyant pellicles was estimated and incorporation ratios were determined and expressed as μg LPS/μg lipid.

The procedure used to radiolabel LPS results in the specific labeling of the carbohydrate residues on the carbon and oxygen atoms (Laude-Sharp *et al.*, 1990). The lability/exchange of ^{3}H associated with ^{3}H-LPS in aqueous milieu follwing separation of unincorporated material and dialysis of the liposomally-incorporated LPS formulations appeared to be minimal. This was demonstrated by repeating the density gradient centrifugation of the liposomally-incorporated LPS preparations which had been stored at 4°C for 5 days, and indicated that there were only small amounts of radiolabel in the infranatant.

RESULTS

Incorporation of LPS and PS of *A.salmonicida* into negatively-charged MLV was studied over a range of lipid concentrations (10-100 mM) in order to determine the optimal lipid concentration for liposome preparation (Fig. 3.1). When liposomes were prepared as 50 mM dispersions, incorporation ratios of LPS and PS appeared to be ideal and therefore this lipid concentration was used in subsequent incorporation studies (Fig. 3.1).

Positively-charged liposomes composed of PC:CH:SA incorporated more *A. salmonicida* LPS in comparison to their negatively-charged counterparts composed of PC:CH:PG ($p < 0.05$, unpaired t-test) as illustrated in Fig. 3.2. +MLV exhibited enhanced trapping compared to -MLV, +$LUVET_{200}$ were better at incorporating LPS than -$LUVET_{200}$, and +SV were more effective than -SV for encapsulating LPS ($p < 0.05$, unpaired t-test). The effects on incorporation of differing liposome formulation methods and liposomal surface charge appeared to be maintained within the range of 1.0 - 5.0 mg/mL LPS in the original stock solution used to hydrate the lipid films (Fig. 3.2). The positively-charged liposomal preparations preferentially incorporated intact LPS within MLV when compared to their unilamellar counterparts, $LUVET_{200}$ and SV, at LPS concentrations of 1.0 and 2.5 mg/mL ($p < 0.05$, unpaired t-test).

FIGURE. 3.1. Effect of lipid concentration on the incorporation of LPS and PS *A. salmonicida* into negatively-charged MLV. Studies were performed using LPS and PS at a concentration of 5 mg/mL and incorporation ratios are presented as μg LPS (or PS)/μg lipid. The symbols plus the vertical bars represent the means $\pm$ 1 SD for N=3 samples.

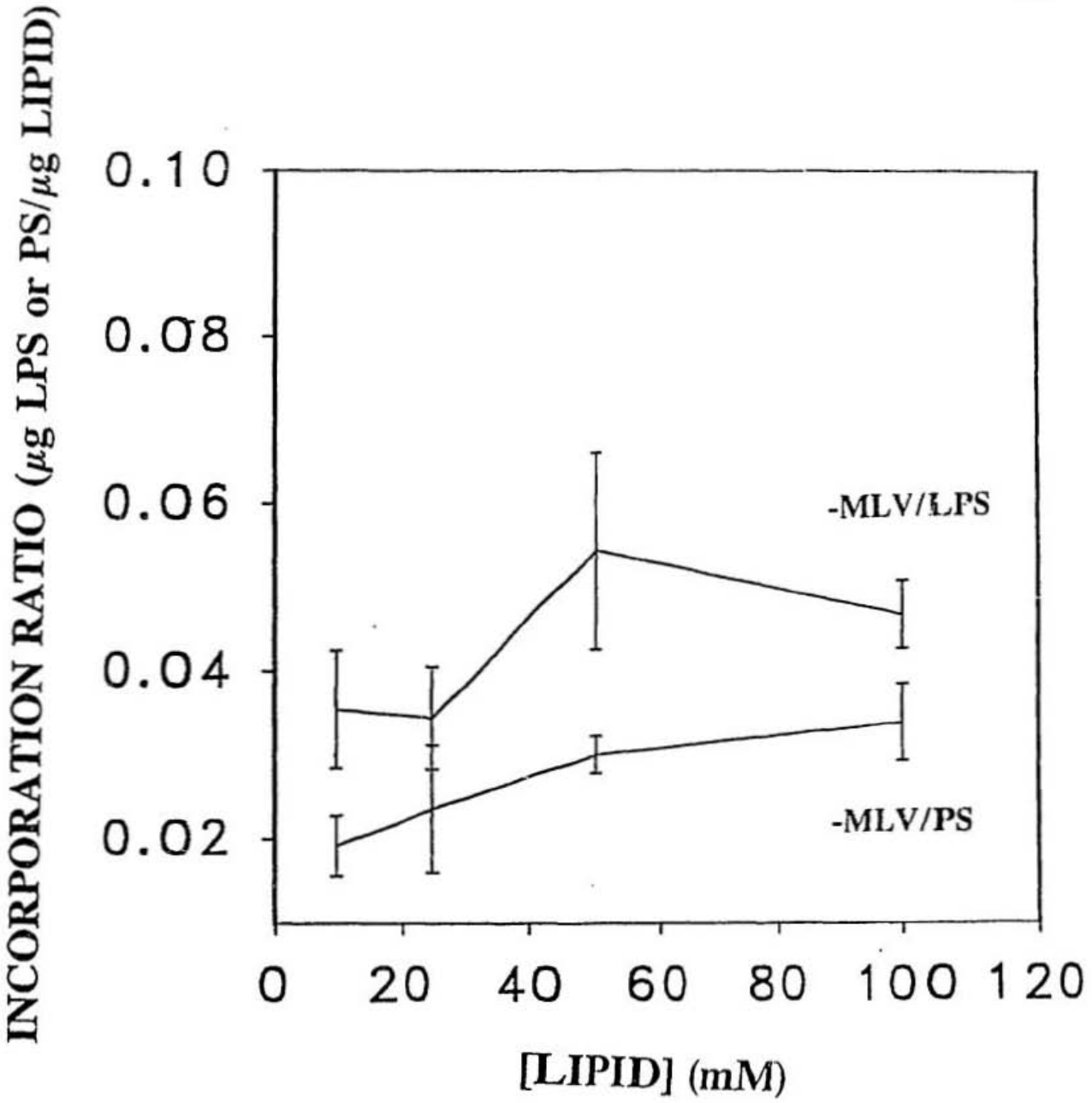
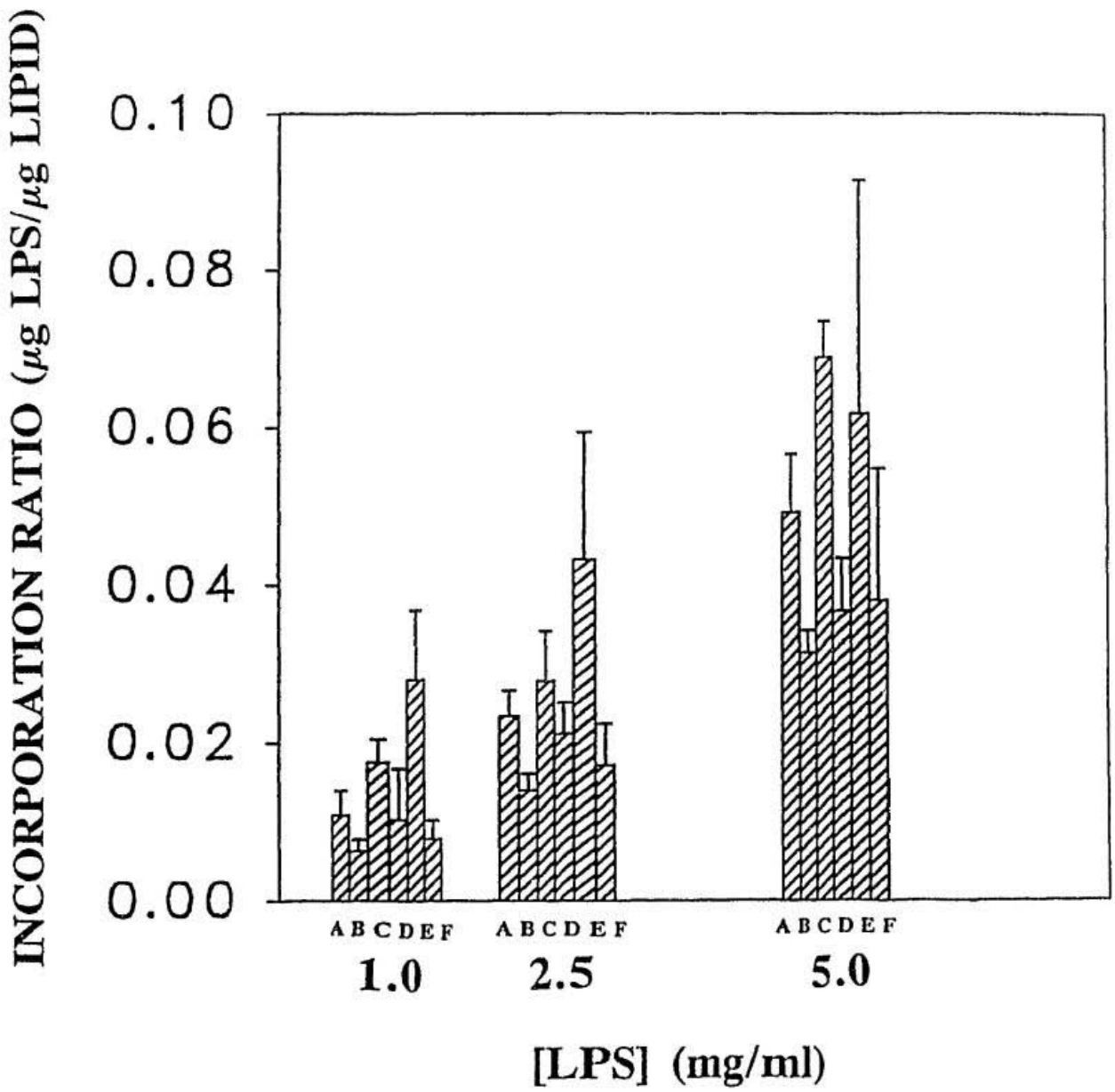

INCORPORATION RATIO (μg LPS or PS/μg LIPID)
0.10
0.08
0.06
0.04
0.02
-MLV/LPS
-MLV/PS
0
20
40
60
80
100
120
[LIPID] (mM)

FIGURE. 3.2. Incorporation of LPS *A. salmonicida* into liposomes of varying lipid composition and lamellarity as a function of concentration of LPS. Incorporation ratios are presented as μg LPS/μg lipid. Vertical bars represent the means and the error bars show 1 SD for N $\geq$ 6 samples. A) +SV/LPS, B) -SV/LPS, C) +LUVET$_{200}$/LPS, D) -LUVET$_{200}$/LPS, E) +MLV/LPS, F)-MLV/LPS. All trials were performed using 50 μmoles total lipid.

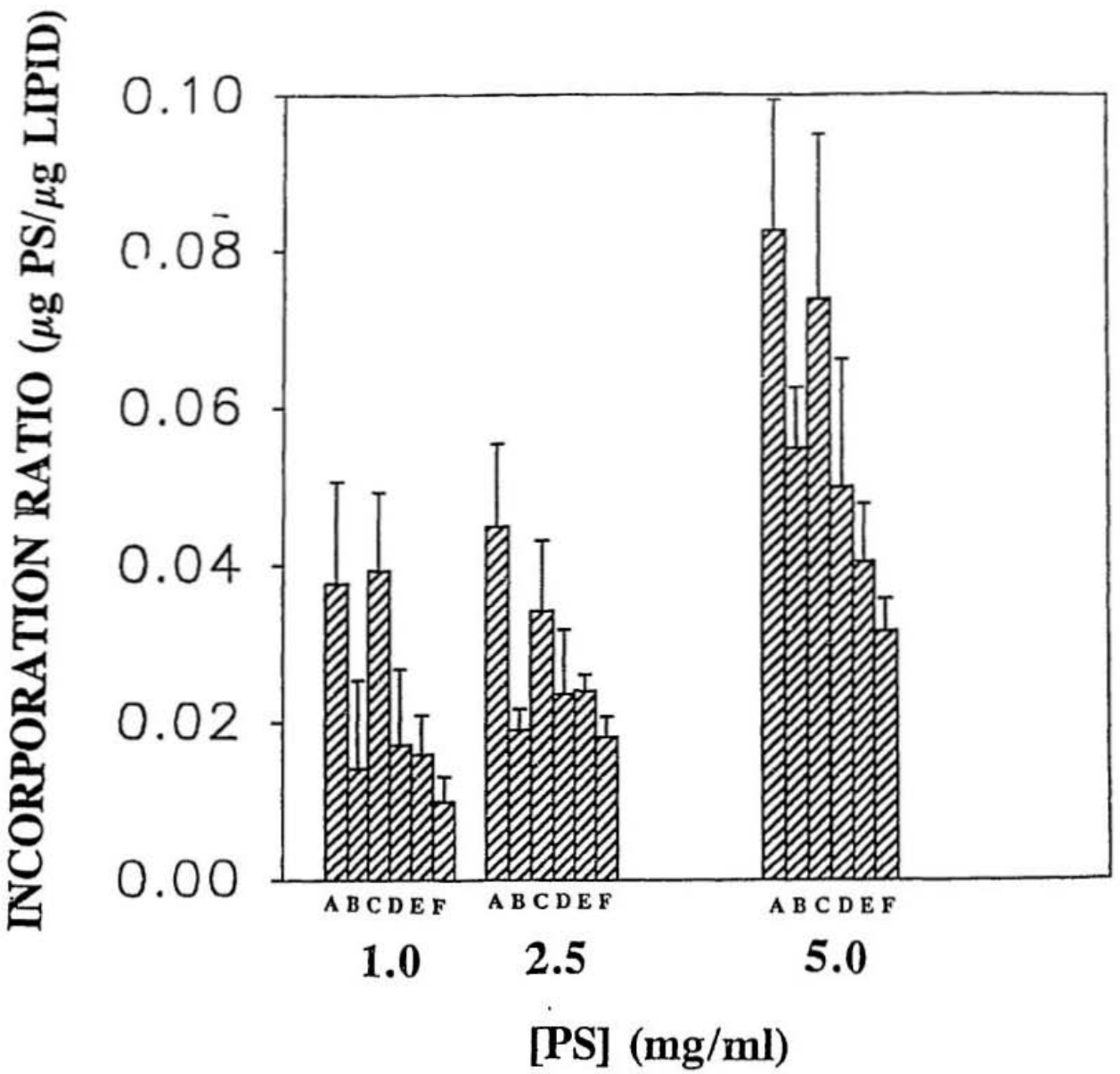

INCORPORATION RATIO (μg LPS/μg LIPID)
0.10
0.08
0.06
0.04
0.02
0.00
A B C D E F
A B C D E F
A B C D E F
1.0
2.5
5.0
[LPS] (mg/ml)

Differences in liposomal incorporation at an LPS concentration of 5.0 mg/mL between multi- and unilamellar liposomes were non-significant ($p > 0.05$, unpaired t-test) (Fig. 3.2). For negatively-charged liposomes, incorporation ratios for LPS into multi- and unilamellar formulations showed no significant differences ($p > 0.05$, unpaired t-test) (Fig. 3.2).

PS and liposomes can be separated by differential centrifugation due to differences in their sedimentation coefficients and hence, radiolabelling of PS was not required to distinguish between incorporated and unincorporated PS. The hydrophilic PS molecule devoid of the lipid A moiety demonstrated better incorporation than LPS within both positively- and negatively-charged sonicated and extruded vesicles ($p < 0.05$, unpaired t-test) (Fig's. 3.2 and 3.3). SV and $LUVET_{200}$, which have larger aqueous compartments per unit of lipid than MLV, appeared to incorporate the water-soluble PS preferentially to LPS (Fig. 3.3). For MLV and SV, but not for $LUVET_{200}$, positively-charged liposomes displayed an enhanced incorporation of PS over their negatively-charged counterparts ($p < 0.05$, unpaired t-test). This pattern was observed over the entire concentration range examined of 1.0 - 5.0 mg PS/mL (Fig. 3.3).

The mean vesicle diameters of the vesicles and their size distributions within the various liposomal preparations were estimated by quasielastic light scattering (QELS), also known as photon correlation spectroscopy or dynamic

FIGURE. 3.3. Incorporation of PS *A. salmonicida* into liposomes of varying lipid composition and lamellarity as a function of concentration of PS. Incorporation is presented as μg PS/μg lipid. Vertical bars represent the means and the error bars show 1 SD for N $\geq$ 6 samples. A) +SV/PS, B) -SV/PS), C)+$LUVET_{200}$/PS, D) -$LUVET_{200}$/PS, E) +MLV/PS, F) -MLV/PS. All trials were performed using 50 μmoles total lipid.


INCORPORATION RATIO (μg PS/μg LIPID)
0.10
0.08
0.06
0.04
0.02
0.00
A B C D E F
A B C D E F
A B C D E F
1.0
2.5
5.0
[PS] (mg/ml)

light scattering. These experiments were kindly performed by Dr. Jordi Hernandez-Borrell at the Unitat de Fisicoquimica, Facultat de Farmacia, Universitat de Barcelona using an Autosizer IIc photon correlation spectrometer (Malvern Instruments, U.K.) equipped with a He-Ne laser as a source of incident light (wavelength = 632.8 nm) operating at 5mW. Data were collected at a scattering angle of 90° and the auto-correlation function was determined via a Malvern 7032-N 72-channel multicorrelator interfaced with an Olivetti PC calculator which was used for data analysis. MLV preparations of either lipid composition were composed of liposomes that had larger mean vesicle diameters and standard deviations than their sonicated and extruded counterparts. QELS also indicated that liposomes produced via extrusion or bath sonication were of similar sizes (data not shown). Regardless of material incorporated or lipid composition, the MLV and SV preparations were more polydisperse than the LUVET, indicating a broader distribution of vesicle diameters within the formulation (data not shown). There appeared to be no distinguishable pattern in regard to vesicle diameter and the nature of the material incorporated when referring to MLV. These results are not surprising as size heterogeneity is a common characteristic of liposomes produced via mechanical agitation. Overall, QELS analysis suggested that the methods of preparation employed in this study resulted in liposomes which displayed mean

vesicle diameters within the ranges that might be expected.

DISCUSSION

Since the description of liposomes by Bangham and Horne (1964), there have been a multitude of drugs and antigens incorporated within liposomes in order to achieve specific delivery to target organs (Bally *et al.*, 1990; Gregoriadis, 1990; Adibzadeh *et al.*, 1992), to reduce toxicity of chemotherapeutic agents (Philips *et al.*, 1991; Gabizon and Papahadjopoulos, 1992; Gabizon, 1992) or to function as immunological adjuvants (Su and van Rooijen, 1986; Liu *et al.*, 1992). As summarized in Table 1.4., there have been several methods developed for the preparation of liposomes in order to optimize the incorporation of particular substances by altering the surface charge or vesicle diameter (Bally *et al.*, 1988; Woodle and Papahadjopoulos, 1989). LPS from *Neisseria meningitidis* (Petrov *et al.*, 1992) *Brucella abortus* (Wong *et al.*, 1992) and *Salmonella typhimurium* (Desiderio and Campbell, 1985) have been incorporated into liposomes to potentiate immune response to LPS in mammals in order to enhance host defence against bacterial infections. Liposomal incorporation appeared to be effective in enhancing immune

responses to the associated LPS in those studies. However, large discrepancies in incorporation rates (defined as a percentage of the material incorporated as compared to that in the initial hydrating solution) ranging from 1.5 to 2.8 % for *Salmonella typhimurium* to 99.9% for the acidic form of LPS of *Neisseria meningitidis* were reported. The incorporations of LPS and PS from *A. salmonicida* were reported as μg antigen/μg lipid as described herein. Incorporation was also calculated as percentages (w/w) of liposome-associated material relative to that included in the original stock solution used to hydrate the lipid films. When expressed as percentages, our values of approximately 30% to 45% for the negatively- and positively-charged liposomes, respectively, were most similar to the values of approximately 45% described for the LPS and PS of *Brucella abortus* (Wong et al., 1992). The large diversity in incorporation of LPS from different bacterial species into liposomes can be attributed to the variations in the carbohydrate chain length of the LPS, solubility properties of the LPS, the types of lipids used to formulate the liposomes, and the methods of preparation of the liposomes. Experiments which involved repeated density gradient centrifugation of our liposomally-incorporated LPS preparations over a 5 day period indicated that retention of LPS was 98% and 89% for positively- and negatively-charged MLV,

respectively, when stored at 4°C.

Positively-charged liposomes composed of PC: CH: SA were more effective at trapping LPS and PS than their negatively-charged counterparts composed of PC: CH: PG and this was exemplified with both multi-and unilamellar vesicle preparations. Presumably, this effect was due to the presence of negatively-charged phosphoryl groups within the macromolecular structure of the LPS. The effects of lipid composition and the consequent difference in liposomal surface charge on incorporation ratios were observed over the concentration range of LPS or PS used to formulate the liposomes.

Incorporation ratios were directly proportional to the LPS or PS concentrations in the hydrating solutions. Because of the structural properties of LPS and PS, these macromolecules can either be trapped in the aqueous compartments between the lipid bilayers, embedded within the lipid bilayer via the hydrophobic moiety (for LPS) or absorbed to the liposomal surface. The fact that LPS was more readily incorporated into MLV than PS is likely due to the lipid A moiety which may serve to secure the LPS within the bilayers of the MLV. Presumably, the carbohydrate portion of the molecule would protrude outwardly into the aqueous environment. When compared to LPS, hydrophilic PS devoid of lipid A was more readily incorporated within $LUVET_{200}$ and SV which is consistent with the larger aqueous compartments relative to lipid mass

in the smaller, unilamellar vesicles.

The effects of liposome incorporation of LPS and PS on immune responses (Desiderio and Campbell, 1985 Petrov *et al.*, 1992; Wong *et al.*, 1992; Laing and Threakston, 1993) or the secretion of inflammatory mediators such as tumor necrosis factor (TNF) (Dijkstra *et al.*, 1988b), interleukin (IL-1) (Dijkstra *et al.*, 1987; Bakouche *et al.*, 1987), and H_2O_2 (Dijkstra *et al.*, 1989) have been studied. However, the mean vesicle diameters of the various liposome formulations were not estimated in any of these studies. We had initially examined the possibility of utilizing techniques such as electron microscopy and flow cytometry, in addition to the use of a Coulter Counter^R instrument to estimate the sizes of our vesicles and their distributions within a given formulation. There was some concern about the use of electron microscopy because of the additional treatment required for the samples. We were unable to standardize the flow cytometer to detect particles at the lower limits (less than 400 nm) and access to the Coulter Counter^R could not be obtained when these trials were performed. Therefore, the QELS technique was used to estimate the vesicle diameters and the size distributions of liposome associated LPS and PS. In comparison to other techniques for measuring liposome sizes, QELS offers the advantages of not disturbing the suspensions

and the capability of covering a fairly large size range of 0.1 to several μm (Ostrowsky, 1993). The conditions employed (e.g. hydration time and temperature, vortexing time and speed) were constant throughout the production of the liposomes in the two analyses of our preparations by QELS. It was observed that incorporation of LPS and PS from *A. salmonicida* into MLV provided heterogenous formulations composed of relatively large liposomes, which might be expected of vesicles prepared by mechanical agitation (Deamer and Uster, 1983). There was variability between the mean vesicle diameters observed for MLV preparations in duplicate trials. QELS, however, is known to be limited by its sensitivity to large particles in a dispersion (Selser *et al.*, 1976), and the results of such analyses are influenced in favor of the larger particles in a mixed dispersion (Lichtenburg and Barenholz, 1988). This appeared to be the case with MLV-incorporated LPS and PS produced by mechanical agitation which, regardless of lipid composition, exhibited large polydispersities compared to the sonicated and extruded vesicles. As expected, the $LUVET_{200}$ and SV preparations exhibited similar mean vesicle diameters that were smaller than the corresponding MLV preparations. Extruded liposome preparations, however, were less polydisperse (indicating less scattering of vesicle diameter sizes within the formulation) which may be due to

the ten successive passages through the pre-sized filters. This is consistent with other studies which found that extrusion and sonication resulted in the generation of more homogenously sized vesicles (Mayer *et al.*, 1986; Bally *et al.*, 1988; Woodle and Papahadjopoulos, 1989). In general, the sizes observed were consistent with those expected for the methods of preparation.

The effects of liposomal LPS formulations on immune responses have been examined in mammalian systems (Desiderio and Campbell, 1985; Petrov *et al.*, 1992; Wong *et al.*, 1992) although studies in fish have been infrequent (Rodgers, 1990). This study demonstrates that LPS and PS from the fish pathogen, *A. salmonicida* can be efficiently incorporated into multi- and unilamellar vesicles composed of either positively or negatively charged lipid to form liposomes of varying surface charge, size and lamellarity. Varying lipid composition appears to be an influential factor for the incorporation of LPS and PS of *A. salmonicida* into liposomes. PS was included in this study for the purpose of comparing the incorporation properties of an amphiphilic and hydrophilic molecule into liposomes of varying size and lamelarity. Incorporation and size distribution studies are important factors to be considered when devising a formulation targeted to the macrophages of the RES. A formulation which is readily taken up by the macrophages of the RES may lead

to the enhancement of immunogenicity of the incorporated material and possibly generate improved protection against bacteriosis.

CHAPTER 4

UPTAKE AND BIODISTRIBUTION OF FREE AND LIPOSOMALLY INCORPORATED LIPOPOLYSACCHARIDE FROM *Aeromonas salmonicida* ADMINISTERED VIA DIFFERENT ROUTES TO RAINBOW TROUT (*Oncorhynchus mykiss*).

INTRODUCTION

A variety of immunogens have been shown to induce some degree of protection against bacterial pathogens in fish (Baba *et al.*, 1988; Hastings and Ellis, 1988; Robertsen *et al.*, 1990; Velji *et al.*, 1990; Wong *et al.*, 1992). Despite some encouraging results regarding immunity to *A. salmonicida* (Olivier *et al.*, 1985; Adams *et al.*, 1988) and the existence of commercially available anti-furunculosis vaccines, heavy losses of cultured salmonids due to furunculosis continue to occur.

In mammals, several liposomal formulations have been shown to be capable of enhancing immune responses to associated antigens, although liposomal LPS vaccines for fish have seldom been tested (Rodgers, 1990). In contrast to the situation in mammals, little is known regarding the fate of exogenously administered liposomes in fishes (Power *et al.*, 1990). It was demonstrated in the previous chapter that LPS and PS from *A. salmonicida* can be efficiently incorporated into multi- and unilamellar vesicles composed of both positively and negatively charged lipids. The fate of free and MLV-incorporated LPS in rainbow trout *vis a vis* differing routes of administration, and altering liposomal surface charge is a principal consideration in developing a liposomal formulation targeted to the RES. MLV were selected as the

delivery vehicles because of the apparent enhanced ability to incorporate LPS when compared to their unilamellar counterparts. In addition, the larger vesicle diameters of MLV preparations makes them likely candidates for uptake by the RES (Allen *et al.*, 1989; Mayer *et al.*, 1989; Allen *et al.*, 1991) which could possibly enhance antigenicity, especially cell-mediated immune responses (Tom, 1981; Garcon and Six, 1991; Harding *et al.*, 1991; Collins *et al.*, 1992) .

^{3}H-labelled LPS, positively- and negatively-charged (^{14}C-labelled) liposomes or ^{14}C-labelled liposomes containing ^{3}H-LPS were administered to trout via intravenous, intraperitoneal, intramuscular, or oral routes. Twenty-four hours following administration, relative uptake of LPS and MLV based on detection of ^{3}H and ^{14}C, respectively, was determined in samples taken from the kidney, spleen, liver, plasma, blood cells and skeletal muscle. Biodistribution studies of this nature have been limited to the comparison of uptake of "empty" MLV and LUVET among the organs and tissues in rainbow trout. Power *et al.* (1990) found a higher degree of localization of liposomes within the spleen and kidney, the major centres of the RES in rainbow trout. The authors also established that there was greater uptake of LUVET by the kidney and spleen as compared to MLV in rainbow trout. These results are analogous to those found in mammals where liposomes are taken up by the cells of the RES, the

liver and spleen (Poste *et al.*, 1982; Senior and Gregoriadis, 1982; Gabizon and Papahadjopoulos, 1988; Allen *et al.*, 1991; Huang *et al.*, 1992; Park *et al.*, 1992). In this section, the aim was to determine whether liposome incorporation exhibited enhanced delivery of LPS to specific organs, especially the kidney and spleen, the principal hemopoietic organs in rainbow trout. The effects of route of administration of free versus liposomal-LPS from *A. salmonicida* on the uptake and distribution of LPS among the organs and tissues of rainbow trout were examined.

MATERIALS AND METHODS

Materials.

Phosphatidylcholine (PC, from frozen egg yolk), phosphatidylglycerol (PG, from egg yolk lecithin), cholesterol (CH), stearylamine (SA) and metrizamide were purchased from Sigma Chemical Co., St. Louis, Mo., USA. NaB^3H_4 (specific activity 490 mCi/mmol) and ^{14}C-cholesteryl oleate ester (specific activity 55 mCi/mmol) for radiolabelling LPS and liposomes, respectively, were obtained from New England Nuclear, Mississauga, Ontario, Canada. Scintillation cocktail (Scintiverse E) and tissue solubilizer (Scintigest)

were from Fisher Scientific, Dartmouth, Nova Scotia, Canada.

Bacterial Cultures and Purification of *A. salmonicida* LPS.

Bacterial cultures of *A.salmonicida* were grown to late stationary phase as briefly described in chapter 2 and in more detail in appendix I.

LPS was extracted from formalin-fixed, lyophilized bacteria by the hot aqueous phenol method (Westphal and Jann, 1965, appendix I). Phenol-extracted LPS was dialyzed against 6 changes of distilled H_2O (3.5 L per change), lyophilized and stored at -15°C prior to use.

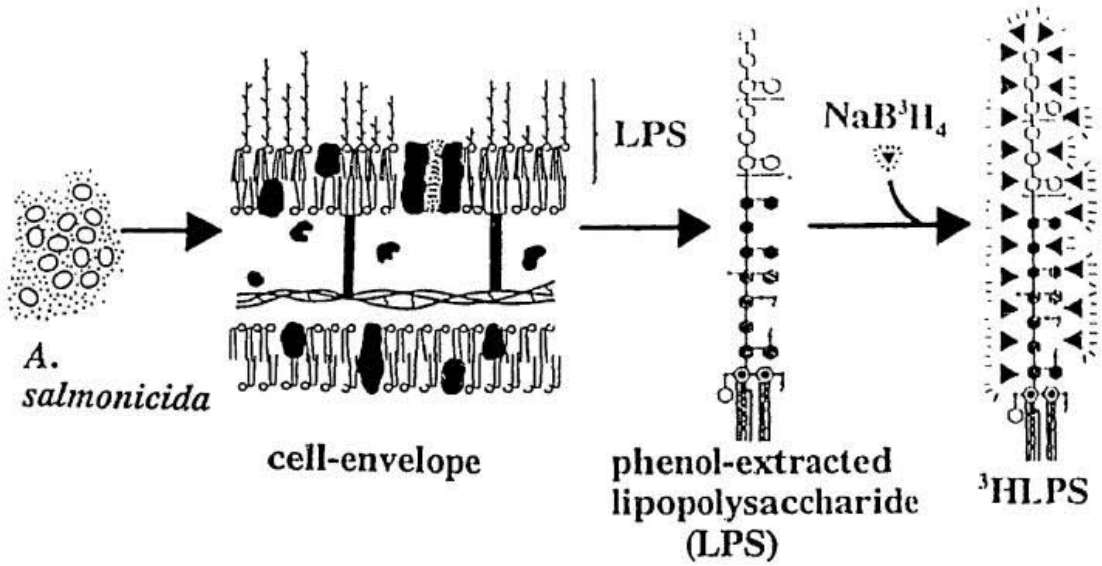
Radiolabelling of LPS.

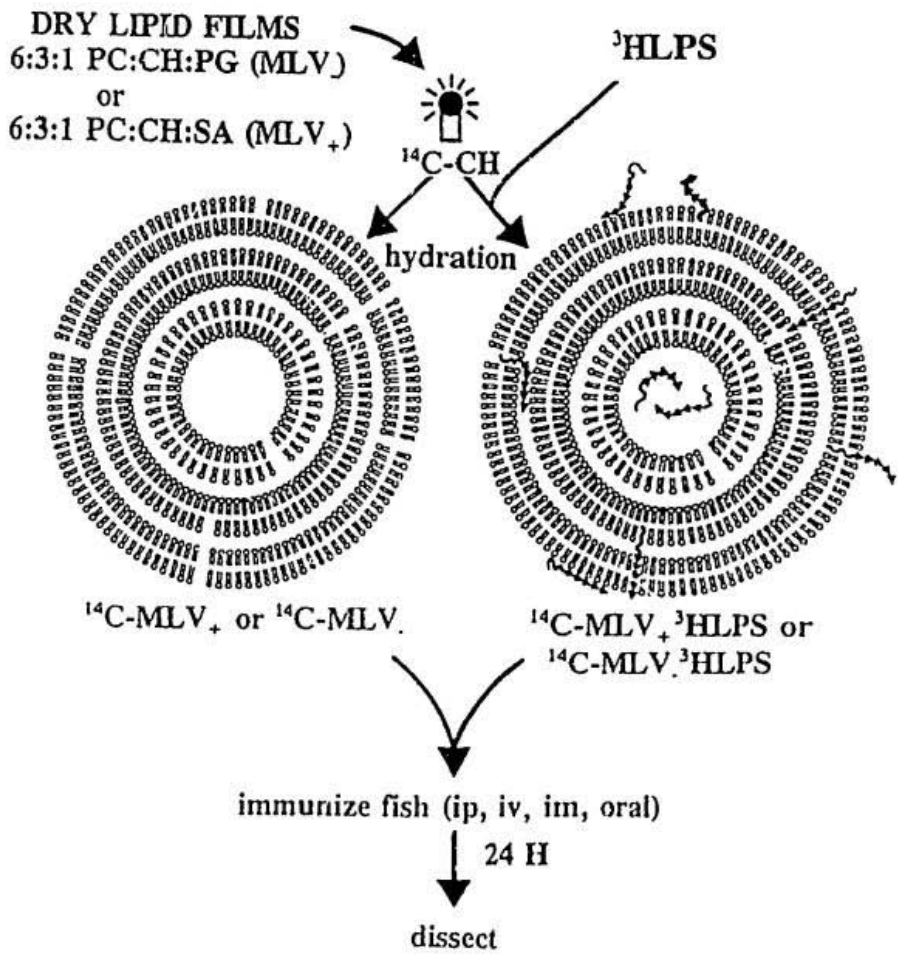
LPS was tritiated using the procedure of Laude-Sharp *et al* (1990) with minor modifications as described in chapter 3 and in appendix II. Purified ^{3}H-LPS had a final specific activity of 0.16 μCi/μg LPS.

Radiolabelling of Liposomes and Encapsulation of ^{3}H-LPS.

Liposomes were prepared as a modification to the procedure described in appendix II (see Fig. 4.1 for schematic representation). Briefly, 25 μL of ^{14}C-cholesteryl oleate ester (5.55 x 10^6 disintegrations per minute, DPM, 27.5 μg)

FIGURE 4.1. Schematic representation of the preparation of radiolabelled LPS and liposomal formulations used in these experiments. Drawn by Dr. A. J. Szalai.


LPS
NaB³H₄
A.
salmonicida
cell-envelope
phenol-extracted
lipopolysaccharide
(LPS)
³HLPS


DRY LIPID FILMS
6:3:1 PC:CH:PG (MLV)
or
6:3:1 PC:CH:SA (MLV+)
³HLPS
¹⁴C-CH
hydration
¹⁴C-MLV+ or ¹⁴C-MLV-
¹⁴C-MLV+ ³HLPS or
¹⁴C-MLV- ³HLPS
immunize fish (ip, iv, im, oral)
24 H
dissect

was added to 50 μmoles lipid, (composed of PC:CH:PG or PC:CH:SA in a 6:3:1 molar ratio) and the solvent was evaporated under a continuous stream of nitrogen. Resultant lipid films were held *in vacuo* overnight and stored at -15°C. To form liposomes, dry lipid films were warmed to room temperature, rehydrated with 1 mL of 0.15 M NaCl or 1 mL of 0.15 M NaCl containing a mixture of LPS (2.5 mg) and ^{3}H-LPS (50 μg, equivalent to 1.08 x 10^7 DPM), and agitated gently at room temperature for 1 hour. Resultant dispersions were vortexed vigorously for 3 one-minute periods to form multilamellar vesicles, MLV or MLV/LPS, respectively.

Separation of Unincorporated LPS from Liposomal LPS.

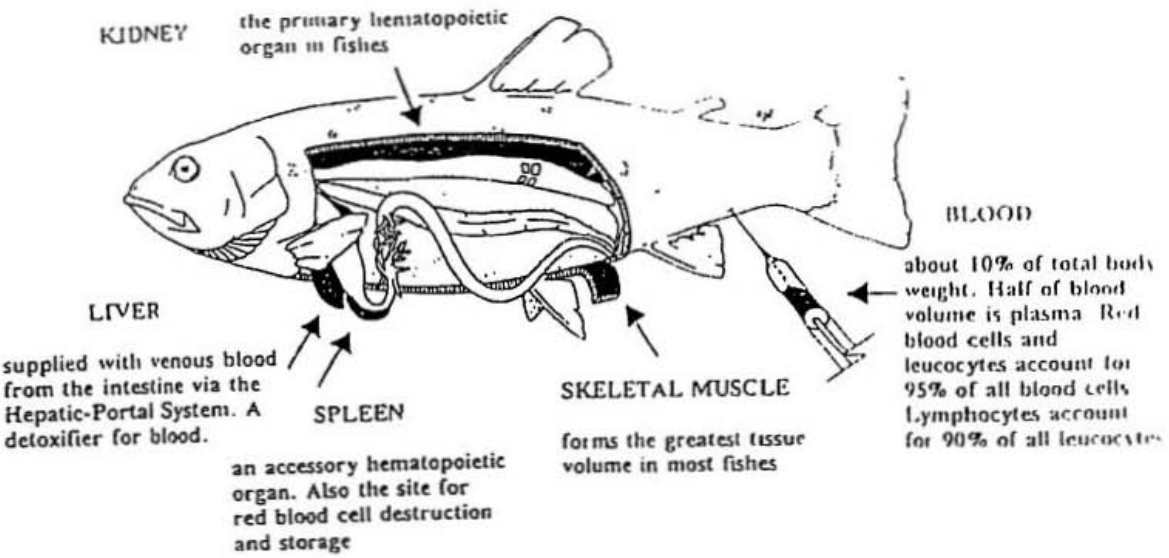
Unincorporated ^{3}H-LPS was separated from liposomal ^{3}H-LPS by discontinuous density gradient centrifugation using the method of Heath (1987) as previously described in chapter 3 and appendix II. The resulting liposomal dispersions were administered to rainbow trout.

Administration of LPS, Liposomes and Liposomal LPS to Rainbow Trout.

LPS, liposomes and liposomal LPS were administered to rainbow trout (body weight, **Wb**= 69 g on average) by intraperitoneal (IP), intravenous (IV),

intramuscular (IM) injections or by oral intubation (OR). Trout received a dose of either a) ^{3}H-LPS (200 μg, equivalent to 1.8x10^{6}dpm); b) ^{14}C-labelled positively-charged, SA-containing MLV (+MLV, 1.87 mg lipid having 1.03x10^{6} dpm); c) ^{14}C-labelled negatively-charged, PG-containing MLV (-MLV, 2.96 mg lipid having 8.06x10^{5} dpm); d) +MLV containing ^{3}H-LPS (+MLV/LPS; 64 μg ^{3}H-LPS that gave 1.93x10^{6} dpm from ^{3}H and 3.78 mg lipid that produced 1.18x10^{6} dpm from ^{14}C); or e) -MLV containing ^{3}H-LPS (-MLV/LPS, 19 μg ^{3}H-LPS which was equivalent to 7.81x10^{5} dpm from ^{3}H and 3.40 mg lipid equivalent to 9.19x10^{5} dpm from ^{14}C). Twenty-four h after administration of materials, fish were killed by lethal anaesthesia with benzocaine and the kidney, spleen, liver and a portion of muscle were removed and weighed (Fig. 4.2). Muscle samples were taken from sites distal to the site of injection (Fig. 4.2). Blood was collected from the caudal sinus and transferred immediately to heparinized tubes (Fig. 4.2). Plasma was separated from blood cells by centrifugation at 2000 x g_{max} at room temperature for 5 min.

FIGURE 4.2. Schematic representation of the regions in rainbow trout from which tissue cross sections were taken for experimental analysis. Drawn by Dr. A. J. Szalai.


KIDNEY
the primary hematopoietic organ in fishes
BLOOD
about 10% of total body weight. Half of blood volume is plasma. Red blood cells and leucocytes account for 95% of all blood cells. Lymphocytes account for 90% of all leucocytes
LIVER
supplied with venous blood from the intestine via the Hepatic-Portal System. A detoxifier for blood.
SPLEEN
an accessory hematopoietic organ. Also the site for red blood cell destruction and storage
SKELETAL MUSCLE
forms the greatest tissue volume in most fishes

Tissue Digestion and Determination of Uptake.

Subsamples of tissue (25-75 mg), packed blood cells (50-100 μL) and plasma (50-100 μL), of weights Ws, were chemically digested with Scintigest, bleached with 30% hydrogen peroxide, and neutralized with acetic acid (appendix III). Scintiverse E was added and the samples were held in the dark for 96 h to reduce chemiluminescence (Long, 1976). DPM attributable to ^{3}H and ^{14}C for each subsample were determined on a Beckman LS 1801 liquid scintillation counter. To account for detection of quenched ^{14}C emissions in the ^{3}H channel and overestimation of ^{3}H emisions, DPM were corrected using the ratio method for quench correction (Minch, 1989). Some samples of liver, blood cells and muscle could not be completely freed of coloured material despite prolonged digestion and addition of 30% hydrogen peroxide. This situation led to high quenching in some of these samples which sometimes led to high variability in DPM. These organs were not however, usually the major targets for the delivered materials.

DPM determined for each subsample were normalized to account for differences in sample weight and total body weight (DPM^{*}) using the formula $DPM^{*}=DPM/Ws/Wb$. Recovery of each isotope (R) from fish was estimated using $R = DPM_{total}/D$ where D is the dose of radiolabel administered, $DPM_{total}= \Sigma [(DPM/Ws) \times Wo]$ over all organs examined for each fish and Wo

(g) is organ weight determined directly for the kidney, liver, and spleen and estimated for the muscle (Wo=0.6 x Wb) and plasma and blood cells (Wo= 0.025 x Wb). DPM*/R, expressed as DPM/g/g, were calculated for each isotope in each organ. This value attempts to account for all main sources of error and is referred to as uptake throughout the text.

All statistical analyses were performed on uptake values using the Statistical Analysis System package (SAS, version 6.01). As the data were skewed, uptake values were transformed prior to parametric analysis using the Power Law (Taylor, 1961). Transformed uptake values were examined using the rank correlation procedure (Spearman, 1904) and analysis of variance, ANOVA. Samples were considered significantly different from one another when $P < 0.05$. When Anova suggested preferential uptake by organs, the Student-Newman-Keuls test, SNK, was performed to arrange the organs by degree of uptake. The data are presented in all figures as untransformed uptake values. Corrected DPM values were used to determine $^{3}H/^{14}C$ ratios for each organ and corresponding 95% confidence limits for sample average ratios were established. Sample ratios were considered different from the ratios in the administered materials if the ratios for the administered materials were outside the 95% confidence limits for the sample averages.

RESULTS

Recovery of ^{3}H from trout administered ^{3}H-LPS was 30-49% and was highest when ^{3}H-LPS was administered via the IP route (Table 4.1). With all routes, ^{3}H was recovered primarily from the kidney and spleen (Fig. 4.3). With IP and IM administration, the majority of ^{3}H uptake was detected in the spleen whereas when ^{3}H-LPS was administered IV, uptake of ^{3}H was predominantly by the kidney (Fig. 4.3). With oral intubation of ^{3}H-LPS, uptake of ^{3}H was lower ($P < 0.05$, ANOVA; SNK) compared to the other routes (Fig. 4.3) although the radiolabel was more evenly distributed among the organs and tissues examined.

Recovery of ^{14}C from fish given +MLV was 24-80% and was highest with the IV route and lowest with the IM and oral routes (Table 4.1). Following administration of -MLV, recovery of ^{14}C was 14-64% and was highest with IP injections and lowest when the IM and oral routes were used (Table 4.1). When liposomes were administered IV, IP, or IM, uptake of ^{14}C by kidney and spleen was enhanced when using -MLV as opposed to +MLV (Fig. 4.4). When +MLV were administered via IV injection there was preferential uptake of the liposomes by the kidney and spleen (Fig. 4.4, left column). IP administration (Fig. 4.4, left column) resulted in the majority of

TABLE 4.1.

Recovery (R)[a] of Radioisotopes from Rainbow Trout 24 h after Administration of Antigens.

		R			
Antigens	Isotope	IV	IP	IM	ORAL
LPS	^{3}H	37.17± 6.15%	48.94±11.33%	30.53± 9.33%	30.59±30.27%
+MLV	^{14}C	80.33±24.69%	56.58±25.79%	26.22± 2.46%	24.05±10.33%
-MLV	^{14}C	33.39±11.41%	63.90±38.76%	15.38± 9.58%	14.23± 6.82%
+MLV/LPS	^{3}H	62.90±27.94%	32.26±13.31%	5.88± 5.46%	16.70± 8.58%
	^{14}C	57.31±66.05%	78.67±26.40%	13.94± 7.73%	30.58±13.16%
-MLV/LPS	^{3}H	93.66±56.13%	13.42± 6.72%	1.70± 0.06%	82.68±46.97%
	^{14}C	30.00±12.99%	39.59±13.13%	25.16± 9.18%	14.36± 4.78%

a. See Materials and Methods for calculation of R. Values are X ± S.D for N=4 fish in each trial.

FIGURE. 4.3. Uptake (expressed as DPM/g/g) of ^{3}H (see Materials and Methods) by organs of rainbow trout 24 hours after administration of ^{3}H-LPS *A. salmonicida* via IV, IP, IM and oral routes. Vertical bars represent the mean plus 1 SD for N=4 organs of each type. Some samples of liver, muscle and blood cells showed high color quenching which resulted in difficulty in accurately estimating DPM. Note changing scales.

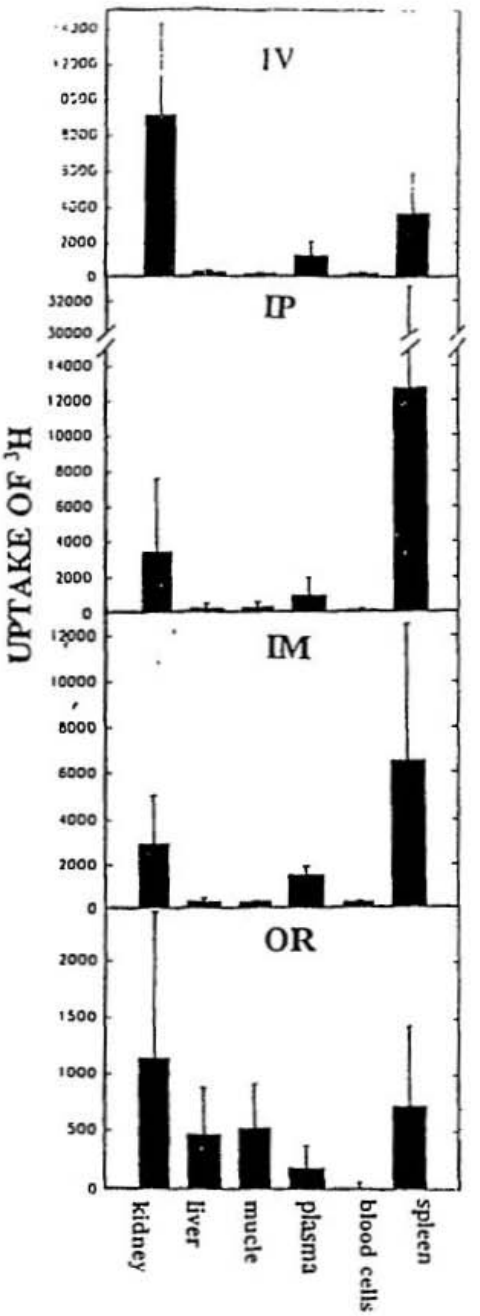
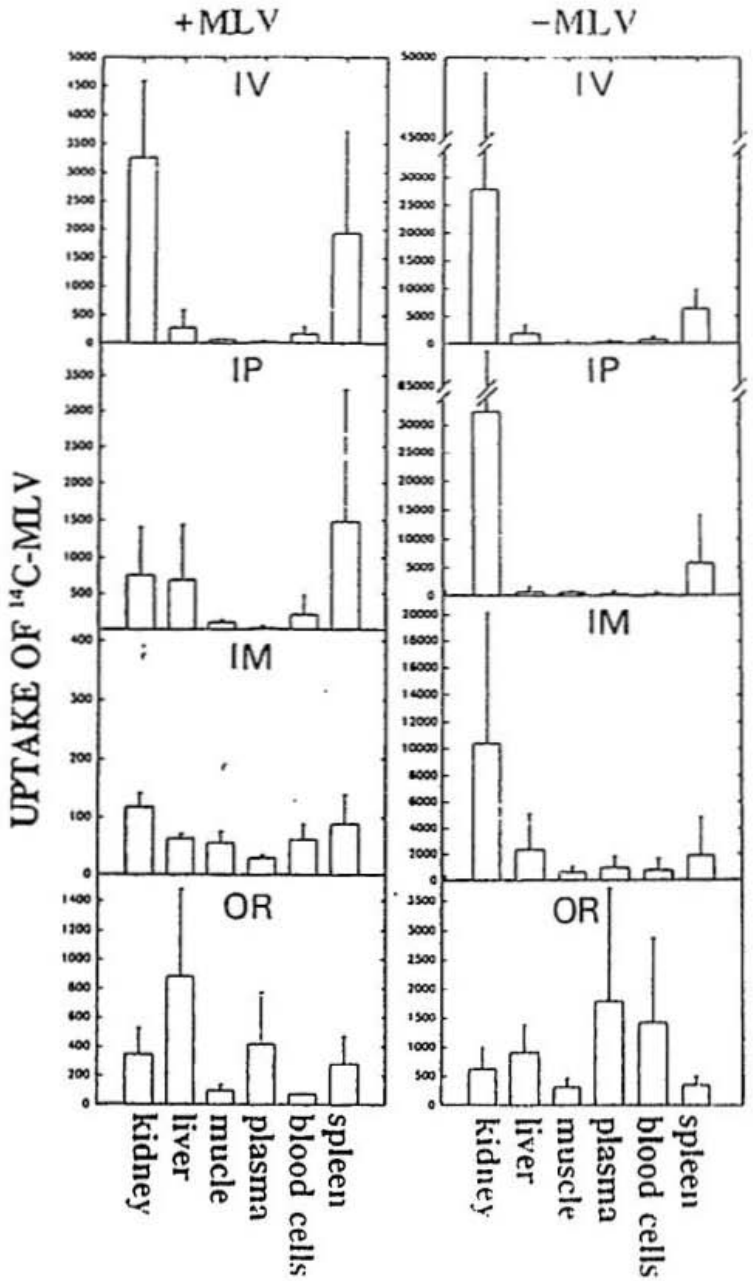

IV
IP
IM
OR
UPTAKE OF ^3H
kidney
liver
mucle
plasma
blood cells
spleen

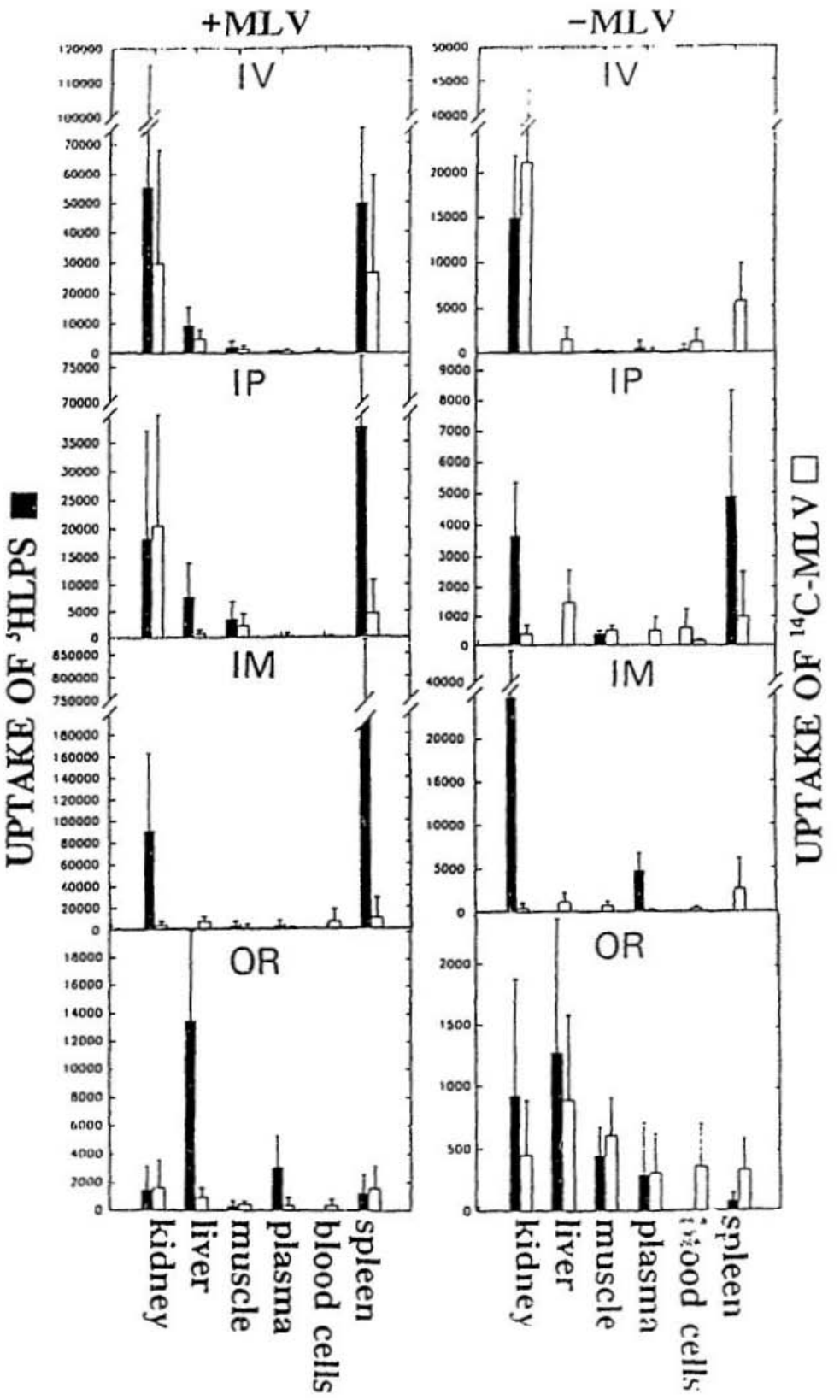
FIGURE. 4.4. Uptake (expressed as DPM/g organ/g body weight) of ^{14}C (see Materials and Methods) by organs of rainbow trout 24 hours after administration of $^{+}$MLV (left) and $^{-}$MLV (right) via IV, IP, IM and oral routes. Vertical bars represent the mean plus 1 SD for N=4 organs of each type. Some samples of liver, muscle and blood cells showed high color quench which resulted in difficulty in accurately estimating DPM. Note changing scales.


+MLV
-MLV
IV
IP
IM
OR
UPTAKE OF ^{14}C-MLV
kidney
liver
mucle
plasma
blood cells
spleen
kidney
liver
muscle
plasma
blood cells
spleen

uptake by the kidney, spleen and liver although the differences in uptake between organs was not significant ($P > 0.05$, ANOVA; SNK). When administered IM or orally, +MLV demonstrated no significant differences ($P > 0.05$, ANOVA; SNK) in uptake among the tissues and organs tested although it was more evenly distributed than with the other routes. In addition, after oral administration, uptake was greatest by the liver among the tissues and organs examined (Fig. 4.4, left column). With -MLV (Fig. 4.4, right column) there was preferential delivery of ^{14}C to the kidney and spleen following IV, IP and IM administration ($P < 0.05$, ANOVA; SNK). Again, delivery of -MLV by oral intubation resulted in a more even distribution of uptake of ^{14}C among the tissues and organs examined than by other routes although overall uptake was lower.

When +MLV/LPS was administered, recovery of ^{3}H was 6-63% and recovery of ^{14}C was 14-79% (Table 4.1). After IV, IP and IM administration of +MLV/LPS (Fig. 4.5, left column), uptake of ^{3}H by the organs demonstrated a pattern that resembled that of fish injected with ^{3}H-LPS (Fig. 4.3), i.e., ^{3}H was localized mainly in the kidney and spleen, although uptake in these organs was much greater than when ^{3}H-LPS was administered. Using IP, IV, and oral routes of administration of +MLV/LPS (Fig. 4.5, left column), uptake of ^{3}H by the liver was higher than when free ^{3}H-LPS was administered

FIGURE. 4.5. Uptake (expressed as DPM/g organ/g body weight) of ^{3}H and ^{14}C (see Materials and Methods) by organs of rainbow trout 24 hours after administration of +MLV/^{3}H-LPS (left) and -MLV/^{3}H-LPS (right) via IV, IP, IM and oral administration. Vertical bars represent the mean plus 1 SD for N=4 organs of each type. Some samples of liver, muscle and blood cells showed high color quench which resulted in difficulty in accurately estimating DPM. Note changing scales. ■ - ^{3}H uptake whereas; □ - ^{14}C uptake.


+MLV
−MLV
IV
IP
IM
OR
UPTAKE OF ³HLPS ■
UPTAKE OF ¹⁴C-MLV □
kidney
liver
muscle
plasma
blood cells
spleen

by the same route (Fig. 4.3). This is especially evident for the oral route. The ^{14}C counts indicate that there were no statistically significant differences ($P > 0.05$, ANOVA; SNK) in uptake of the MLV by the organs or tissues tested although the data suggest a tendency toward preferential uptake in the kidney and spleen with IV and IP administration. After administration of +MLV/LPS to fish, the ratio of $^{3}H/^{14}C$ in particular organs was altered compared to the$^{3}H/^{14}C$ ratio in the initial dispersion (Table 4.2). When +MLV/LPS was administered IV, the $^{3}H/^{14}C$ ratio in the plasma, blood cells and spleen increased suggesting that there was more LPS (or LPS metabolites) present as compared to MLV. IP injection showed no significant differences in the $^{3}H/^{14}C$ among the organs as compared to that in the original formulation. When +MLV/LPS was injected IM, the $^{3}H/^{14}C$ was increased in the spleen whereas when orally ingested, $^{3}H/^{14}C$ was elevated in the liver and plasma relative to that in the initial preparation (Table 4.2).

When -MLV/LPS was administered, recovery varied from 2-94% for ^{3}H and 14-40% for ^{14}C (Table 4.1). Uptake of the ^{3}H was primarily by the kidney and spleen when the liposomes were administered IV and IP (Fig. 4.5, right column) in a distribution pattern quite similar to that when free ^{3}H-LPS was administered (Fig. 4.3). Uptake of ^{3}H after IM injection of -MLV/LPS was greatest in kidney (Fig. 4.5, right column) which is in contrast to administration

TABLE 4.2.

Ratios of Radiolabel Uptake ($^3H/^{14}C$) in Organs of Rainbow Trout 24 h following Administration of +MLV/LPS and -MLV/LPS via Different Routes.

	$^3H/^{14}C$ RATIOS[a]							
	+MLV/^{3}H-LPS[b]				-MLV/^{3}H-LPS[c]			
ORGANS	IV	IP	IM	ORAL	IV	IP	IM	ORAL
Kidney	4.62±2.33	4.79±4.32	3.31±0.95	0.53±0.06	3.22±2.48	2.95±0.86*	5.27±0.64*	10.64±1.11*
Liver	6.92±4.63	4.68±3.08	-[d]	7.41±1.40*	3.50±1.62	-	-	3.81±0.60*
Muscle	5.38±3.32	1.06±0.52	0.71±0.40	0.32±0.18	3.22±2.57	0.35±0.27	-	4.10±1.20*
Plasma	3.88±0.12*	0.68±0.29	0.24±0.03	4.46±1.29*	3.53±0.02*	-	1.98±0.22*	3.35±0.74*
Blood Cells	10.24±1.95*	-	-	-	0.54±0.01	1.26±0.99	-	-
Spleen	7.68±2.60*	4.66±2.52	2.49±0.23*	0.41±0.02	0.89±0.23	2.85±0.70*	0.78±0.32	2.20±2.59

a. calculated from quench corrected DPM as described in material and methods. Values are X± S.D for N=4 fish in each trial. Asterisks indicate that the $^3H/^{14}C$ ratios in the initial dispersion are outside the lower 95% confidence limit.

b. $^3H/^{14}C$ ratio in original dispersion was 1.64.

c. $^3H/^{14}C$ ratio in original dispersion was 0.85.

d. Samples showed high color quenching and were omitted from these analyses because they gave anomalously low ratios (see materials and methods).

of ^{3}H-LPS by the corresponding route (Fig. 4.3). Uptake by all organs of ^{3}H after oral administration of -MLV/LPS was very low and had a comparable pattern of distribution as did the uptake of ^{14}C when $^{+}$MLV was administered orally (Fig. 4.4, left column). Uptake of ^{3}H after administration of -MLV/LPS was significantly less ($P < 0.05$, ANOVA; SNK) for all organs compared to uptake of ^{3}H after administration of +MLV/LPS but similar to that obtained following administration of free ^{3}H-LPS. Interestingly, distribution of ^{14}C among organs after -MLV/LPS treatment (Fig. 4.5, right column) was similar to that of the +MLV (Fig. 4.4, left column). The ^{3}H/^{14}C ratios were increased compared to the original dispersion in the plasma following IV administration and in the kidney and spleen following IP administration of -MLV/LPS (Table 4.2). In addition, the ^{3}H/^{14}C ratios were elevated in the kidney and plasma after IM injection and in the kidney, liver, muscle and plasma when -MLV/LPS was administered orally (Table 4.2).

DISCUSSION

In teleosts, and certainly in trout, kidney and spleen are the primary myeloid tissues in which hemopoiesis occurs. The anterior portion of the kidney in teleosts is especially rich in cells with immune functions including macrophages, which are highly phagocytic for antigenic substances and efficient antigen-presenting cells (Ellis, 1977; Yasutake, 1983). Our results suggest that, 24 h after administration to trout, uptake of exogenously administered LPS, MLV and MLV/LPS (or their metabolites) was primarily by the kidney and spleen. The IV route afforded the most effective delivery of LPS to the kidney and spleen, followed by the IP and IM routes. Although IV administration of vaccines to fish may have limited commercial application, this route was incorporated within the experimental protocol for the purpose of expanding the understanding of the uptake of liposomal LPS since it may be considered the most direct route of delivery. Uptake of LPS following oral administration was minimal in terms of the total material recovered after 24 h, but it yielded the greatest delivery of LPS to the liver among the routes examined. The low overall recovery can possibly be attributed to inefficient transport of material across the intestine, whereas the direct supply of venous blood from the intestine to the liver via the hepatic portal system might explain the relatively

enhanced delivery to the liver after oral administration (Yasutake, 1983).

Negatively-charged MLV were taken up better by the kidney and spleen than were their positively-charged counterparts. It is noted that there was 37% less lipid per dose in the positively-charged MLV which may have contributed to difference in uptake. Similar behaviour for negatively-charged liposomes being taken up by the macrophages of the RES is well documented in mammalian systems. For instance, phosphatidylserine imparts a negative charge to the liposome surface and has been implicated in direct recognition by the RES (Schroit *et al.*, 1984; Allen *et al.*, 1988). Interestingly, the opposite scenario occurs with positively- and negatively-charged MLV/LPS in rainbow trout where we observed that there was better uptake of ^{3}H (from LPS) by the kidney and spleen when +MLV/LPS was administered as compared to -MLV/LPS. Due to differences in incorporation LPS observed by positively and negatively-charged MLV (see chapter 3), the amounts of LPS and lipid per dose were different in the groups which had recieved +MLV/LPS and -MLV/LPS. The preparations were not standardized for dose of LPS and lipid because this would have required the addition of empty liposomes which could affect the biodistribution. In addition, the distribution of ^{14}C (from liposomes) among the organs when +MLV and -MLV/LPS were administered was similar.

LPS is an amphipathic negatively-charged molecule and its incorporation into MLV may alter the net charge on the liposome surface and consequently be an important factor for targeting to the organs of the RES in rainbow trout. It is also interesting that encapsulation of LPS within positively-charged MLV resulted in enhanced uptake by the kidney and spleen when compared to uptake of free LPS, whereas encapsulation within negatively-charged MLV caused comparable uptake and does not appear to enhance delivery to the kidney and spleen. The recovery of radioisotope from rainbow trout after administration of MLV and MLV containing LPS was generally higher when they were given IV and IP rather than IM and orally. It is most likely that the low recovery 24 h following IM administration is due to slow release of material from the site of injection, which was distal to the sampling site. The $^{3}H/^{14}C$ ratios in some organs and tissues were altered 24 hours after administration in comparison to that in the original liposome preparation, and certain routes and formulations resulted in better uptake of ^{3}H-LPS by the kidney or spleen or both. This is perhaps due to an enhanced delivery of LPS to those organs with the MLV serving as a "chaperone". It is also possible that both LPS and MLV are being delivered but MLV or their lipid components are catabolized at a different rate than LPS. It is likely that uptake and the $^{3}H/^{14}C$ ratios would be different at time points other than 24 h. Certain organs (particularly muscle, liver and

blood cells) showed high color quenching as compared to the other organs and resulted in difficulty accurately estimating DPM. These organs also demonstrated elevated chemiluminescence as compared to the kidney, spleen and plasma, even after prolonged incubation in the dark. Uptake values for these organs may be somewhat overestimated, reinforcing the fact that the major uptake is by kidney and spleen. Uptake values for the organs were not corrected for their blood volumes which could lead to some degree of overestimation of levels of uptake, especially in hemopoietic organs.

In mammals, it has been suggested that, in contrast to "conventional" liposomes, formulations of long-circulating liposomes are dependent on the shielding of negative surface charges by compounds composed of neutral long-chain saturated phospholipids and cholesterol (Allen *et al.*, 1991; Lasic *et al.*, 1991). Since incorporation of LPS altered the uptake of "empty" liposomes, the results presented here indicate that the uptake of liposomes and liposomal LPS in rainbow trout may also be dependent on surface charge.

Ferguson *et al.*, (1982) reported that the uptake of bacteria by salmonids is primarily by the kidney and spleen. We observed that uptake of the LPS component of *A. salmonicida* was primarily by the kidney and spleen and that this uptake was significantly enhanced in those organs when LPS was

incorporated into liposomes. Recently, it has been reported that a vaccine composed of *A. salmonicida* whole cells, 'toxoided' extracellular products and LPS incorporated into liposomes was capable of conferring some degree of protection against *A. salmonicida* in rainbow trout (Rodgers, 1990). The results presented here suggest that liposomally-incorporated LPS from *A. salmonicida* might have potential as an anti-furunculosis vaccine in rainbow trout. Immunization experiments in rainbow trout using free and liposomal LPS will be discussed in the following chapter.

CHAPTER 5

SERUM ANTI-LPS ANTIBODY PRODUCTION BY RAINBOW TROUT (*Oncorhynchus mykiss*) IN RESPONSE TO THE ADMINISTRATION OF FREE AND LIPOSOMALLY-INCORPORATED LPS FROM *Aeromonas salmonicida*.

INTRODUCTION

The numerous efforts to develop an effective, inexpensive and easily administered anti-furunculosis vaccine have produced some encouraging data *vis a vis* immunity against *Aeromonas salmonicida* infections (Olivier *et al.*, 1985; Hastings and Ellis, 1988; Tatner, 1990; Lund *et al.*, 1991; Niki *et al.*, 1991). Most of the currently available anti-furunculosis vaccines, however, have demonstrated limited effectiveness in preventing disease (Ellis, 1988) and the search for more potent vaccines continues (Niki *et al.*, 1993; Vaughan *et al.*, 1993; Thornton *et al.*, 1994; Hirst and Ellis, 1994; AAkre *et al.*, 1994). LPS is believed to be a major factor of bacterial virulence and has been shown to induce diverse immunological and pathophysiological changes in mammalian (Morrison and Ryan, 1979; Rietschel and Brade, 1992) and fish systems (Porreau *et al.*, 1986; Lee and Ellis, 1991). As a TI antigen in mammals (Morrison and Rudbach, 1981) and fish (Clem *et al.*, 1990; Arkoosh and Kaattarri, 1991), LPS induces immune responses that are antibody mediated, without activating immune mechanisms involving T_H cells. Certainly, humoral immunity plays a significant role in resisting some of the harmful effects of LPS although the immune response to LPS appears to be faint and limited to IgM antibodies and does not lead to immunological memory (Elkins *et al.*,

1989). T-cell mediated immunity is considered to be of prime importance to the enhancement of protective immunity against bacterial disease (Hahn and Kaufman, 1981). It is believed that association of some antigens with liposomes can influence the generation of T cell mediated immunity in mammals (Tom, 1981; Garcon and Six, 1991; Harding *et al.*, 1991; Collins *et al.*, 1992). Wong *et al.* (1992) examined the IgM and IgG levels in sera of mice immunized with free and liposomal LPS and PS from *Brucella abortus* and found that liposomal antigen generated significantly higher IgG levels than free antigen. Tatner *et al.* (1990) observed that modifying the LPS from *A. salmonicida* by coating it onto polystyrene beads rendered the LPS stimulatory for lymphocyte proliferation in Atlantic salmon. According to these studies, it appears that liposomal incorporation may serve to modify the LPS and additionally act as a delivery vehicle and an immunological adjuvant.

It was established in the preceding section that liposomes may serve as efficient delivery vehicles for the LPS from *A. salmonicida* to the primary hemopoietic organs of rainbow trout, and consequently, these formulations may also function to enhance the immunological response to bacterial endotoxin. In mammals, it has been demonstrated that liposomal LPS formulations are capable of enhancing immunogenicity to the associated LPS from various

bacterial strains (Desiderio and Campbell, 1985; Petrov *et al.*, 1992; Wong *et al.*, 1992). Accordingly, LPS-based liposomal vaccines may be of value as a safe, economic and practical delivery system which could improve fish quality and quantity by reducing bacterial fish diseases and enhancing growth. We believe that these trials are the first to examine the delivery vehicle and adjuvant capabilities of liposomal LPS formulations in rainbow trout, although there has been a study using liposomes which employed a similar approach to that described here. Rodgers (1990) immunized rainbow trout with liposomes composed of "egg" PC which contained a mixture of formalin-killed whole cells of *A. salmonicida*, inactivated toxin from *A. salmonicida*, and LPS from *A. salmonicida*. The author observed increased survival rates among the group which received the liposomal vaccine. The group which received liposomal vaccine had a cumulative mortality of 11.4% by the end of the 18 week experimental period as compared to mortalities of 15.1% and 37.3% for the groups which had received a non-liposomal form of the vaccine and the non-vaccinated controls, respectively.

Immunization experiments were performed using intraperitoneally (IP) injected free and liposomal LPS in order to determine whether enhanced delivery of LPS to the primary heompoietic tissues in rainbow trout resulted in

a concomitant potentiation of the antibody-mediated immune response to LPS. In this chapter, the results of those immunization studies are presented and discussed.

MATERIALS AND METHODS

Materials.

Phosphatidylcholine (PC, from frozen egg yolk), phosphatidylglycerol (PG, produced from egg yolk PC by transphosphatidylation) cholesterol (CH), stearylamine (SA), metrizamide and all immunological reagents were purchased from Sigma Chemical Co., St. Louis, Mo., USA. All other materials were of reagent grade or better, and were purchased from various suppliers.

Bacterial Cultures and Isolation of *A.salmonicida* LPS.

Bacterial cultures of *A.salmonicida* were grown to late stationary phase as briefly described in chapter 2 and in more detail in appendix I.

LPS was extracted from formalin-fixed, lyophilized bacteria by the hot aqueous phenol method (Westphal and Jann, 1965, appendix I). Phenol-extracted LPS was dialyzed against 6 changes of distilled H_2O (3.5 L per

change), lyophilized and stored at -15°C prior to use.

Preparation of Liposome-Associated LPS.

LPS from *A. salmonicida* was incorporated into MLV and $LUVET_{200}$ composed of either PC:CH:PG or PC:CH:SA in a 6:3:1 molar ratio as described in chapter 3 and in appendix II. Liposomally-incorporated LPS with initial lipid composition of PC:CH:PG are referred to as -MLV/LPS or -$LUVET_{200}$/LPS whereas those with initial lipid composition of PC:CH:SA are referred to as +MLV/LPS or +$LUVET_{200}$/LPS. Free LPS was separated from liposomal LPS by density gradient centrifugation using a discontinuous metrizamide gradient (Heath, 1987) as described in chapter 3 and in appendix II. Following separation of unincorporated LPS, liposome-associated LPS was quantified by the phenol-sulphuric acid method (Dubois *et al.*, 1956, appendix II) using *A. salmonicida* LPS as a standard and corrected with appropriate lipid blanks. -MLV/LPS and +MLV/LPS suspensions had final concentrations of 250 and 680 μg LPS/mL, respectively, whereas the -$LUVET_{200}$/LPS and +$LUVET_{200}$/LPS dispersions had final concentrations of 378 and 1000 μg/mL, respectively. Free LPS used to immunize trout was a 1 mg/mL dispersion in 0.15 M NaCL.

Immunization of Rainbow Trout and Collection of Sera.

Groups of 25 rainbow trout weighing 40 to 80 g were kept in 60 L holding tanks supplied with a continuous flow of oxygen and filtered water. Fish were allowed to acclimate to the environment for one week prior to immunization. Some of the immunization experiments were performed in the tank room facilities at the Department of Fisheries and Oceans, Canada, which received water from the city water supply. Water was filtered and continuously flowed through the system. Temperature regulators were attached to each tank and were adjusted to maintain the temperature between 10-15°C. However, the experimental conditions, particularly the temperature and water purity, were difficult to conserve over the duration of the experiments and only the data presented in Fig. 5.1 were collected from experiments conducted at the Department of Fisheries and Oceans. The remainder of the immunization experiments described in this chapter were performed in the aquatics room of the Animal Care Facilities at Memorial University. The setup consisted of a circulatory water system maintained between 8-13°C with a biological filter attached. The system maintained consistent measures of Ph and water hardness and negligible levels of ammonia. In all instances, 100 μL of blood was collected from the caudal sinus of the rainbow trout by venipuncture using a 1

cc syringe fitted with a 21 gauge needle prior to immunization to evaluate existing serum anti-LPS antibody levels. This was followed immediately by administration of IP injections or by oral intubations of doses equivalent to 50 μg of LPS in the free or liposomal form. Doses were standardized only for LPS since standardizing for lipid would require addition of empty liposomes to some preparations which may have affected adjuvanticity. Times of antigen administration to rainbow trout are indicated in the figures, and all doses were equivalent to 50 μg free or liposomal LPS. At various times after immunization, trout (N = 3-8) from each group were randomly chosen and blood samples were obtained as described above. Following incubation of the blood samples at 4°C overnight, serum was separated from blood cells by centrifugation at 2000 x g_{max} for 5 minutes at room temperature. Serum was assayed for anti-LPS activity as described below. NaN_3 was added to the serum at a final concentration of 0.02% and the serum was stored at 4°C.

Evaluation of Immune Response Using ELISA.

Serum was evaluated for the presence of anti-LPS antibody using an ELISA as described in chapter 2 and appendix I with minor modifications. The titre of anti-LPS antibodies in rainbow trout serum was determined by using the

LPS from *A. salmonicida* as the immobilized antigen. Rainbow trout antiserum samples were used as the primary antibody. Previously tested trout anti-LPS serum and diluent were added to each plate as positive and background controls. In some instances, pre-bleed sera (normal trout serum, NTS) were added to the plates to serve as negative controls. As described in chapter 2, MAb 1.14 was used as the secondary antibody and a conjugate of rabbit anti-mouse immunoglobulin and horseradish peroxidase was used as the reporter. The remainder of the assay was performed as described in chapter 2 and appendix I.

RESULTS

The results of the immunization experiments performed using free and liposomally-incorporated from *A. salmonicida* are presented here. The data is expressed as the mean $\pm$ 1 SD of antibody titres for 3-8 serum samples. Appendix V contains figures representing the same data presented as either individual data points (figures designated a) or mean, medium, and mode (figures designated b). For the remainder of this chapter, the data expressed as mean $\pm$ 1 SD will be discussed.

After IP administration of three doses of free LPS to trout over an 8 week period, an increase in serum anti-LPS antibody titres was detected by week 10, although the response was markedly reduced by week 12 (Fig. 5.1,A). In contrast, when +MLV/LPS was administered first in the immunization programme, anti-LPS antibody titres were elevated in trout sera by week 7 or 8 (Fig. 5.1, C and D). In addition, serum antibody levels for rainbow trout primed with +MLV/LPS (Fig 5.1, B and D) rose to higher levels within shorter time intervals than did those achieved without liposomal LPS included in the protocol (Fig 5.1, A). Most importantly, following subsequent challenge with free LPS, rainbow trout that were administered two IP injections of +MLV/LPS mounted a prolonged response against LPS (Fig. 5.1, D). Other immunization protocols using +MLV/LPS (Fig. 5.1, B and C) did not appear to produce a prolonged response against LPS.

Immunization experiments that included -MLV/LPS in the protocol demonstrated that incorporation of LPS into negatively-charged liposomes also succeeded in substantially prolonging serum anti-LPS antibody titres (Fig. 5.2). Anti-LPS levels in the serum of rainbow trout were monitored over 22 weeks. It appeared that, in contrast to the behaviour when +MLV/LPS was injected, only one IP injection of -MLV/LPS prior to IP injection of free LPS was necessary to generate a sustained level of serum anti-LPS activity; this response

FIGURE 5.1. The titre of anti-LPS reactivity seen after administration of free *A. salmonicida* LPS and +MLV/LPS intraperitoneally to rainbow trout as a function of time. All injections contained 50 μg of LPS either in the free (LPS) or liposomal form (LLPS) as indicated by arrows in the figure. First injections were administered at day 0. Panels A-D represent different immunization regimes. Serum anti-LPS values were determined by ELISA and are expressed as mean $\pm$ 1 SD for N=7-8 fish per sampling time.

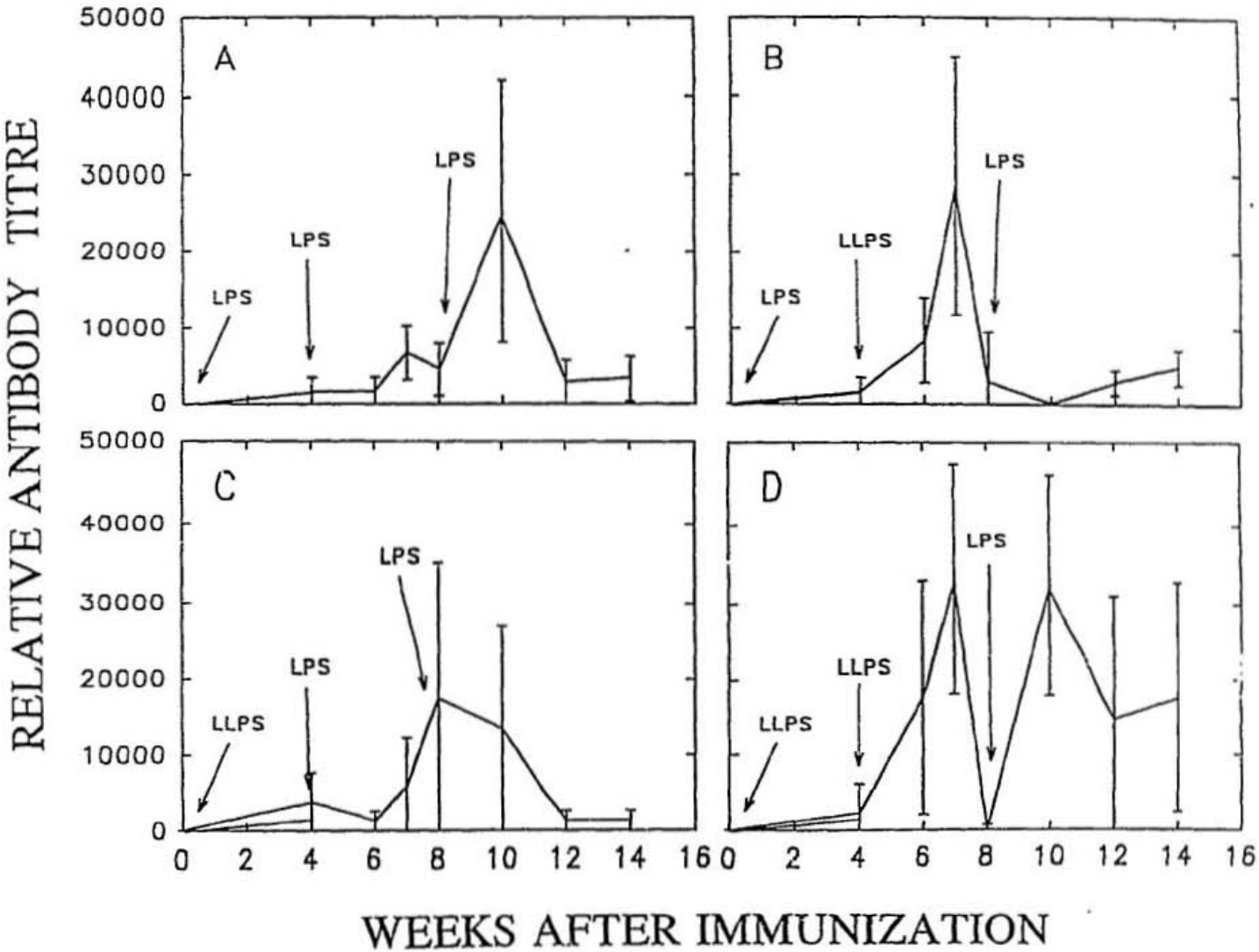
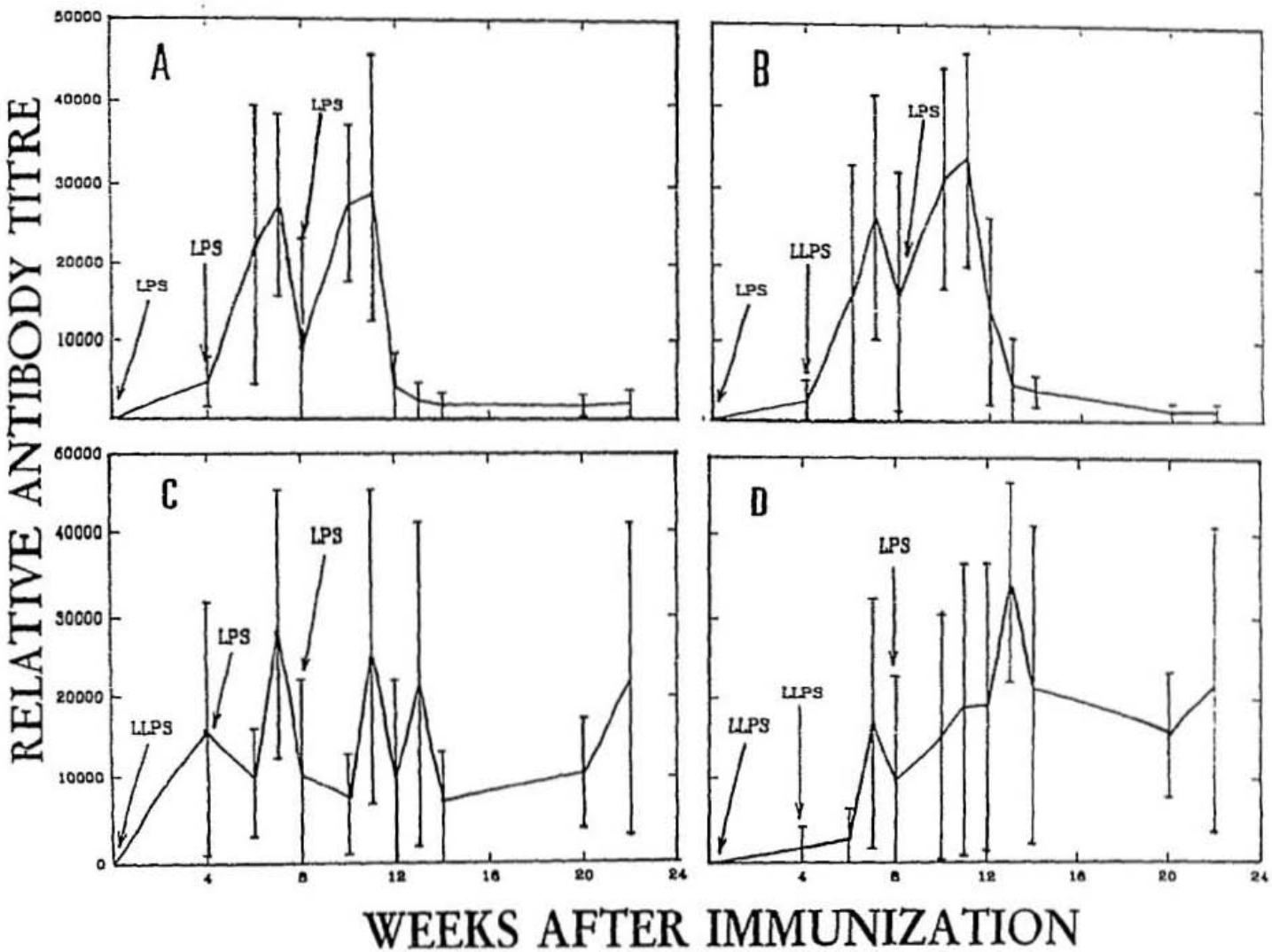

RELATIVE ANTIBODY TITRE
50000
40000
30000
20000
10000
0
A
B
C
D
LPS
LLPS
0 2 4 6 8 10 12 14 16
WEEKS AFTER IMMUNIZATION

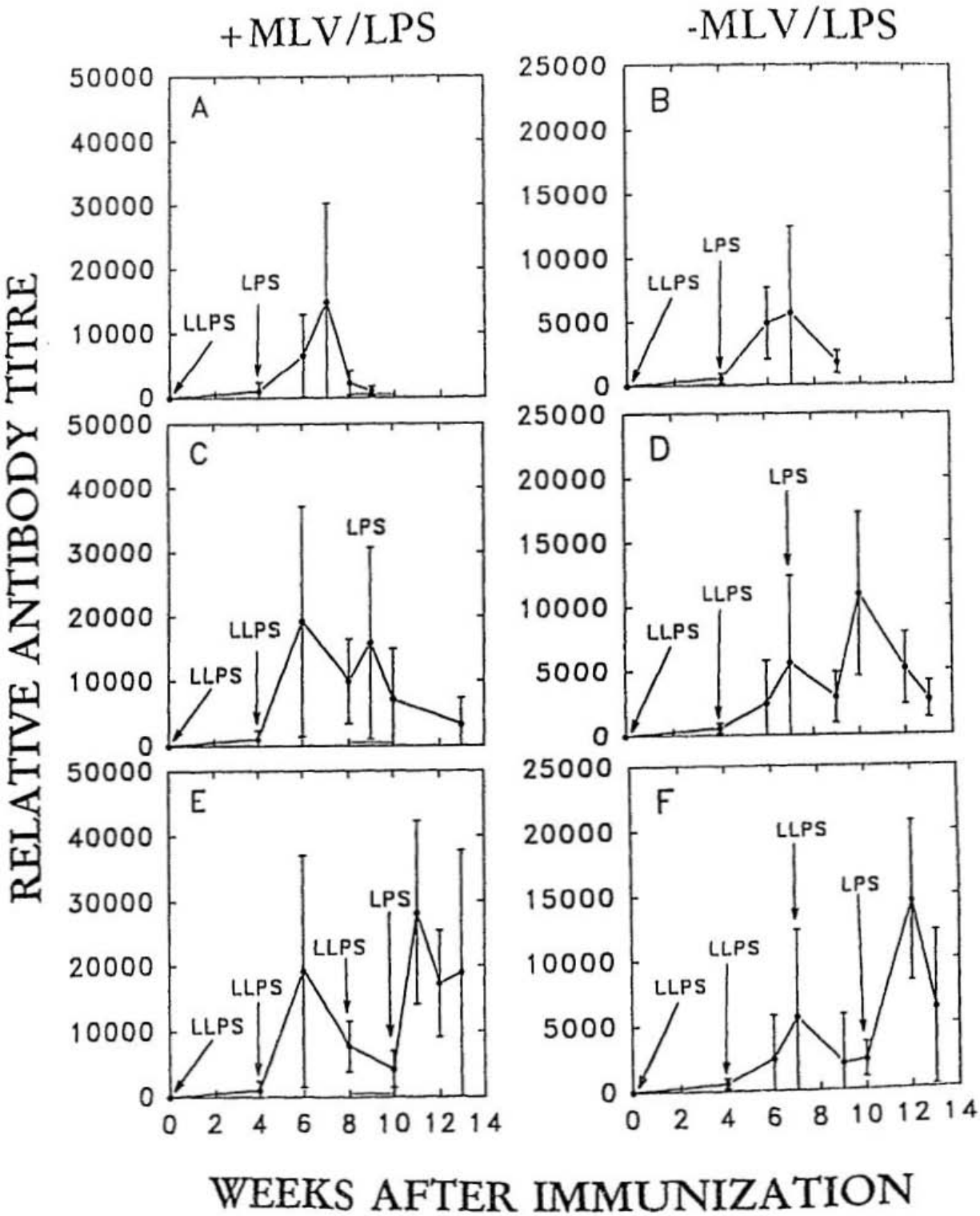
FIGURE 5.2. The titre of anti-LPS reactivity seen after administration of free *A. salmonicida* LPS and -MLV/LPS intraperitoneally to rainbow trout as a function of time. All injections contained 50 μg of LPS either in the free (LPS) or liposomal form (LLPS) as indicated by arrows in the figure. First injections were administered at day 0. Panels A-D represent different immunization regimes. Serum anti-LPS values were determined by ELISA and are expressed as mean $\pm$ 1 SD for N=3-8 fish per sampling time.


A
B
C
D
LPS
LLPS
RELATIVE ANTIBODY TITRE
WEEKS AFTER IMMUNIZATION
50000
40000
30000
20000
10000
60000
0
4
8
12
16
20
24

was still elevated 14 weeks following exposure to LPS (Fig. 5.2, C). As was the case when the trout were primed with +MLV/LPS, a sustained immune response to LPS resulted from two IP injections of -MLV/LPS prior to challenge with LPS (Fig. 5.2, D). Following challenge with free LPS, the anti-LPS responses were maintained between week 8 and week 22. An injection of -MLV/LPS following priming with free LPS (Fig. 5.2, B) resulted in considerable anti-LPS activity once a third IP injection of free LPS was administered, although the responses against LPS were not prolonged (Fig 5.2, A). Trout which received repeated IP injections of free LPS (Fig. 5.2, A) displayed different humoral response profiles than the analogous treatments illustrated in Fig. 5.1, A. This may likely be the effect of variations in environmental conditions (see discussion).

The relationship between the number of IP injections of liposomal LPS prior to administration of free LPS and the serum anti-LPS antibody response is illustrated in Fig. 5.3. A primary response was produced by week 7 in rainbow trout administered one IP injection of +MLV/LPS or -MLV/LPS prior to exposure to free LPS although this response had subsided by week 9 (Fig. 5.3, A and B). However, profiles suggestive of a prolonged response to LPS were exhibited when either two or three IP injections of +MLV/LPS (Fig. 5.3, C and E respectively) or -MLV/LPS (Fig. 5.3, D and F, respectively) were

FIGURE 5.3. Serum anti-LPS antibody titres as a function of the number of doses of +MLV/LPS (A, C, and E) or -MLV/LPS (B, D, and F) prior to challenge with free LPS. All injections contained 50 μg of free (LPS) or liposomal LPS (LLPS) as indicated by arrows in the figure. First injections were administered at day 0. Serum anti-LPS values were determined by ELISA and are expressed as mean $\pm$ 1 SD for N=5-8 fish per sampling time.


+MLV/LPS
-MLV/LPS
A
B
C
D
E
F
LLPS
LPS
RELATIVE ANTIBODY TITRE
WEEKS AFTER IMMUNIZATION
0
5000
10000
15000
20000
25000
30000
40000
50000
0 2 4 6 8 10 12 14

administered previous to challenge with free LPS. It is possible that the duration of serum anti-LPS responses might be related to the number of doses of liposomal LPS administered prior to exposure to free LPS.

Immunization experiments performed with +MLV/LPS and -MLV/LPS (Fig. 5.4, A and B, respectively) administered via oral intubation to rainbow trout suggested that neither of these formulations were as potent in eliciting considerable serum anti-LPS antibody responses in rainbow trout as were IP injections. The levels of antibody were still relatively low following exposure of rainbow trout to LPS and only a small response was elicited in either group. Given the difficulty with controlling these systems and the limited studies done, it may be premature to eliminate this route as a potential means for delivery.

The results from immunization experiments using the extruded unilamellar liposomal LPS formulations in rainbow trout are illustrated in Fig. 5.5. The protocol consisted of each trout receiving two IP injections of either +$LUVET_{200}$/LPS or -$LUVET_{200}$/LPS within the first 4 weeks prior to subsequent exposure to free LPS during week 8, as was conducted in panel D in Fig. 5.1 and 5.2. As this experiment was devised to compare the abilities of multi- and unilamellar LPS formulations to potentiate the immune responses to LPS, there were no groups that received treatments parallel to those described in panels A, B, and C in Fig. 5.1 and 5.2. The rainbow trout exhibited similar

FIGURE 5.4. The titre of anti-LPS reactivity seen after administration of free *A. salmonicida* LPS or MLV-incorporated LPS orally to rainbow trout as a function of time. Doses administered contained 50 μg of LPS in the free (LPS) or liposomal form (LLPS) as indicated by arrows in the figure. Serum anti-LPS values were determined by ELISA and are expressed as mean $\pm$ 1 SD for N=3-8 fish per sampling time.

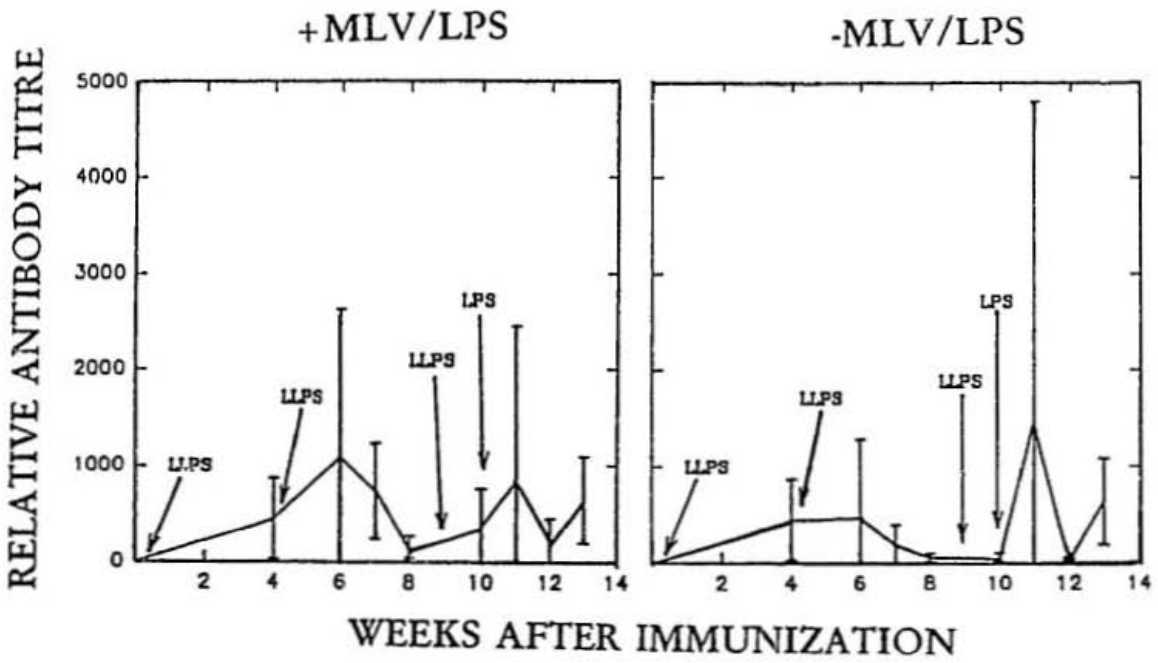
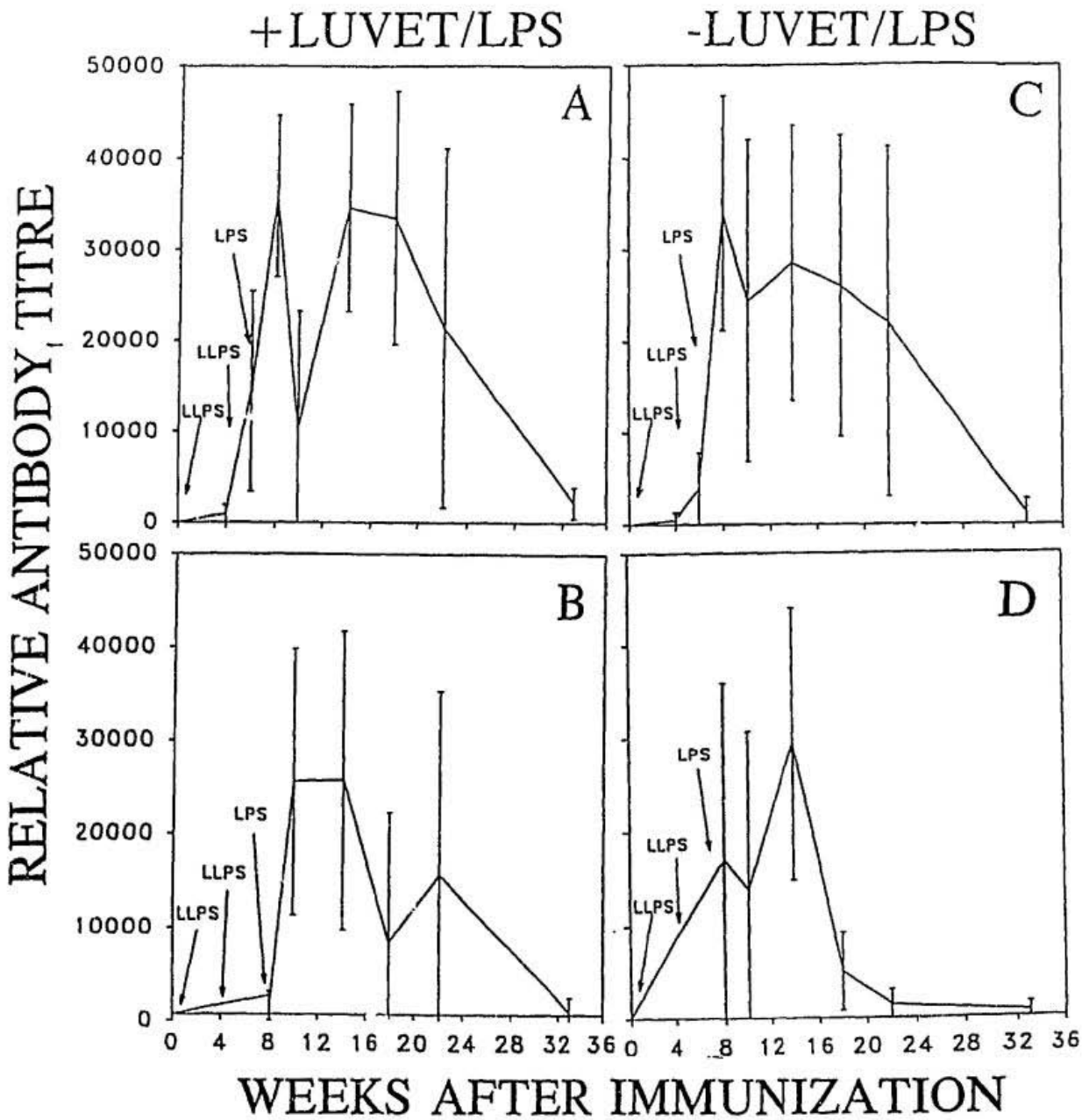

+MLV/LPS
-MLV/LPS
RELATIVE ANTIBODY TITRE
5000
4000
3000
2000
1000
0
LPS
2
4
6
8
10
12
14
WEEKS AFTER IMMUNIZATION

FIGURE 5.5. The titre of anti-LPS (A and C) and anti-lipid A (B and D) reactivity seen after administration of free *A. salmonicida* LPS and +LUVET$_{200}$/LPS or -LUVET$_{200}$/LPS intraperitoneally to rainbow trout as a function of time. Doses administered contained 50 μg of LPS in the free (LPS) or liposomal form (LLPS) as indicated by arrows in the figure. Serum anti-LPS values were determined by ELISA and are expressed as mean $\pm$ 1 SD for N=8 fish per sampling time.


+LUVET/LPS
-LUVET/LPS
A
B
C
D
LPS
LLPS
LLPS
LLPS
RELATIVE ANTIBODY TITRE
50000
40000
30000
20000
10000
0
0 4 8 12 16 20 24 28 32 36
WEEKS AFTER IMMUNIZATION

profiles of serum anti-LPS antibody responses (Fig. 5.5, A and C) as those described in Fig. 5.1, D and 5.2, D. Elevated serum anti-LPS antibody responses were displayed for at least 15 weeks following exposure to free LPS before declining as shown by the response at week 33. The presence of a slowly declining antibody response upon a second exposure to antigen is one of the characteristics of a secondary immune response. Given the current protocol that included two priming injections of liposomal LPS, the persistence of the serum anti-LPS response seems to be between 14-25 weeks after exposure to free LPS. Thus, it appears that liposomes are capable of potentiating the immune response to LPS in rainbow trout when administered IP, and that these events are independent of the liposome composition or lamellarity. In addition to the serum anti-LPS antibody responses, the concentrations of anti-lipid A antibodies were evaluated throughout these immunization trials (Fig. 5.5, B and D). Administrations of $+LUVET_{200}$/LPS prior to exposure to free LPS appeared to induce a prolonged anti-lipid A antibody response before it subsided between week 22 and 32 (Fig. 5.5, B). Priming with $-LUVET_{200}$/LPS before exposure to free LPS indicated that the anti-lipid A antibody response had declined by week 18 (Fig. 5.5, D).

DISCUSSION

Many avenues have been explored for the development of vaccines against bacterial diseases of fish. In salmonid fishes, these strategies include immunizations with whole-cell bacterial preparations (Anderson and Jenney, 1991; Niki *et al.*, 1991), extracellular products (Hastings and Ellis, 1988; Lund *et al.*, 1991) and endotoxins (Velji *et al.*, 1990) in addition to adjuvant formulations (Tatner, 1990; Bogwald *et al.*, 1991), polysaccharides (Yano *et al.*, 1991), glycoconjugates (Banoub *et al.*, 1989) and antigens which have been coated onto sugar (Wong *et al.*, 1992) or polystyrene beads (Adams *et al.*, 1988; Tatner, 1990) in order to enhance their immunogenicity. Although liposomal LPS vaccines have been infrequently tested in fish (Rodgers, 1990), it has been observed that liposomes can serve as efficient delivery vehicles for LPS to the kidney and spleen (see chapter 4), the primary hemopoietic organs in teleost fish (Ellis, 1982).

The results presented in this chapter demonstrate that both positively- and negatively-charged liposomes are capable of prolonging the immune response against LPS from *A. salmonicida* in rainbow trout. It has been demonstrated that liposomal surface charge may influence uptake by the macrophages of the RES in mammals (Allen *et al.*, 1989; Allen *et al.*, 1991;

Gabizon and Papahadjopoulos, 1992; Yamauchi *et al.*, 1993). For instance, negatively-charged phospholipids such as phosphatidylglycerol and phosphatidylserine are known to enhance liposome uptake by the cells of the RES in mammals (Raz *et al.*, 1981). Senior *et al.* (1991) demonstrated that there is a positive correlation between the molar ratio of positively-charged lipid included in a liposomal formulation and the interactions of those liposomes with blood components *in vitro*. There appeared to be an effect of surface charge on the uptake of liposomes by the kidney and spleen in rainbow trout (chapter 4). Under the current protocols, there was no marked difference in the humoral responses against LPS generated by liposomal LPS formulations that included either positively-charged (SA) or negatively-charged (PG) components in rainbow trout. *In vivo* experiments in rainbow trout (Power *et al.*, 1991) and *in vitro* experiments using murine macrophages (Allen *et al.*, 1991) suggested that there was an inverse relationship between liposome size and uptake by the RES. These observations suggest that liposomes, particularly the formulations containing vesicles with smaller diameters, may enhance immune responses to an associated antigen. Our results suggest that multi- and unilamellar liposomes containing LPS generated comparable humoral responses against LPS in rainbow trout.

In mammals, the prominent characteristics of memory (anamnestic) responses in mammals are a shorter lag period, an extended plateau, and a slower decline of antibody titre upon a second exposure to the priming antigen. In addition, antibody levels in response to second exposure to the priming antigen are much higher. However, if the priming antigen is a TI antigen such as LPS is known to be in mammals (Morrison and Rudbach, 1981) and in fish (Miller *et al.*, 1985; Tatner, 1990), immunological memory is not induced. LPS can directly trigger B cells to antibody formation which is almost exclusively IgM and apparently does not require collaboration of T cells. There is evidence that liposome-encapsulated antigens demonstrate enhanced antigen presentation and recruitment of macrophages, that may result in the generation of T-cell responses in mammals (Tom, 1981; Garcon and Six, 1991; Harding *et al.*, 1991; Collins *et al.*, 1992). In addition, it is believed that the lipid A moiety of LPS can abolish suppressor T-cell activity, resulting in increased immune responses to polysaccharide antigens in mammals (Alving, 1993). Combined, the immunomodulating effects of both liposomes and LPS may provide an efficacious formulation for resisting bacterial diseases. For instance, the incorporation of the LPS and PS of *Brucella abortus* into liposomes resulted in the potentiation of humoral responses to those antigens in

mice (Wong *et al.*, 1992). Desiderio and Campbell (1985) reported that liposomal LPS from *Salmonella typhimurium* was an efficient modulator of the host immune response in favor of cell-mediated immunity, resulting in increased levels of protection against murine salmonellosis. Recently, immunizations with liposomally incorporated LPS from *Neisseria meningitidis* increased the number of anti-LPS plaque forming cells in the spleens of mice, in addition to an exhibiting elevated serum anti-LPS antibody responses in comparison to free LPS (Petrov *et al.*, 1992).

When rainbow trout were injected intraperitoneally with liposomal LPS and then exposed to free LPS, we observed that the humoral immune responses exhibited some of the criteria associated with secondary immune responses in mammals. In comparison to those instances when fish where primed with free LPS, serum anti-LPS antibody responses were prolonged when the priming antigen was liposomal LPS prior to injections of free LPS. Prolonged response is a characteristic of memory responses in mammals. However, it appears that two priming injections of liposomal LPS prior to challenge with free LPS were necessary to generate the prolonged response. Typically, the sustained antibody responses of a secondary response might be expected after only one priming injection. An *in vitro* study conducted with spleen cells from rainbow trout

established that more than a single injection of the priming antigen (TNP-KLH, a T-dependent antigen) was required to produce a secondary response to TNP using TNP-KLH (Arkoosh and Kaattari, 1991). Curiously, those authors found that the T-independent form of the antigen, TNP-LPS, induced a secondary response when only one priming injection of TNP-KLH was administered. The ratio between the titres obtained in the primary and secondary responses in mammals can be 10-100 fold, although it appears that the ratio between primary and secondary responses do not reach those levels in fish (Dorson, 1984). Secondary immune responses have been reported against BSA in carp (Avtalion, 1969), goldfish (Trump and Hildemann, 1970) and channel catfish (Clem and Siegel, 1965) as well as against DNP in carp (Ambrosius and Frenzel, 1972). In rainbow trout, properties associated with the anamnestic responses in mammals were observed when primary (100 μg/fish) and secondary (20 μg/fish) injections of TNP-KLH were administered IP (Arkoosh and Kaattari, 1991). The ratio of serum anti-TNP antibody levels between primary and secondary responses, however, was approximately 3.5. Although we observed prolonged serum anti-LPS antibody responses, our results did not manifest marked differences between the antibody levels induced by primary and secondary IP injections. The heightened antibody levels observed during a secondary response in mammals is due to an increase in monomeric IgG while

the levels of multimeric IgM are not considerably elevated. This situation is not likely to occur in teleost fish since the antibody responses consist of only the IgM isotype. Large increases in antibody-secreting cells during secondary responses have been observed in rainbow trout (Arkoosh and Kaattari, 1991) and channel catfish (Miller and Clem, 1984) and it has been suggested that memory responses in fish are due to increases in the number of antigen-specific B cells rather than increased antibody secretion (Arkoosh and Kaattari, 1991; Kaattari, 1994).

Shorter lag times were observed in Fig. 5.1 when peak anti-LPS antibody levels were attained more rapidly when the liposomal LPS formulations, as compared to free LPS, was included in the protocols. Shorter lag periods, however, were not observed in Fig. 5.2. This discrepancy may be due to the potential variations in the temperatures at which the immunization experiments were conducted. Initially, all *in vivo* studies were performed in the tank room facilities at the Department of Fisheries and Oceans, Canada, which received water from the city water supply (see materials and methods). However, experimental conditions were difficult to conserve over the duration of the experiments and there were instances when temperatures fluctuated to rise as high as 18°C and drop as low as 4°C. In addition, there were frequent

problems with water purity at those facilities. As a result, some experiments were never concluded because of large losses of fish. The remainder of the immunization experiments described in this chapter were performed in the aquatics room of the Animal Care Facilities at Memorial University. These facilities consisted of a circulatory water system located in a cold-room that was maintained between 8-13°C. The system had a biological filter attached that allowed for consistent levels of Ph, water hardness and ammonia. Further, this system allowed the fish to be outside of the water for less time when being bled, which would likely reduce stress. Stolen *et al.* (1984) suggested that the primary response of teleosts may be slower at lower temperatures. This may explain the differences in the times at which the peak levels of anti-LPS antibody were attained in Fig. 5.1.

The data presented in Fig. 5.3 demonstrates the relationship between the number of doses of MLV/LPS prior to subsequent exposure to free LPS and the serum anti-LPS antibody titres. Arkoosh and Kaattari (1991) confirmed the existence of immunological memory in rainbow trout albeit without many of qualitative changes in antibody or B cell function associated with the expression of memory responses in mammals. Those authors suggested that memory response in rainbow trout, in the absence of isotype switching and affinity

maturation, may be due to an expansion of the antigen-specific precursor B lymphocyte pool. It has been recently reported however, that a greater than ten-fold increase in antibody affinities were observed during secondary immune responses using TNP-KLH in rainbow trout (Kaattari, 1994). Our results tend to suggest that at least two IP injections of liposomal LPS were necessary prior to exposure to free LPS in order to prolong serum anti-LPS antibody levels. Teleost fish are known to produce only tetrameric IgM (Voss *et al.*, 1980; Lobb and Clem, 1983; Atanassov and Botev, 1988) and therefore isotype switching is not likely to occur. The existence of affinity maturation of B lymphocytes or activation of T-cell mechanisms were not examined in these studies. The antibodies generated in our immunization trials in rainbow trout appeared to be IgM (see chapter 6) and the levels of anti-LPS antibodies raised following one or multiple IP injections of LPS or liposomally-incorporated LPS were not considerably different, indicating that our results conform to the model concerning expansion of the antigen-specific precursor B lymphocyte pool as proposed by Arkoosh and Kaattari (1991).

Administration of the liposomal LPS formulations by oral intubation followed by exposure to free LPS produced a diminished serum anti-LPS antibody response to LPS when compared with IP injection. The results using

the oral administration were not surprising considering the outcome of the biodistribution studies described in chapter 4. Those studies indicated that, when compared to IP injection, oral intubation resulted in a lower overall uptake of liposomal LPS by the tissues and organs examined from rainbow trout, particularly by the kidney and spleen. There were disadvantages to the oral intubation method, such as stress from extended handling and loss of administered material by regurgitation, which may have been important factors in these results. It would appear to be more practical to administer formulations orally by incorporation into feed.

The lipid A moiety of LPS, is structurally, the most conserved component of LPS. Lipid A is composed of a glucosamine disaccharide with attached phosphate(s) and fatty acids and only the number of fatty acids and the sites of attachment are variable (Brade *et al.*, 1986). Studies in mammals have produced evidence of a conserved, possibly immunodominant, epitope among lipid A moieties from many bacterial species (Galanos *et al.*, 1985; Su *et al.*, 1990). The lipid A segment of the LPS molecule, therefore, is considered an ideal target for the generation of antibodies that might cross-react with LPS from different bacterial species (Rietschel *et al.*, 1994). In fact, monoclonal antibodies have been produced that cross-react with a large variety of free lipid

A of distinct bacterial origin (Kuhn *et al.*, 1992). We observed that there were substantial levels of anti-lipid A antibodies produced by the immunizations with liposomal LPS in rainbow trout. These anti-lipid A responses were comparable in magnitude to the anti-LPS responses although they declined more rapidly than the anti-LPS antibody levels in one of the two experimental groups. Although other researchers have examined the ability of liposomal LPS formulations to enhance immune responses against LPS (Desiderio and Campbell, 1985; Petrov *et al.*, 1992; Wong *et al.*, 1992), anti-lipid A antibody responses were not monitored along with the anti-LPS antibody responses. It has been demonstrated in rabbits that substantial levels of anti-lipid A activity (reported as radioimmunoassay units, RIA) could be generated by injections of bacterial cells coated with lipid A or liposomal lipid A (Alving *et al.*, 1980; Alving 1993). The generation of anti-lipid A antibodies, potentially directed against common antigenic determinants inherent to several LPS molecules, suggests that liposomal LPS formulations may be valuable for use as vaccines against a variety of bacterial infections.

The standard deviations associated with the mean anti-LPS antibody titres were large and this may be attributable, at least in part, to the variation from individual to individual. It is likely that individuals within a population will

display dissimilar abilities to produce immune responses to a given antigen. Blood samples were obtained from rainbow trout that were randomly chosen (N = 3-8, from test groups of approximately 25) at the time points indicated, and in some instances, one or more of the serum samples displayed relatively low anti-LPS antibody titres. Attempts were made to tag the trout but this appeared to induce high stress, sometimes even causing death, so this process was not continued. Instead, a random sample was selected at each time point from the population of fish immunized. The experimental protocol for the ELISA consisted of serial double dilutions of the test sera ranging from 40-fold to 40,960-fold. The serial dilution procedure contributes some of the variability because the titre chosen was the last dilution step with a readable value on the ELISA microtitre plate. Using the ELISA, antibody titres were determined by discerning absorbance values of greater than 0.02 above the optical density of the controls as the end-point for positive reactions. This cutoff was 30-40% above the background level. In addition, the profiles of the anti-LPS antibody responses as a function of time were similar when absorbance values of greater than 0.05 (approximately 50-75%) above the background controls were chosen as the end-point for positive reactions, although the titres were lower. Approximately 5% of the serum samples did not react in the ELISA, suggesting that some fish did not develop anti-LPS antibody responses. The non-

responding fish could have been a result of the immunogens being delivered to areas other than the i.p. cavity or simply due to individual fish that were not capable of generating an immune response or had developed tolerance. If the i.p. cavity was bypassed, the most likely destination of the injected dose would be the intestine. Stress may have also been a factor in the large standard deviations as the differences in stress levels among individuals may have resulted in a variability in the humoral immune responses. Despite the variability, it appears that the antibody responses exhibited by trout administered IP injections of liposomal LPS were prolonged compared to those which were administered LPS in the free form.

The current paradigm in mammalian immunology is that liposomally-encapsulated antigens demonstrate enhanced antigen presentation and uptake by macrophages compared to free antigens, resulting in the generation of T-cell mediated responses (Alving, 1992; Collins *et al.*, 1992). Harding *et al.* (1991) demonstrated that liposomally-encapsulated antigens are processed in lysosomes, recycled and presented to T cells. Antigen presentation events in fish are believed to be similar to those in mammals, although they have not been as extensively studied (Vallejo *et al.*, 1992). The possibility that liposomally-incorporated LPS may enhance antigen presentation and recruitment

of macrophages in rainbow trout is worthy of further investigation. *In vitro* assays for T cell proliferation are necessary to evaluate T-cell responses induced by liposomally-incorporated LPS in rainbow trout. Challenge experiments are also required to determine the level of protection induced in rainbow trout primed IP with liposomal LPS.

It has been demonstrated in mammals that liposomes serve as efficient delivery vehicles for a variety of antigens (for a review see Poznansky and Juliano, 1984) and as immunological adjuvants (Desiderio and Campbell, 1985; Gregoriadis, 1990; Petrov *et al.*, 1992; Wong *et al.*, 1992; Laing and Threakston, 1993). The data presented here suggest that liposomally-incorporated LPS can also be successfully administered to fish while safely functioning as delivery vehicles and as immunological adjuvants.

CHAPTER 6

PARTIAL CHARACTERIZATION OF ANTIBODIES GENERATED BY IMMUNIZATIONS WITH FREE AND LIPOSOMAL LPS FROM *Aeromonas salmonicida.*

INTRODUCTION

In the previous chapter, it was demonstrated that serum anti-LPS antibody responses in rainbow trout *(Oncorhynchus mykiss)* were prolonged when liposomal LPS from *A. salmonicida* was included in immunization programmes. The serum anti-LPS antibody titres were evaluated by an ELISA, in which a mouse anti-trout IgM monoclonal antibody (mAb I-14) was used as a secondary antibody. Initially, we did not have access to the hybridoma secreting this antibody, and because a secondary antibody of this nature was not commercially available, we intended to purify trout IgM in order to raise anti-trout IgM antibodies in rabbit, which would subsequently be used as a secondary antibody in the ELISA. We applied a technique previously described for the isolation of immunoglobulin (Ig) from human and murine sera to rainbow trout immune sera. The protocol was designed in conjunction with Dr. Alexander Szalai, and the method was used to isolate and subsequently characterize the antibodies from trout serum raised against free LPS from *A. salmonicida* or LPS incorporated in negatively- or positively-charged multilamellar liposomes.

The primary serum Ig in teleost fishes is a tetrameric, IgM-like molecule with a mass of approximately 700 kDa (Voss *et al.*, 1980; Lobb and Clem,

1983; Atanassov and Botev, 1988). Despite several methods available for the purification of Ig from mammalian sera or cell culture supernatants (Campbell *et al.*, 1951; Dissanayake and Hay, 1975; Ey *et al.*, 1978; Brooks *et al.*, 1992), most of the methods used to isolate large amounts of purified IgM from fish sera are tedious (Sanchez *et al.*, 1991). Purification methods utilizing immunoadsorbent Sepharose beads have been successful for isolation of small amounts of highly purified serum antibodies or IgM from fish (Lobb and Clem, 1983; Sanchez *et al.*, 1989; Sanchez *et al.*, 1991; Sanchez and Dominguez, 1991). This procedure, however, may afford only a portion of the total population of antibodies and IgM present in fish sera. In addition, mAb generated against fish IgM may recognize only a portion of the total IgM (Sanchez *et al.*, 1989; Sanchez and Dominguez, 1991). Another technique for IgM isolation from fish sera involves preparing γ-globulin enriched fractions by precipitation with saturated ammonium sulphate, followed by anion exchange chromatography and gel filtration to isolate a high-molecular mass fraction enriched in IgM (Sanchez *et al.*, 1991). A drawback of this technique is that material isolated in this manner from fish sera contains many proteins in addition to IgM.

In this chapter, a procedure to isolate trout IgM from serum is described

which relies on the adherence of IgM (in the presence of a large excess of protein contaminants) to a mixed-mode ion-exchange chromatographic matrix (ABx resin) originally employed for the isolation of mammalian Ig. ABx chromatography presents an economical alternative to immunoaffinity chromatography for the rapid purification of substantial amounts of rainbow trout IgM. With minor modification, this protocol likely can be adapted to the isolation of Ig from any fish species.

MATERIALS AND METHODS

Materials.

The bicinchoninic acid (BCA) Protein Assay Kit was from Pierce Chemical Co., Rockford, IL. Desalting columns (PD-10, Sephadex G-25M, 1.5 x 8 cm), gel filtration resin (Sephacryl S200-HR) and molecular weight standards for SDS-PAGE were obtained from Pharmacia LKB Biotechnology, Uppsala, Sweden. Electro-blotting equipment (Trans-Blot Cell) was purchased from Bio-Rad Laboratories (Richmond, CA). Ultrafiltration devices (Centriprep-10) were from Amicon (Danvers, MA). Nitrocellulose was from Schleicher & Schuell, Inc. (Keene, NH). Bakerbond ABx resin was from J.T.

Baker Canada (Toronto, ON, Canada). Ammoniacal silver stain used for staining gels was from Mandel Scientific Company Ltd. (Guelph, ON, Canada). All other chemicals were of reagent grade or better and were purchased from Sigma Chemical Co. (St. Louis, MO).

Collection of Immune Sera.

IgM was isolated from sera which had been generated via immunizations of rainbow trout *(Oncorhynchus mykiss)* with free or liposomal LPS from *A. salmonicida* (see chapter 5, appendix IV). Sera from groups of immunized trout were pooled and clarified by high speed centrifugation (10,000 x g_{max}, 5 min). Total protein concentration for duplicate samples of pooled serum were estimated using a BCA based protein assay (see appendix IV).

Precipitation of Trout Serum Proteins with Ammonium Sulphate.

Serum proteins were concentrated by precipitation with ammonium sulphate according to the method described by Bolag and Edelstein (1991) (appendix IV). Pooled, clarified sera (2 mL) from rainbow trout immunized with LPS, +MLV/LPS or -MLV/LPS (having protein concentrations of 31.5, 21.3 and 33.5 mg/mL, respectively) were added drop-wise while stirring to an

equal volume of saturated ammonium sulphate and the resulting solutions were stirred slowly on ice for 1 h. After standing overnight at 4°C, the solutions were centrifuged at 48,200 x g_{max} (20,000 rpm in a Sorvall SS-34 rotor) for 30 min at 4°C. The resulting supernatants were aspirated and the pellets of precipitated proteins were resuspended in Tris buffered saline (TBS: 10 mM Tris-HCl, 140 mM NaCl, pH 7.4) followed by dialysis overnight against 4 changes of TBS (3.0 L per change). Residual ammonium sulphate was removed from the protein concentrates by passage through PD-10 desalting columns (Sephadex G-25M, 1.5 x 8 cm) that had previously been equilibrated with TBS. Fractions of effluent containing serum proteins were pooled (25 mL) and concentrated by ultrafiltration using Centriprep-10 device by way of repeated centrifugation at 5000 x g_{max} for 30 min at 25°C in a Sorval SS-34 rotor until the final volume of the pooled fractions was 3.5 mL (Appendix IV). Final protein concentrations of the concentrated serum samples to be gel filtered were 2.13, 3.74 and 2.96 mg/mL protein for sera raised against LPS, +MLV/LPS, and -MLV/LPS, respectively.

Gel-filtration Chromatography of Concentrated Trout Serum Proteins.

Gel-filtration of concentrated serum protein solutions was performed at room temperature using a column (2.6 x 65 cm, bed volume = 345 mL) of

Sephacryl S200-HR equilibrated with TBS (appendix IV). The column was calibrated with proteins of known molecular masses: bovine serum albumin (BSA), 66 kDa; alcohol dehydrogenase, 150 kDa; and β-amylase, 200 1kDa. Fractions (2.5 mL) of effluent were collected (flow rate = 100 drops/5 min/fraction) and were monitored for the presence of protein based on absorbance at 280 nm using a Beckman DU-50 spectrophotometer. The major protein peaks were identified and fractions likely containing trout IgM (> 200 kDa) were pooled and concentrated by ultrafiltration using Centriprep-10 devices to a final volume of 2 mL. The concentrated materials contained protein concentrations of 3.45, 1.84, and 3.80 mg/mL and were subjected to further chromatography on a column of Bakerbond ABx antibody exchanging resin.

Isolation of Trout Serum IgM on Bakerbond ABx.

A column (1.5 x 12 cm) of Bakerbond ABx antibody exchanging resin was packed (bed volume = 21.2 mL) and was washed with 100 mL of distilled H_2O (appendix IV). The column was then washed with 100 mL of 2M sodium acetate followed by washing with 100 mL of distilled H_2O and 100 ml 1% acetic acid. Finally the column was equilibrated with starting buffer (10 mM

2[N-morpholino]-ethanesulfonic acid, or MES, at pH 5.6). Gel-filtered, IgM-enriched concentrates in 100 mM MES, pH 5.6 were applied to the equilibrated ABx column and 2.0 mL fractions (flow rate = 70 drops/1.38 min/fraction) were collected until the absorbance of the effluent measured at 280 nm was < 0.02. Proteins adherent to the column were eluted by subjecting the column to a linear gradient formed by mixing starting buffer with limiting buffer (10 mM sodium acetate, 200 mM ammonium sulphate, pH 5.6). Fractions from the void volumes and fractions collected from ABx exposed to 0-100% v/v of limiting buffer were pooled and concentrated by ultrafiltration to a final volume of 2.0 mL. Final protein concentrations of the pooled, ABx-adherent materials were 1.825, 1.52, and 2.10 mg/mL protein for sera raised against LPS, +MLV/LPS, and -MLV/LPS, respectively.

Polyacrylamide Gel Electrophoresis (PAGE).

Aliquots (equivalent to 35 μg protein according to the BCA assay) of the pooled, ABx-adherent material or of the non-bound fractions were boiled (5 min) in the presence of 2% (w/v) sodium dodecyl sulfate (SDS) and 5% (v/v) 2-mercaptoethanol (2-ME) to achieve denaturation and reduction (appendix I). Alternatively, samples were boiled in the absence of 2-ME to observe the electrophoretic mobility of ABx-adherent material under non-reducing

conditions. In addition, 25 μL aliquots of the pooled, void-volume fractions were subjected to the reducing and non-reducing conditions described above. SDS-PAGE was performed according to the procedures of Laemmli (1970) in vertical slab gels containing 5 or 12.5% (w/v) polyacrylamide and 0.1% SDS. Polyacrylamide contained 0.8% w/w *bis*-acrylamide as a cross-linking agent. Bands of protein in gels were visualized by staining with ammoniacal silver. Proteins of known molecular mass used as markers for PAGE were: α-lactoglobulin, 14.4 kDa; soybean trypsin inhibitor, 20.1 kDa; carbonic anhydrase 30 kDa; ovalbumin, 43 kDa; bovine serum albumin, 67 kDa; and phosphorylase b, 94 kDa.

Western Blots.

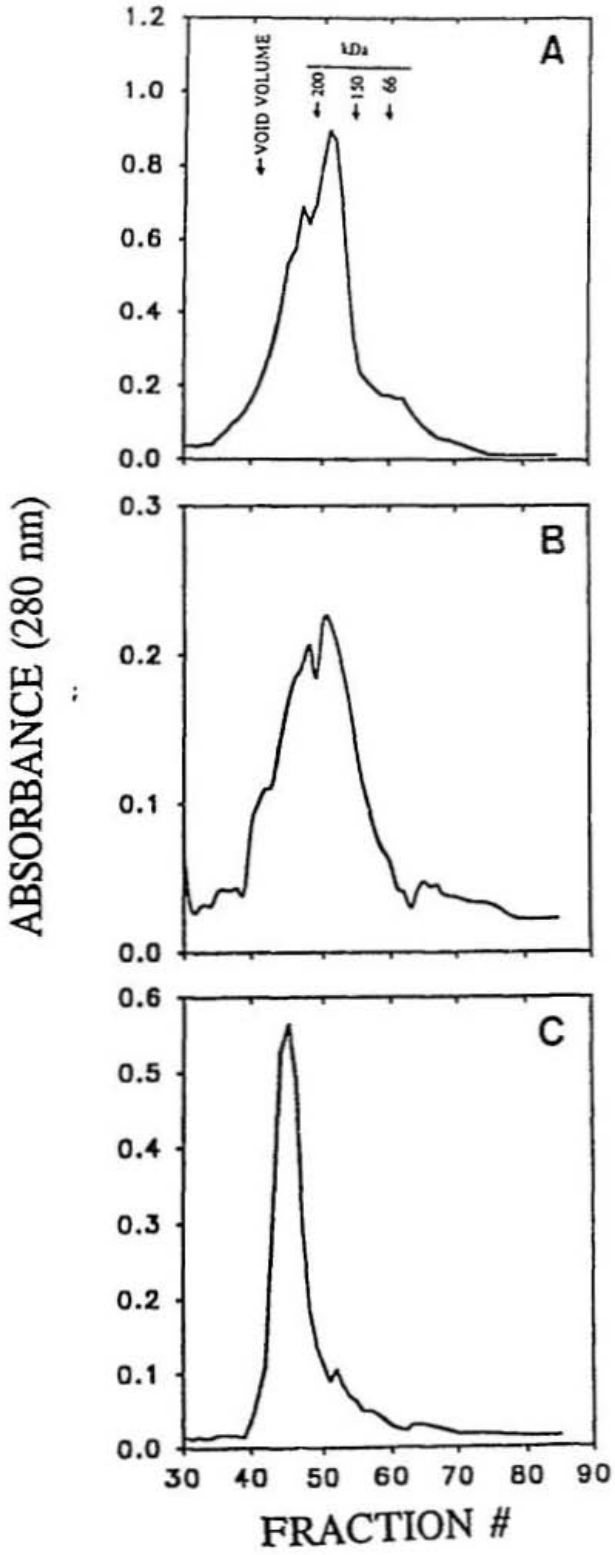
Western blots were done according to the procedure described by Towbin *et al.* (1979) (appendix IV). Samples containing non-reduced or 2-ME-reduced protein were separated on SDS-PAGE and subsequently blotted by electro-transfer (200 mA, 4 h, 4°C) onto nitrocellulose sheets (see appendix IV). Residual proteins remaining in the gels after electro-elution were visualized by staining with ammoniacal silver. Bands of protein transferred to nitrocellulose were visualized by staining with Ponceau S to determine their

positions relative to marker proteins. Ponceau S was removed by repeated washing with distilled H_2O and non-specific binding sites were blocked by immersion in a 3% solution (w/v in TBS containing 0.05% Tween 20 [TBST]) of gelatin for 1 h at room temperature. Membranes were washed with TBST (3 x 2 min) prior to incubation in diluent (1.5% gelatin-TBST) containing 3% (v/v) solutions of culture supernatant for 1.5 h at room temperature. Culture supernatants were from hybridomas secreting monoclonal antibodies (mAb 1.14 is a IgG_1, κ-class antibody, see materials and methods in chapter 2 and appendix I) reactive to the heavy chain (H-chain) of rainbow trout IgM (DeLuca *et al.*, 1983; Sanchez *et al.*, 1991). Membranes were washed with TBST (3 x 2 min) and reacted (1 h at room temperature) with a 1/2000 (v/v) solution of goat anti-mouse IgG (whole molecule) alkaline phosphatase conjugate in diluent. Finally the membranes were washed again and incubated in substrate buffer A (33 μg/mL nitro-blue-tetrazolium, NBT, plus 16.5 μg/mL 5-bromo-4-chloro-3-indonyl phosphate, BCIP, dissolved in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). After sufficient colour development, the reaction was terminated by removal of excess substrate via repeated rinsing with distilled water. The proteins constituting the molecular weight markers were not immunoreactive.

RESULTS

The pooled, clarified sera from rainbow trout immunized with LPS, positively-charged MLV containing LPS (+MLV/LPS), and negatively-charged MLV containing LPS (-MLV/LPS) contained 62.2, 42.6 and 67.0 mg protein, respectively, as determined by BCA protein assay. After ammonium sulphate precipitation and desalting, 10.4, 7.5, and 13.1 mg of the protein was recovered for sera raised against LPS, +MLV/LPS, and -MLV/LPS, respectively. These values represented 16.6%, 17.6%, and 19.6%, respectively, of the protein originally subjected to ammonium sulphate precipitation. The concentrated protein samples were then subjected to gel filtration on columns of Sephacryl S200HR from which they eluted either as one major peak (Fig. 6.1, c) or displayed asymmetrical elution profiles which may be indicative of two or more major components (Fig. 6.1, a and b). In instances of asymmetrical elution profiles, fractions from the first peak corresponded to proteins with molecular masses of greater than 200 kDa (Fig. 6. 1. a and b), and were pooled and concentrated to a final volume of 2 mL prior to ABx chromatography. BCA assay indicated that the gel filtered material contained 6.9, 3.7, and 7.6 mg of protein for sera raised against LPS, +MLV/LPS, and -MLV/LPS, respectively. These values represented 11.1%,

FIGURE 6.1. Gel filtration (Sephacryl S200-HR) profile for ammonium sulphate precipitated serum proteins from rainbow trout immunized with a) LPS, b) +MLV/LPS, and c) -MLV/LPS. Void volume and elution volumes for marker proteins with known molecular mass (kDa) are indicated.


A
B
C
VOID VOLUME
kDa
200
150
66
ABSORBANCE (280 nm)
FRACTION #
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.3
0.2
0.1
0.0
0.6
0.5
0.4
0.3
0.2
0.1
0.0
30
40
50
60
70
80
90

8.6%, and 11.3%, respectively, of the protein originally subjected to ammonium sulphate precipitation.

IgM-enriched trout serum was separated into 2 major components by chromatography on ABx antibody exchanging resin (Fig. 6.2). Non-bound fractions (Fig. 6. 2, a, b, c; first peak) and ABx-adherent fractions (Fig. 6. 2, a, b, c; second peak) were pooled and concentrated to a final volume of 2.0 mL. Under the conditions employed, material that had adhered to the ABx was eluted only upon exposure to 50 mM ammonium sulphate (Fig. 6. 2, a, b, c; second peak). ABx-adherent material, presumably trout IgM, was assayed and found to contain 3.6, 3.0, and 4.2 mg protein for sera raised against LPS, +MLV/LPS, and -MLV/LPS, respectively. These values represented 5.9%, 7.1%, and 6.3%, respectively, of the protein in the original serum samples.

When subjected to 12% SDS-PAGE under reducing conditions, ABx-adherent trout Ig (equivalent to 35 μg protein) isolated from the various sera displayed virtually identical banding patterns (Fig. 6.3, a, lanes 4-6). Trout Ig was routinely resolved into major bands which could be attributed, in order of electrophoretic mobility to; the heavy chain (H) of trout IgM corresponding to a molecular mass of approximately 72 kDa and three light chain (L) variants corresponding to molecular masses of approximately 31 kDa, 24 kDa, and 20 kDa. The H band gave a broad appearance which indicated that it may actually

FIGURE 6. 2. Elution profile for gel-filtered, IgM-enriched trout sera raised against a) LPS, b) +MLV/LPS, and c) -MLV/LPS applied to ABx antibody exchanging resin. The samples were applied and the column (1 x 20 cm) was washed extensively with starting buffer (10 mM MES, pH 5.6) to remove any unbound material (first peak). Trout serum IgM was subsequently eluted (second peak) by exposure to a linear gradient (dotted line) of limiting buffer (200 mM ammonium sulphate, 10 mM sodium acetate, pH 5.6). Protein contents for each fraction were monitored using absorbance at 280 nm.

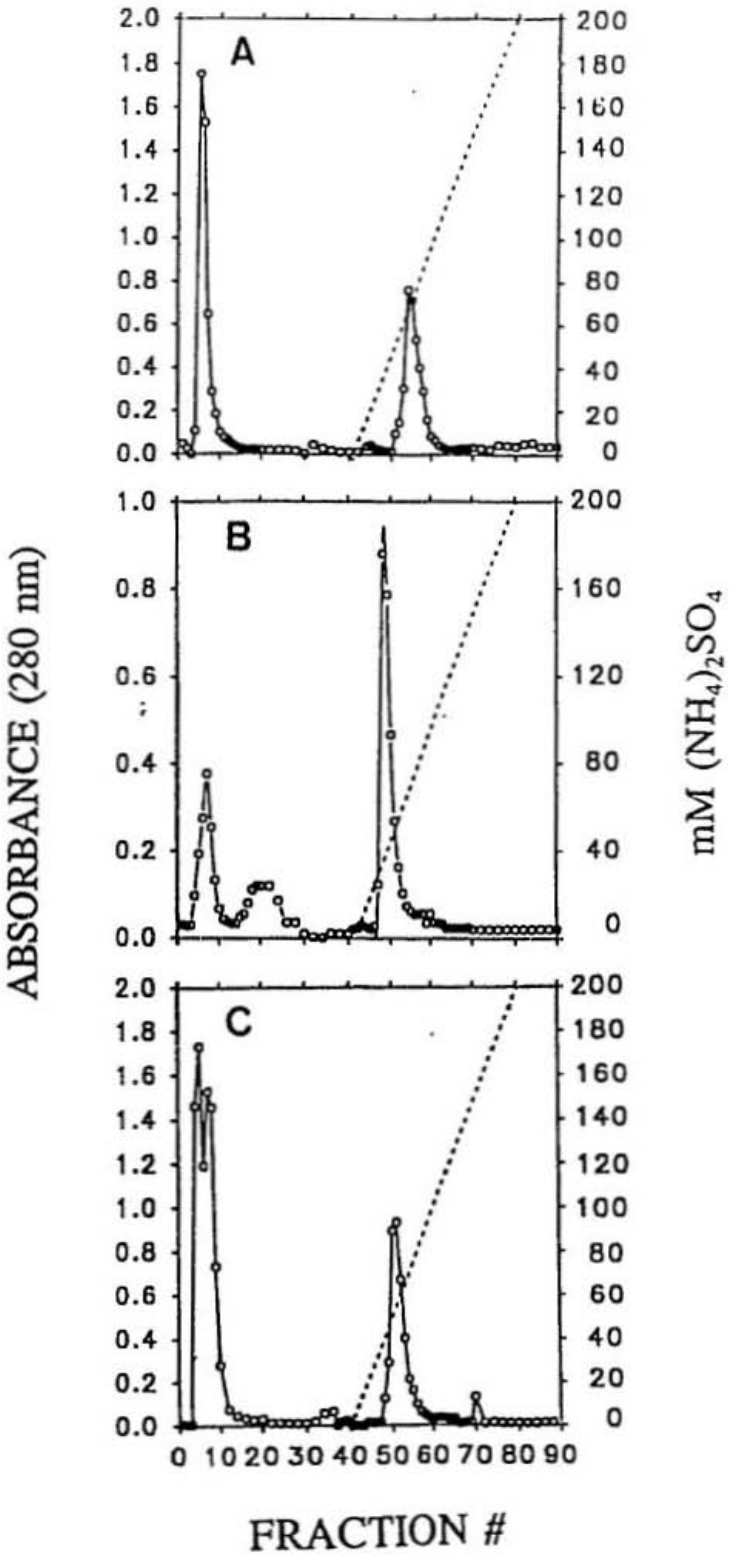
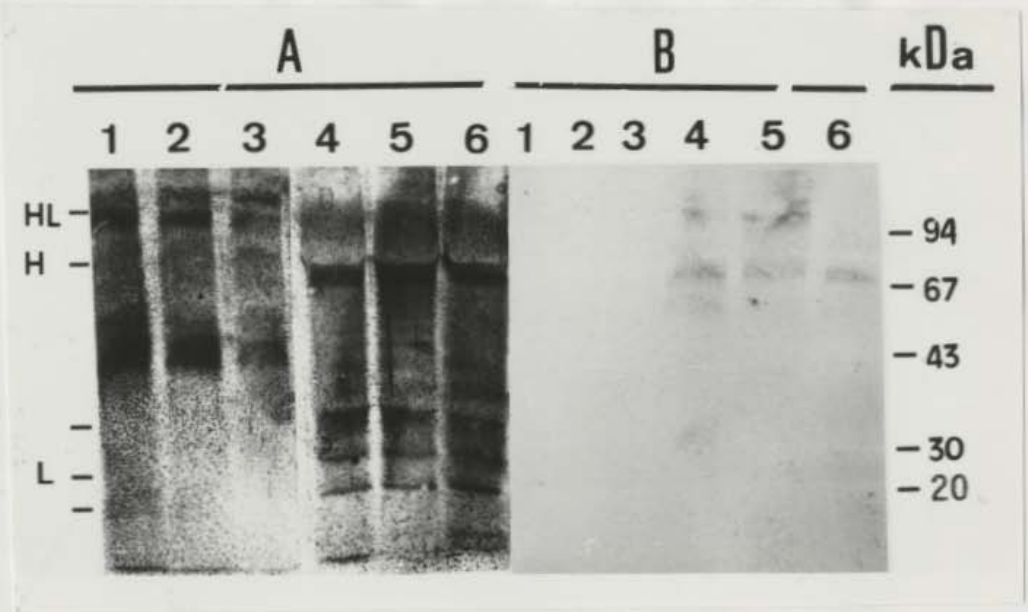

A
B
C
ABSORBANCE (280 nm)
mM $(NH_4)_2SO_4$
FRACTION #
0 10 20 30 40 50 60 70 80 90

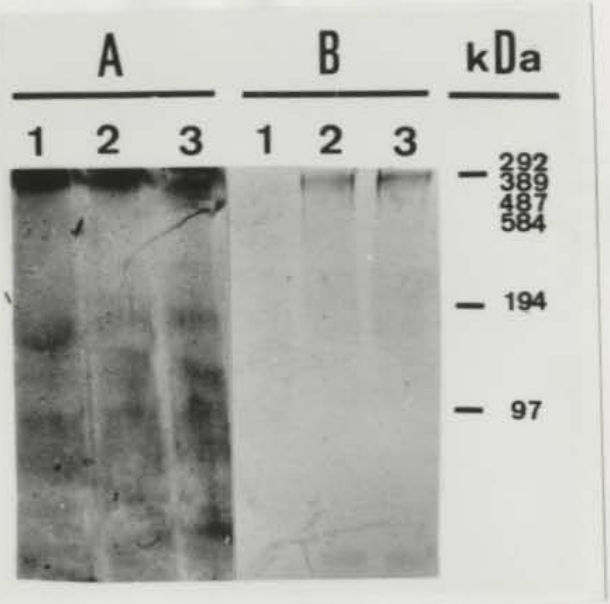
FIGURE 6. 3. The non-bound (lanes 1-3) and ABx-retained (lanes 4-6) fractions from concentrated, gel-filtered sera generated by immunizations with LPS, +MLV/LPS, and -MLV/LPS, respectively, following reducing 12% SDS-PAGE (A) and corresponding Western immunoblot (B). Aliquots of 35 μg of protein was applied to the gels which were subsequently blotted by electro-transfer onto nitrocellulose sheets. Bands corresponding to trout IgM heavy-chains (H) and light-chains (L) and the approximate positions of molecular mass markers (kDa) are indicated. "HL" indicate putative H-L dimers.


A
B
kDa
1 2 3 4 5 6 1 2 3 4 5 6
HL
H
L
94
67
43
30
20

be a closely spaced doublet. There also appeared to be a broad band corresponding to a molecular mass of 96 kDa which could be attributed to combination of H and L chains (HL). The non-retained fractions from the ABx chromatography of the concentrated, gel-filtered sera exhibited at least three distinct bands (Fig. 6.3, a, lanes 1-3) although they were not assigned. The corresponding Western immunoblot demonstrated that both the putative H and HL bands were reactive with mAb 1.14 whereas the L bands were not immunoreactive with mAb 1.14 (Fig. 6.3, b, lanes 4- 6). None of the bands observed on the SDS-PAGE analysis of the non-retained fractions from the ABx chromatography of the concentrated, gel-filtered sera were immunoreactive with mAb 1.14 (Fig. 6.3, b, lanes 1-3).

Under non-reducing conditions on 5% SDS-PAGE, the banding patterns of the ABx-adherent trout IgM isolated from the various immune sera indicated that the majority of the material was unable to migrate from the wells (Fig. 6.4, a, lanes 1-3). There was also the presence of weak bands at approximately 97 and 194 kDa, corresponding to H-L chain combinations and L-H-H-L subunits, respectively (Fig. 6.4, a, lanes 1-3). This observation suggests that the material which was unable to migrate from the wells and was not seen on SDS-PAGE under reducing conditions, may correspond to the intact tetrameric IgM molecule. These gels indicated that optimal resolution of bands HL, H and L

Figure 6. 4. The ABx-retained fractions from concentrated, gel-filtered sera generated by immunizations with 1) LPS, 2) +MLV/LPS, and 3) -MLV/LPS, following non-reducing 5% SDS-PAGE (A) and corresponding Western immunoblot (B). The equivalent of 35 μg of protein was applied to the gels which were subsequently blotted by electro-transfer onto nitrocellulose sheets. The approximate positions of molecular mass markers (kDa) are indicated.


A
B
kDa
1 2 3 1 2 3
292
389
487
584
194
97

on SDS-PAGE could only be achieved after reduction of IgM with 5% 2-ME. The corresponding Western immunoblot (Fig. 6.4, b, lanes 1-3) indicated that only the materials in the wells were immuno-reactive with mAb 1.14.

DISCUSSION

Tetrameric IgM from salmonid fishes has been well characterized (Cisar and Fryer, 1974) and its subunit composition has been established (Kobayashi *et al.*, 1982). In this chapter, we describe a protocol utilizing ABx chromatography, which provides the rapid isolation of substantial quantities of purified IgM from rainbow trout serum. This method is a modification to the affinity chromatography (Lobb and Clem, 1983; Sanchez and Dominguez, 1991; Sanchez *et al.*, 1991) or conventional chromatographic procedures (Kobayashi *et al.*, 1982; Sanchez *et al.*, 1989) which have previously been applied to the isolation of the tetrameric IgM molecule from fishes.

Our method for isolation of IgM from rainbow trout serum consisted of precipitation of serum proteins with ammonium sulphate, followed by gel filtration and ABx chromatography. As expected there was a considerable decrease in protein concentration resulting from each of these enrichment steps.

Based on recovery of ABx-adherent protein, presumably IgM, we estimate that the serum that we analyzed from rainbow trout that had been immunized with free or liposomally-incorporated LPS from *A. salmonicida*, contained between 1.52 - 2.10 mg IgM per mL. This accounts for 5.87-7.14% of the total protein in pooled rainbow trout serum, well within the ranges estimated for other salmonids (Voss *et al.*, 1980; Kobayashi *et al.*, 1982; Sanchez *et al.*,1989; Sanchez *et al.*, 1991).

The molecular masses, the behaviours in reducing and non-reducing SDS-PAGE, and reactivities with anti-trout IgM heavy-chain mAb 1.14 indicate that all of the major bands observed on SDS-PAGE for the ABx-adherent fraction from trout serum can be attributed to IgM or its constituent polypeptide chains. The band with a molecular mass of approximately 72 kDa is consistent with the heavy-chain (H) of salmonid IgM originally characterized by Cisar and Fryer (1974). The reactivity of the 72 kDa band with mAb 1.14 strongly suggests that this band represents the heavy (H) chain of salmonid IgM, since mAb 1.14 is specific for the H chain of rainbow trout IgM (DeLuca *et al.*, 1983). The 72 kDa band observed in SDS-PAGE under reducing conditions is broad and may actually be a closely spaced doublet, which is consistent with the two types of heavy-chains known to exist in salmonid fish (Kobayashi *et*

al., 1982; Sanchez *et al.*, 1989; Sanchez *et al.*, 1991). It has been reported that rainbow trout contain two light-chain molecular mass variants of 26 kDa and 24 kDa (Sanchez *et al.*, 1989), although we observed three bands in that molecular mass range on reducing SDS-PAGE, corresponding to masses of approximately 31 kDa, 24 kDa, and 20 kDa. These bands were not reactive with mAB 1.14, suggesting that they may represent the rainbow trout IgM light (L) chains. Three light chain molecular mass variants (26kDa, 24kDa, 22kDa) have been found in other fish species, including catfish (Lobb *et al.*, 1984; Lobb, 1986). A band with a molecular mass of approximately 96 kDa on reducing SDS-PAGE, most likely represents the existence of intact H-L chain combinations. H-L chain combinations are not unusual as disulfide interchange is a behaviour characteristic of many types of fish IgM (Cisar and Fryer, 1974; Kobayashi *et al.*, 1982; Lobb and Clem, 1983). The reactivity of the H-L chain combination on Western blots is not surprising although it has been suggested that reactivity to mAb may be affected by the structure (ie. denatured vs. native, reduced vs. non-reduced) of the trout IgM being analyzed (DeLuca *et al.*, 1983). As expected, the individual heavy and light chains were not detectable by silver staining on non-reducing SDS-PAGE and the major band, unable to migrate from the wells in a 5% gel, was the only band

immunoreactive with mAb 1.14. There were faint bands in the molecular mass ranges expected for a HL chain combination (97 kDa) and a monomeric subunit (194 kDa) (Cisar and Fryer, 1974) which were not immunoreactive.

The results discussed in this chapter demonstrate that ABx chromatography appears to be a practical procedure for the purification of rainbow trout Ig from serum. It was observed that immunizations with LPS, +MLV/LPS or -MLV/LPS resulted in the generation of the same Ig isotype, putatively tetrameric IgM, and that the serum concentrations of IgM generated were comparable with either immunogen. We believe that this protocol may be adapted for the isolation of total IgM from any fish species for whom mAbs reactive with Ig are not yet available.

CHAPTER 7

SUMMARY AND CONCLUSIONS

The vertebrate immune system must confront a variety of pathogens, viral, bacterial and parasitic, that present a wide array of pathogenic mechanisms and immune evasion techniques. Vaccination represents the most effective approach to control the majority of infectious diseases. Protective immunity induced by vaccination is dependent on the capacity of the vaccine to elicit the appropriate immune response to either resist, control or eliminate the pathogen. Depending on the pathogen, host defense may require a cell-mediated or humoral immune response. For instance, it has been reported that mice vaccinated with killed *Salmonella* bacterins or with soluble *Salmonella* antigens were not sufficiently protected from a lethal challenge inoculum despite the presence of a substantial amount of specific antibody (Collins and Mackaness, 1968; Collins, 1969) and that cell-mediated immunity in host defences against facultative intracellular bacteria is considered to be of utmost importance (Collins and Campbell, 1982; Desiderio and Campbell, 1985). On the other hand, other researchers have observed that a significant degree of protection against *Salmonella* in mice was provided by humoral mechanisms (Ornellas *et al.*, 1970; Angerman and Edelstein, 1980).

For fishes, it has been established that both humoral (Avtalion, 1969; Tatner, 1986) and cell-mediated immune mechanisms (Tatner, 1990; Arkoosh

and Kaattari, 1991) are critical for the generation of protection against infectious agents. Many approaches have been explored for development of vaccines against various bacterial pathogens. Immunization protocols have included killed bacterins, whole-cell preparations adjuvenated with various immunostimulants, or soluble bacterial antigens that have been administered to fishes via different routes of administration in attempts to induce protection against several pathogens. These strategies have produced some encouraging results. For instance, Velji *et al.* (1990) reported that protection could be induced in juvenile coho salmon (*Oncorhynchus kisutch*) by immunization with either extracellular or cell wall LPS from *Vibrio ordali* or live *Vibrio ordali* cells via both the IP and immersion routes. Another study demonstrated that protection against *Aeromonas salmonicida* could be induced in coho salmon when they were immunized with killed bacterin adjuvenated with polysaccharide immunostimulants via one IP injection (Niki *et al.*, 1991). Interestingly, Oliver *et al.* (1985) found that immunity to *A. salmonicida* could be reinforced by one IP injection of modified complete Freund's adjuvant alone, and suggested that the component of the adjuvant that was responsible for this non-specific immunity was *Mycobacterium butyricum*. Coating the extracellular products of *A. salmonicida* onto polystyrene beads was

demonstrated to induce a measure of protective immunity in salmon when the material was used in a bath vaccine. The material was found to be a less efficient antigen when injected into rainbow trout as compared to the bath immersion (Adams *et al.*, 1988). Wong *et al.* (1992) developed a vaccine formulation composed of whole cells of *Vibrio anguillarum* coated onto dextrose beads, followed by a coating with a copolymer to protect against gastric degradation (enteric coated vaccine). When the enteric coated vaccines were administered orally to coho salmon, serum and mucus antibody levels were enhanced compared to the corresponding positive controls, which consisted of whole cells of *Vibrio anguillarum*. The enteric coated vaccines however, were no more efficient at inducing protective immunity than the positive controls. The studies described above have demonstrated some of the many strategies that have been examined for the development of vaccines against bacterial diseases of fish. Despite some encouraging results concerning immunity against *A. salmonicida,* and the existence of commercially available anti-furunculosis vaccines, outbreaks of infections continue to occur, resulting in large losses of cultured salmonids (Ellis, 1988).

The objectives of the research presented in this thesis concerned the potential of liposomes to function as a delivery vehicle and as an immunological

adjuvant for LPS from *A. salmonicida* in rainbow trout (*Oncorhynchus mykiss).* LPS is a prominent factor in determining virulence of Gram-negative bacteria (Luderitz *et al.*, 1966 a,b) and is thought to be essential to bacterial cell viability. The important role and exposed position of LPS represents an ideal target on disease-producing bacteria for antibodies and suggests that LPS may be a reasonable choice as a major component for the development of a formulation to enhance immunity against *A. salmonicida.* It has been demonstrated that LPS is capable of inducing the production of protective antibodies in rats against *Neisseria meningitidis* (Saukkonen *et al.*, 1988). In mice, monoclonal antibodies against the polysaccharide moiety of the LPS from *Brucella abortus* (Montaraz *et al.*, 1986) and *Salmonella typhimurium* (Colwell *et al.*, 1984) have been shown to be capable of conferring protection. However, other studies in mammals have suggested that bacterial multiplication is controlled by the presence of activated macrophages generated as a result of T-cell mediated response (Hahn and Kaufman, 1981). In fish, antibody production may not necessarily generate protection, and vaccination trials have indicated a poor correlation between the levels of specific antibodies against *A. salmonicida* and the degree of protection (Olivier *et al.*, 1985; Hastings and Ellis, 1988). Both the humoral (Ellis *et al.*, 1988) and cellular (Olivier *et al.*,

1986) immune responses have been implicated in the immunity against *A. salmonicida* in fish. *A. salmonicida* is regarded as an efficient pathogen because it can modulate host immune mechanisms (Kennedy-Stoskopf, 1993) and it has been suggested that this microorganism may be immunosuppressive (Evenberg *et al.*, 1986; Porreau *et al.*, 1986), and that certain cell surface epitopes may stimulate putative T suppressor cells (Tatner, 1990). LPS from *A. salmonicida,* as a component of the whole-cell antigen, was demonstrated to be unable to stimulate lymphocyte proliferation in the head kidney in Atlantic salmon (Tatner, 1990). However, it was shown in the same report that LPS from *A. salmonicida* can induce lymphocyte proliferation when coated onto polystyrene beads. These results tend to suggest that by modifying LPS, it may be possible to alter its immunogenicity.

Liposomal LPS formulations have been demonstrated to enhance humoral immune responses against LPS in mammals (Petrov *et al.*, 1992; Wong *et al.*, 1992; Threakston and Laing, 1993). In addition, it has been suggested that liposomal LPS formulations may induce the generation of cell-mediated immunity in mice (Desiderio and Campbell, 1985). Liposome-based formulations, however, have been infrequently used in fishes (Power *et al.*, 1990; Rodgers, 1990). During the term of this research project, we have

investigated a number of aspects concerning the development of liposomal LPS formulations which were considered to be important.

An early objective was to investigate the structure of the LPS from *A. salmonicida* using ^{1}H-NMR and GC-EIMS. Using those spectroscopic techniques, we found that the structure of the LPS from our aqueous-phenol extractions was consistent with structures that have previously been proposed for the O-antigen (Shaw *et al.*, 1983) and core-oligosaccharide (Shaw *et al.*, 1992) moieties using conventional chemical techniques. We also attempted to observe which, if any, of these resonances from the ^{1}H-NMR spectrum of the LPS the could be shifted in the presence of paramagnetic metal ions in order to attempt to calculate outside/inside ratios of LPS incorporated into liposomes. We considered that the outside/inside ratios might be imporatant factors in characterizing the liposomal formulations to be used in the subsequent *in vivo* studies. However, this approach was not effective because we could not reliably qualitate shifted or broadened signals in the presence of the complex ^{1}H-NMR spectrum of the LPS.

An unusual signal resonating at approximately 0 ppm was observed in the ^{1}H-NMR spectrum of the LPS and lipid A from *A. salmonicida*, suggestive of the presence of silicon containing matter in our preparations. We

investigated the possibility that our LPS preparations included silicon-containing contaminants from the silicon-based antifoaming agent (Antifoam A™) used in growing bacterial cultures. Using ^{1}H-NMR, GC-EIMS, and SDS-PAGE, we analyzed LPS extracted from cells grown in the presence or absence of Antifoam A™, in addition to material extracted from Antifoam A™. The data indicated that Antifoam A™ might not be responsible for the unusual resonance at approximately 0 ppm in the ^{1}H-NMR spectra of our LPS preparations. Further, the data suggested that Antifoam A™ contained material that had some characteristics which were comparable to the lipid A moiety of the LPS from *A. salmonicida*. We observed that some commercially obtained phenol-extracted LPS preparations that were grown in the presence of antifoaming agents also showed an unusual high-field ^{1}H-NMR resonance at approximately 0 ppm, while a commercially available trichloroacetic acid extracted preparation of LPS from *E. coli* did not exhibit a resonance near 0 ppm. Additional extraction of the phenol-extracted LPS preparations with trichloroacetic acid could diminish the strength of the high field signal observed in some of the ^{1}H-NMR spectra.

Combined, the data obtained from the structural studies of the LPS from *A. salmonicida* indicated that a) the ^{1}H-NMR and GC-EIMS data for the LPS were consistent with the structures previously proposed b) the signal observed

at approximately 0 ppm in the ^{1}H-NMR spectra may relate to both the LPS from *A. salmonicida* and to a component of Antifoam A™, c) this signal could be altered depending on the method of extraction employed and e) LPS isolated from bacterial cells via the aqueous phenol extraction cultivated in the presence of Antifoam A™, may contain materials which have some properties coinciding with those of native LPS.

The behaviour of liposomes *in vivo* is dependent on lipid composition, surface charge and vesicle size. The diversity of materials which can be incorporated into liposomes and their associated applications (see table 1.3.) has resulted in modifications to the classical MLV (see table 1.4.) originally described by Bangham and Horne (1964). Such flexibility provides the possibility of designing formulations tailored to specific objectives. In mammals, liposomal LPS formulations from a variety of bacterial species have been shown to be effective in enhancing host defence by potentiating immune response to LPS (Desiderio and Campbell, 1985; Petrov *et al.*, 1992; Wong *et al.*, 1992; Threakston and Laing, 1993) and in modifying the regulation of cytokine release from phagocytic cells (Bakouche *et al.* 1987; Dijkstra *et al.*, 1987; Dijkstra *et al.*, 1988a,b; Dijkstra *et al.*, 1989). The incorporation ratios of LPS and PS from *A. salmonicida* into liposomes with respect to varying lipid

compositions and methods of preparation were examined. The incorporation rates of LPS and PS from *A. salmonicida* into liposomes as a function of lipid composition have not, to our knowledge, been previously examined. We observed that positively-charged liposomes were more efficient at incorporating the LPS and PS from *A. salmonicida* than were negatively-charged liposomes, which was not surprising, considering the presence of negatively-charged phosphoryl groups within the macromolecular structure of the LPS. The incorporation rates estimated in these studies were most similar to the values of approximately 45% reported for the LPS and PS of *Brucella abortus* (Wong et al., 1992). In addition, it was observed that positively-charged MLV incorporated LPS preferentially to vesicles produced by sonication or extrusion. Negatively-charged liposomes exhibited similar incorporation rates for LPS regardless of the method of preparation. In contrast to the liposomal incorporation of LPS, it was observed that sonication or extrusion of MLV enhances the incorporation of the PS devoid of the lipid A moiety, which may be due to the larger aqueous compartments relative to lipid mass of the sonicated or extruded vesicles. Light-scattering studies indicated that the vesicle diameters and size distributions of the liposomes obtained by the various techniques employed were within the ranges expected.

In mammals, liposomes have been shown to be eliminated rapidly from the circulation and distributed among the macrophages of the liver and spleen (Senior and Gregoriadis, 1982; Gabizon and Papahadjopoulos, 1988; Allen *et al.*, 1991; Huang *et al.*, 1992; Park *et al.*, 1992), an important factor when devising a vaccine targeted to the RES. MLV-incorporated LPS preparations, having the advantage of a rapid and simple method of preparation and the highest incorporation ratios, were viewed to be the most practical formulations and were subsequently chosen as the formulations to be employed in biodistribution studies.

The fate of exogenously administered liposomal LPS formulations in rainbow trout has not previously been examined. We investigated the uptake and biodistribution of radiolabel on free and MLV-incorporated LPS by the organs and tissues in rainbow trout as a function of differing routes of administration and liposomal surface charge. It has been reported that uptake of bacteria by salmonids is predominantly by the kidney and spleen, the primary hemopoietic tissues in rainbow trout (Ferguson *et al.*, 1981). Conventional liposomes composed of egg phosphatidylcholine, cholesterol and a small molar ratio of charged lipid have been shown to be taken up by the macrophages of the RES in mammals (Poste *et al.*, 1982; Allen *et al.*, 1989)

more readily than long-circulating or Stealth™ liposomes (Allen *et al.*, 1991; Gabizon and Papahadjopoulos, 1992; Yamauchi *et al.*, 1993). It was not surprising that LPS, MLV and MLV-incorporated LPS were recovered primarily in the kidney or spleen or both of rainbow trout, when the IV, IP, and IM routes of administration were used. Oral administration of the immunogens did not display preferential uptake of radiolabel in any of the organs and tissues examined and overall uptake was low possibly due to loss of material by regurgitation after administration of immunogen by intubation. Neumann and Tripp (1986) examined the effects of IM, IP and intraesophageal routes of administration on the humoral immune responses of channel catfish (*Ictalarus punctatus*) to *Yersinia ruckeri*. The authors found that the levels of serum anti-*Y. ruckeri* antibodies elicited were substantial when the IP and IM routes were employed whereas the response was considerably lower when the intraesophageal route was employed. Our studies indicated that the uptake of LPS, MLV and MLV-incorporated LPS was greatest when the IV route was used, which is the most direct route of delivery into the circulation but is the least commercially applicable. The reduced uptake of the antigenic materials by the kidney and spleen displayed by the IM route might be explained by slow release from the site of injection which was distal to the site of sampling.

When the oral route was used, there appeared to be considerably more radiolabel in the liver when LPS was incorporated into liposomes, which may be due to the limited passage of the antigenic materials across the intestine. Perhaps incorporation of the liposomes into the feed would be a more appropriate method for oral administration than intubation. The IP route displayed substantial uptake by the kidney and spleen, and is a less harmful and labour-intensive route of delivery than the IV route. Accordingly, IP injection was chosen as the route of administration in subsequent immunization trials.

Studies in mammals have demonstrated that conventional liposomes containing negatively-charged lipids enhance uptake by the cells of the RES compared to neutral liposomes (Raz *et al.*, 1981). Senior *et al.*, 1991 showed that, when compared to neutral liposomes, the molar ratio of positively-charged lipid in a liposomal preparation was correlated to the interactions of liposomes with blood components. Our studies suggest that liposomal surface charge is a factor in the uptake of liposomes by the kidney and spleen in rainbow trout. In the absence of LPS, it appeared that negatively-charged MLV displayed increased uptake by the kidney and spleen as compared to positively-charged MLV. When LPS was incorporated into MLV, however, the opposite correlation was observed, suggesting that the positively-charged MLV

containing LPS behaved *in vivo* as did the negatively-charged MLV without LPS. This observation suggests that the LPS, which contains negatively-charged phosphoryl groups, may alter the liposomal surface charge, although the basis for this is unclear.

Power *et al* (1991) demonstrated that "empty" MLV and LUV composed of phosphatidylcholine and cholesterol were taken up primarily by the kidney and spleen in rainbow trout, consistent with the findings presented here. Our biodistribution studies suggest that liposomes can efficiently serve as delivery vehicles for LPS to the hemopoietic organs in rainbow trout. Rodgers (1990) performed immunization studies with rainbow trout that had been immersed in a liposomal preparation which contained a mixture of formalin-killed whole cells of *A. salmonicida*, inactivated toxin from *A. salmonicida*, and LPS from *A. salmonicida*. This approach resulted in increased survival rates among the group which received the liposomal vaccine as compared to the control groups which were treated with non-liposomal vaccine or were untreated.

The most interesting characteristic of liposomes with respect to this study was their ability to function as immunological adjuvants (Poznansky and Juliano, 1984; Gregoriadis, 1990; Buiting *et al.*, 1992; Fries *et al.*, 1992). Immunization experiments were performed in rainbow trout in order to

determine if the incorporation of the LPS from *A. salmonicida* into liposomes could enhance the immunogenicity of the LPS. Repeated IP injections of free LPS resulted in elevated, albeit short-lived serum anti-LPS activity which resembled a typical primary immune response, similar to that observed by Ingram and Alexander (1980). When rainbow trout received a priming dose of liposomally-incorporated LPS intraperitoneally, prolonged serum anti-LPS antibody levels were observed following exposure to free LPS. A typical secondary immune response in mammals (see chapter 1) is characterized by a shorter lag period, a higher level of specific antibodies, and an extended duration of antibody levels. Prolonged serum antibody responses to LPS were observed when either positively- or negatively-charged, multilamellar or unilamellar liposomal LPS were used as the priming antigen in rainbow trout. In most instances, it appeared that two priming IP injections of liposomal LPS prior to challenge with free LPS were required to produce prolonged serum anti-LPS titres. Generally during secondary responses, sustained antibody titres might be expected after only one priming injection of antigen. Our results have some similarities with another study in rainbow trout that reported that more than a single injection of the priming antigen (TNP-KLH) was required to produce a secondary response to TNP (Arkoosh and Kaattari, 1991). It was

also reported by those authors that only one priming injection of the TNP-LPS was required to produce a secondary response to TNP.

In some instances it was observed that lag periods were shorter following exposure to free LPS when trout were primed with liposomal LPS. However, this was not consistent throughout the trials. Stolen *et al.* (1984) suggested that the primary response of teleosts may be slower at lower temperatures and the relevance of this observation to our results is explained in more detail in chapter 5. The discrepancy in environmental temperature may have contributed to the differences observed in immunization profiles between Fig. 1a and Fig. 2a; these were duplicate trials in which the protocol consisted of successive IP injections of free LPS. In contrast to mammals, Dorson (1984) reported that although a secondary immune response has been claimed several times in fish, the ratio between the titres obtained in the secondary and primary response is not nearly as pronounced as it is in mammals. Although we observed prolonged serum anti-LPS antibody responses, our results did not demonstrate notable differences between the antibody levels induced by primary and secondary IP injections.

The number of doses of liposomal LPS administered IP prior to the subsequent exposure to free LPS appeared to be correlated with the prolonged

serum antibody responses observed in rainbow trout. The results from that immunization trial, together with the others presented herein, suggest that at least two IP injections of liposomal LPS were necessary prior to exposure to free LPS in order to prolong serum anti-LPS antibody responses. The results coincide with the theory that immunological memory in rainbow trout may be due to the expansion of the precursor B lymphocyte pool (Arkoosh and Kaattari, 1991). Isotype switching, which is a characteristic of memory responses in mammals, is unlikely since teleost fish are known to produce only an IgM-like molecule. Affinity maturation, also a characteristic process involved with immunological memory in mammals, was not examined in this study. Arkoosh and Kaattari (1991), however, observed no significant differences in average affinity in the antibodies generated during the primary and secondary *in vivo* responses against TNP in rainbow trout.

Administration of the liposomal LPS formulations by oral intubation followed by exposure to free LPS produced only a modest immune response to LPS compared to IP administration. The results using the oral administration were not surprising considering the outcome of the biodistribution studies performed previously. It might be more practical to administer formulations orally by incorporation into feed.

Overall, the results of the immunization trials using IP administered liposomal LPS in rainbow trout were encouraging. We observed that the immune responses to LPS were prolonged when trout were primed with liposomal LPS, showing some resemblance to a secondary immune response. Wong *et al.*, (1992) reported that enhanced humoral immune responses in mice to LPS and PS from *Brucella abortus* were obtained when the liposomally-incorporated antigens was used as opposed to the free antigens. The immune responses generated in that study were consistent with the criteria of secondary immune responses, including a more pronounced IgG response following reexposure to antigen as compared to IgM response. However, those experiments were maintained for only six weeks and it was not possible to determine if prolonged antibody responses resulted from immunizations with liposomally-incorporated antigens. Other studies in mammals have shown that liposome incorporation can enhance antigen presentation (Alving, 1992; Collins *et al.*, 1992) and macrophage recruitment (Harding *et al.*, 1991) to an associated antigen. The possibility that incorporation of LPS from *A. salmonicida* into liposomes may enhance antigen presentation and recruitment of macrophages in rainbow trout is worthy of further investigation. *In vitro* assays for T cell proliferation and *in vivo* challenge experiments are necessary

to evaluate T-cell response and level of protection, respectively, induced by liposomally-incorporated LPS in rainbow trout. In addition to prolonged serum anti-LPS antibody levels, it appears that immunization with liposomal LPS can also generate a considerable level of anti-lipid A antibodies in rainbow trout.

Throughout the course of this study, we have used an ELISA assay to evaluate specific serum anti-LPS antibody titres. The secondary antibody used in this assay was a mouse anti-trout IgM monoclonal antibody (mAb I-14). However, we did not initially have access to the hybridoma producing mAb 1-14, a dilemma which led to the purification of trout IgM for the purpose of generating polyclonal antibodies to be used as secondary antibodies in the ELISA. As a consequence, we developed a method for the purification of rainbow trout IgM from globulin enriched, gel-filtered immune-sera. We utilized this method, originally designed for the isolation of Ig from mammalian serum or culture supernatants, to purify and characterize the antibodies from trout serum generated from the immunization studies using free and liposomal LPS. This method, which relies on the adherence of IgM to a mixed-mode ion-exchange chromatographic matrix (ABx resin) previously used for the isolation of mammalian Ig, has the advantage of being rapid, inexpensive and less tedious than the other techniques used to purify IgM from fish serum. In addition, it appears that a substantial quantity of IgM can be purified, including

antigen-specific antibodies (Szalai *et al.*, 1995).

We estimated that ABx-adherent trout IgM accounted for approximately 6% of the total protein in pooled rainbow trout serum, a value which correlated well with the serum IgM concentrations previously reported for other salmonids (Voss *et al.*, 1980; Kobayashi *et al.*, 1982; Sanchez *et al.*,1989; Sanchez *et al.*, 1991). In addition, our data indicates that all of the major bands observed on SDS-PAGE for the ABx-adherent fraction from trout serum can be attributed to IgM or its constituent polypeptide chains. The results generated by this technique demonstrate that ABx chromatography is an alternative and practical procedure for the purification of rainbow trout Ig from serum. We believe that this protocol may be adapted for the isolation of total IgM from any fish species for whom mAbs reactive with Ig are not yet available.

In conclusion, it has been demonstrated that the LPS from *A. salmonicida* can be efficiently incorporated into liposomes. It appears that liposomal LPS formulations are capable of facilitating the delivery of the LPS to the primary lymphoid organs of rainbow trout. Immunization trials using free and liposomally-incorporated LPS formulations indicated that prolonged serum anti-LPS antibody responses were obtained if rainbow trout were primed with liposomally-incorporated LPS prior to exposure to free LPS. In addition,

it appears that considerable levels of anti-lipid A antibodies, potentially directed against common antigenic determinants inherent to several LPS molecules, could be generated by immunizations with liposomal LPS. Liposomally-incorporated LPS dispersions can safely be administered to fish as delivery vehicles and immunological adjuvants and are worthy of further investigation in terms of their potential and application as anti-furunculosis formulations.

REFERENCES

Aakre, R., Wergeland, H. I., Assjord, P. M., Endresen, C. 1994. Enhanced antibody response in Atlantic salmon (*Salmo salar L*) to *Aeromonas salmonicida* cell wall antigens using a bacterin containing B-1,3 glucan as adjuvant. Fish and Shellfish Immunol. 4:47-61.

Abramson, M. B., Katzman, R., and Gregor, H. P. 1964. Aqueous dispersions of phosphatidylserine. J. Biol. Chem. 239:70-76.

Adams, A., Auchinachie, N., Bundy, A., Tatner, M. F., and Horne, M. T. 1988. The potency of adjuvated injected vaccines in rainbow trout (*Salmo gairdneri* Richardson) and bath vaccines in Atlantic salmon (*Salmo salar* L.) against furunculosis. Aquaculture. 69:15-26.

Adibzadeh, M., Weder, H.G., Rehbein, S., Schwulera, U., Obermeier, K., and Pawelec, G. 1992. Activity of liposomal interleukin-2 *in vitro*. Mol. Biother. 4:24-28.

Ahmad, I., Longnecker, M., Samuel. J., and Allen, T. M. 1993. Antibody-targeted delivery of doxorubicin entrapped in sterically stabilized liposomes can eradicate lung cancer in mice. Cancer Res. 53:1484-1488.

Alberts, B., Bray, D., Lewis, J., Raff, M. Roebrts, K., and Watson, J. D. 1989. The Immune System. In " Molecular Biology of the Cell." Garland Publishing Inc., New York. pp.1001-1057.

Allen, T. M., Hansen, C., Martin, F., Redemann, C., and Yau-Yong, A. 1991. Liposomes containing synthetic derivatives of poly(ethylene glycol) show prolonged circulation half-lives *in vivo*. Biochim. Biophys. Acta. 1066:29-36.

Allen, T.M., Austin, G. A., Chonn, A., Lin, L., and Lee, K. C. 1991. Uptake of liposomes by cultured mouse bone marrow macrophages: influence of liposome composition and size. Biochim. Biophys. Acta 1061:56-64.

Allen, T. M., Hansen, C., and Rutledge, J. 1989. Liposomes with prolonged circulation times: factors affecting uptake by reticuloendothelial and other

tissues. Biochim. Biophys. Acta 981:27-35.

Allen, T. M., Williamson, P. and Schlegel, R. A. 1988. Phosphatidylserine as a determinant of reticulonedothelial recognition of liposome models of the erythrocyte surface. Proc. Natl. Acad. Sci. 85:8067-8071.

Allen, T. M., and Chonn, A. 1987. Large unilamellar liposomes with low uptake into the reticuloendothelial system. FEBS lett. 223:42-46.

Allison, A., and Gregoriadis, G.. 1974. Liposomes as immunological adjuvants. Nature 252:252.

Alving, C. R. 1993. Lipopolysaccharide, lipid A, and liposomes containing lipid A as immunological adjuvants. Immunobiol. 187:430-446.

Alving, C. R. 1992. Immunologic aspects of liposomes: presentation and processing of liposomal protein and phospholipid antigens. Biochim. Biophys. Acta. 1113:307-322.

Alving, C. R. 1987. Liposomes for carriers as vaccines. In "Liposomes: from

biophysics to therapeutics". Ostro, M. J. (Ed.). Marcel Dekker Inc., New York. pp.195-218.

Alving, C. R., Banerji, B., Clements, J. D., and Richards, R. L. 1980. Adjuvanticity of lipid A and lipid A fractions in liposomes. In "Liposomes and Immunobiology". B. H. Tom, and H. R. Six (Eds.). Elsevier, North-Holland, Amsterdam. pp.67-78.

Amorosius, H., and Frenzel, E. V. 1972. Anti-TNP antibodies in carps and tortoises. Immunochem. 9:65-71.

Anderson, D. P., and Jeney, G. 1991. Responses to *in vitro* and *in vivo* immunizations with *Aeromonas salmonicida* O antigen bacterins in rainbow trout (*Oncorhynchus mykiss*). Fish and Shellfish Immunol.

Angerman, C. R. and Edelstein, T. K. 1980. Correlation of the duration and magnitude of protection against *Salmonella* infections afforded by various vaccines with antibody titres. Infect. Immun. 27:435-443.

Arkoosh, M. R., and Kaattari, S. L.. 1991. Memory in rainbow trout (*Oncorhynchus mykiss*). I. An immunochemical and cellular analysis of the B cell response. Dev. Comp. Immunol. 15:279-293.

Atanassov, C. L. and Botev, B. A., 1988. Isolation and partial characterization of IgM-like immunoglobulins from the serum of carp (*Cyprinus carpio L.*) frog (*Rana ribiduna* Pall.) and tortoise (*Testudo graeca* Pall.). Comp. Biochem. Physiol. 89:737-741.

Avtalion, R. R. 1969. Temperature effect on antibody production and immunological memory in carp (*Cyprinus carpio*) immunized against bovine serum albumin (BSA). Immunol. 17:927-931.

Awake, T., Egusa, S., Kimura, T., and Watanabe, T. 1971. Detection of R factors involved in naturally occurring *Aeromonas salmonicida* strains. Appl. Microbiol. 22:716-717.

Baba, T., Imamura, J., Izawa, K., and Ikeda, K. 1988. Cell-mediated protection in carp, *Cyprinus caprio L.*, against *Aeromonas hydrophila*. J.

Fish Dis. 11:171-178.

Bakker-Woudenberg, I. A. J. M., Lokerse, A .F., van den Berg, J. C. V., Roerdink, F. H., and Michel, M. F. 1985. Free versus liposome-entrapped ampicillin in the treatment of infection due to *Listeria monocytogenes* in normal and athymic (nude) mice. J. Infect. Dis. 151:917-924.

Bakouche, O., Koff, W. C., Brown, D. C., and Lachman, L. B. 1987. Interleukin 1 release by human monocytes treated with liposome-encapsulated lipopolysaccharide. J. Immunol. 139:1120-1126.

Bally, M. B., Nayar, R., Masin, D., Hope, M. J., Cullis, P. R. and Mayer, L. D. 1990. Liposomes with entrapped doxorubicin exhibit extended blood residence times. Biochim. Biophys. Acta. 1023:133-139.

Bally, M. B., Hope, M. J., Mayer, L. D., Madden, T. D. and Cullis, P. R. 1988. Novel procedures for generating and loading liposomal systems. In "Liposomes as Drug Carriers". G. Gregoriadis (Ed.). John Wiley &

Sons Ltd., London, p.841.

Bangham, A. D. and Horne, R. W. 1964. Negative staining of phospholipids and their structural modification by surface-active agents as observed in the electron microscope. J. Mol. Biol. 8:660-664.

Banoub, J. H., Shaw, D. H., Nakhla, N. A. and Hodder, H. J. 1989. Synthesis of glycoconjugates derived from various Lipopolysaccharides of the *Vibrionaceae* family. Eur. J. Biochem. 179:651-657.

Banoub, J. H., Michon, F. and Hodder, H. J. 1987. Structural elucidation of the O-specific polysaccharide of the phenol-phase soluble lipopolysaccharide of *Vibrio anguillarum*. Biochem. Cell Biol. 65:19-26.

Banoub, J. H., and Hodder, H. J. 1985. Structural investigation of the lipopolysaccharide core isolated from a virulent strain of *Vibrio ordali*. Biochem. Cell Biol. 63: 1199-1205.

Barenholz, Y., Amselem, S., and Lichtenberg, D. 1979. A new method for

preparation of phospholipid vesicles. FEBS lett. 99:210-215.

Bartlett, G. R. 1959. Phosphorous assay in column chromatography. J. Biol. Chem. 234:466-468.

Belland, R. and Trust, T. J. 1988. DNA reassociation analysis of *Aeromonas salmonicida*. J. General Microbiol. 134:307-315.

Benjamini, E., and Leskowitz, S. 1991. The role of MHC in the immune response. In " Immunology: A Short Course.", second edition. Wiley-Liss, New York. pp. 193-210.

Bergelson, L. D. 1978. In "Methods in Membrane Biology", vol 9. (Korn, E. D., ed.). Plenum Press, New York, pp.275-335.

Berzci, I., Bertok, L., and Bereznai, T. 1966. Comparative studies on the toxicity of *Escherichia coli* lipopolysaccharide endotoxin in various animal species. Can. J. Microbiol. 12:1070-1071.

Beutler, B., Krochin, N., Milsark, J. W., Luedke, C., and Cerami, A. 1986. Control of cachectin (tumor necrosis factor) synthesis: mechanisms of endotoxin resistance. Science. 232:977-980.

Blenkharn, J. I, and Wood, S. 1987. A low-cost antifoam additive for agar-based culture media. J. Applied. Bacteriology. 63:465-468.

Bogwald, J., K. Stensvag, J. Hoffman, and T. Jorgensen. 1991. Antibody specificities in Atlantic salmon, *salmo salar L.*, against the fish pathogens *Vibrio salmonicida* and *Vibrio anguillarum*. J. Fish Dis. 14:70-87.

Bollag, D.M. and Edelstein, S.J., 1991. Protein Methods. Wiley-Liss, New York, p.79.

Brade, L., Rietschel, E. T., Kusomoto, S., Shiba, T., and Brade, H. 1986. Immunogenicity and antigenicity of synthetic *Escherichia Coli* lipid A. Infec. Immunity. 51:110-114.

Brett, J. R. 1958. Implications and assessments of environmental stress in the investigation of fish power problems. H. R. MacMillan Lectures. University of British Columbia.

Brooks, D.A., Bradford, T.M. and Hopwood, J.J., 1992. An improved method for the purification of IgG monoclonal antibodies from culture supernatants. J. Immunol. Meth. 155:129-132.

Buiting, A. M. J., Van Rooijen, N. and Classen, E. 1992. Liposomes as antigen carriers and adjuvants *in vivo*. Research in Immunology. 143(5):541.

Campbell, C. M., Duncan, D., Price, N. C., and Stevens, L. 1990. The secretion of amylase, phospholipase and protease from *Aeromonas salmonicida*, and the correlation with membrane-associated ribosomes. J. Fish Dis. 13:463-474.

Campbell, D.H., Luescher, E. and Lerman, L.S. 1951. Immunological adsorbents. I. Isolation of antibody by means of a cellulose-protein

antigen. Proc. Natl. Acad. Sci. USA 37:575-578.

Chiller, J. M., Skidmore, B .J., Morrison, D. C., and Wiegle, W. O. 1973. Relationship of the structure of bacterial lipopolysaccharide to its function in mitogenesis and adjuvanticity. Proc. Natl. Acad. Sci. 70:2129.

Cipriano, R. C. 1982. Immunogenic potential of growth products extracted from cultures of *Aeromonas salmonicida* for brook trout (*Salvenilus fontinalis*). Can. J. Fisheries. Aquat. Sci. 39:1512-1518.

Cisar, J.O. and Fryer, J.L., 1974. Characterization of anti-*Aeromonas salmonicida* antibodies from coho salmon. *Inf.Imm.* 9:236-243.

Clem, L. W., Miller, N. W., and Bly, J. E. 1990. Evolution of lymphocyte subpopulations, their interactions, and temperature sensitivities. In "Phylogenesis of immune functions." Warr, G. W. and Cohen, N. (Ed.s). CRC Press, Boca Raton, USA. 191-213.

Clem, L. W., and Siegel, M. M. 1965. Antibody responses of lower vertebrates to bovine serum albumin. Fed. Proc. 24:54.

Coleman, R. M., Lombard, M. F., Sicard, R. E., and Renricca, N. J. 1984. Cells and tissues of the immune system. In " Fundamental Immunology." Jaffe, E. G. (Ed.). Wm. C. Brown Publishers, Dubuque, Iowa, USA. pp. 58-82.

Coleman, R. M., Lombard, M. F., Sicard, R. E., and Renricca, N. J. 1984. Elements of humoral immunity. In " Fundamental Immunology." Jaffe, E. G. (Ed.). Wm. C. Brown Publishers, Dubuque, Iowa, USA. pp. 129-161.

Collins, D. S., Findlay, K., and Harding, C. V. 1992. Processing of exogenous liposome-encapsulated antigens *in vivo* generates class I MHC-restricted T cell responses. J. Immunol. 148(11):3336-3341.

Collins, F. M. and Campbell, S. G. 1982. Immunity to intracellular bacteria. Vet. Immunol. Immunopathol. 3:5-66.

Collins, F. M. 1969. Effect of specific immune mouse serum on the growth of *Salmonella enteritidis* in mice preimmunized with living or ethyl alcohol-killed vaccines. J. Bacteriol. 97: 676-683.

Collins, F. M. and Mackaness, G. B. 1968. Delayed hypersensitivity and arthus reactivity in relation to host resistance. J. Immunol. 101:830-845.

Colwell, D. E., Michalek, S. M., Briles, D. E., Jirillo, E., and McGhee, J. R. 1984. Monoclonal antibodies to *Salmonella* lipopolysaccharide: anti-O-polysaccharide antibodies protect C3H mice against challenge with virulent *Salmonella typhimurium*. J. Immunol. 133:950-957.

Cowens, J. W., Canter, P., Brenner, D .E., Ginsberg, R. S., Mayer, L. D., Bally, M. B., Cullis, P. R., Pravalic, Z., Douglass, H., Petrelli, N., Karakousis, C., Craven, P. J. 1989. Phase I study of doxorubicin encapsulated in liposomes. Am. Soc. Clin. Oncol. 8:A268.

Cullis, P. R., Mayer, L. D., Bally, M. B., Madden, T. D., and Hope, M. J. 1989. Generating and loading of liposomal systems for drug-delivery

applications. Adv. Drug Delivery Rev. 3:267-282.

Darveau, R. P. and Hancock, R. E. W. 1983. Procedure for isolation of bacterial lipopolysaccharide from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. J. Bacteriol. 155:831-838.

Davis, P. J., and Keough, K. M. W. 1986. Gel to liquid- crystalline transitions of aqueous dispersions of positional isomers of a heteroacid unsaturated phosphatidylcholine mixed with epicholesterol and cholesterol. Biochim. Biophys. Acta. 856:395-398.

Davis, P. J., and Keough, K. M. W. 1985. Chain arrangements in the gel state and the transition temperatures of phosphatidylcholines. Biochem. J. 48:915-918.

Davis, P. J., and Keough, K. M. W. 1984. Phase diagrams of bilayers of dimyristoyl lecithin plus heteroacid lecithins. Che. Phys. Lipids. 35:299-308.

Davis, P. J., and Keough, K. M. W. 1984. Scanning calorimetric studies of aqueous dispersions of bilayers made with cholesterol and a pair of positional isomers of 3-*sn*-phosphatidylcholine. Biochim. Biophys. Acta. 778:305-310.

Deamer, D. W., and Uster, P. S. Liposome preparation: methods and preparation. In "Liposomes". Ostro. M. J. (ed.). Marcel Dekker, New York. PP. 27-51.

DeLuca, D., Wilson, M. and Warr, G.W., 1983. Lymphocyte heterogeneity in the trout, *Salmo gairdneri*, defined with monoclonal antibodies to IgM. Eur. J. Immunol. 13:546-551.

Desiderio, J. V., and Campbell, S. G. 1985. Immunization against murine Salmonellosis with liposome-associated O-antigen. Infect. Immun. 48:658-663.

Dijkstra, J., Mellors, J. W., and Ryan, J. L. 1989. Altered *in vivo* activity of liposome-incorporated lipopolysaccharide and lipid A. Infect. Immun.

57:3357-3363.

Dijkstra, J., Ryan, J. L. and Szoka, F. C. 1988a. A procedure for the efficient incorporation of wild-type lipopolysaccharide into liposomes for use in immunological studies. J.Immun. Methods 114:197-205.

Dijkstra, J., Larrick, J. W., Ryan, J. L. and Szoka, F. C. 1988b. Incorporation of LPS in liposomes diminishes its ability to induce tumoricidal activity and tumor necrosis factor secretion in murine macrophages. J. Leukocyte Biol. 43:436-444.

Dijkstra, J., Mellors, J. W., Ryan, J. L. and Szoka, F. C. 1987. Modulation of the biological activity of bacterial endotoxin by incorporation into liposomes. J. Immunol. 138:2663-2670.

Dinarello, C. A. 1984. Interleukin-I. Rev. Infect. Dis. 6:51-95.

Dissanayake, S. and Hay, F.C., 1975. Isolation of pure normal IgG1 from mouse serum. Immunochemistry 12:101-103.

Dorson, M. 1984. Applied immunology of fish. In " Symposium on Fish Vaccination." P. Kinkelin (Ed.). Bulletin de L' Office Internationale des Epizooites, Paris, p.39-74.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. 1956. Colorimetric determination of total hexoses. Anal. Chem. 28(3):350-356.

Duff, D. C. B. 1942. The oral immunization of rainbow trout against *Bacterium salmonicida*. J. Immunol. 44:87-93.

Elkins, K. L., Stashak, P. W., and Baker, P. J. 1989. Specific immunological unresponsiveness to bacterial lipopolysaccharide develops in a cyclic manner. Infect. Immun. 57:2253-2255.

Ellis A. E. 1991. An appraisal of the extracellular toxins of *Aeromonas salmonicida* ssp. *salmonicida*. J. Fish Dis. 14:265-277.

Ellis, A. E. 1988. Current aspects of fish vaccination. Dis. aquat. Org. 4:159-

164.

Ellis, A. E. 1982. Differences between the immune mechanisms of fish and higher vertebrates. In "Microbial Diseases of Fish." R. J. Roberts (Ed.). Academic Press, New York, p.1-29.

Ellis, A. E. 1977. The leucocytes of fish. A review. J. Fish Biol. 11:453-491.

Ellsaesser, C. F., Bly, J. E., and Clem, L. W. 1988. Phylogeny of lymphocyte heterogeneity: the thymus of the channel catfish. Develop. Comp. Immunol. 12:787-799.

Emmerich, R. and Weibel, E. 1894. Ueber eine durch Bakterien erzengte Seuche unter den Forellen. Archives fur Hygiene und Bakteriologie. 21:1-21.

Epstein, M. A., Morgan, A. J., Finerty, S., Randle, B. J., and Kirkwood, J. K. 1985. Protection against cottontop tamarins agaist Epstein-Barr virus-induced malignant lymphoma by a prototype subunit vaccine. Nature. 318:287-289.

Evenberg, D., de Graaf, P., Fleuren, W, and van Muiswinkel, W. B. 1986. Blood changes in carp (*Cyprinus carpio*) induced by ulcerative *Aeromonas salmonicida* infections. In "Fish Immunology". Stolen, J. S., Anderson, D. P., and van Muiswinkel, W. B. (ed's.). Elsevier, Amsterdam, p.321.

Ey, P.L., Prowse, S.J. and Jenkin, C.R., 1978. Isolation of pure IgG1, IgG2a, and IgG2b immunoglobulins from mouse serum using protein A-Sepharose. Immunochemistry 15:429-436.

Ferguson, H. W., Claxton, M. J., Moccia, R. D., and Wilkie, E. J. 1982. The quantitative clearance of bacteria from the bloodstream of rainbow trout (*Salmo gairdneri*). Vet. Pathol. 19:687-699.

Fountain, M. W., Dees, M., and Schultz, C. R. 1981. Enhanced intracellular killing of *Staphylcoccus aureus* by canine monocytes treated with liposomes containing amikacin, gentamicin, kanamycin, and tobramycin. J. Inf. Dis. 6:373-376.

Fuller, D. W., Pilcher, K. S., and Fryer, J. L. 1977. A leucocytolytic factor isolated from cultures of *Aeromonas salmonicida*. J. Fisheries Research Board of Canada. 34:1118-1125.

Fries, L. F., Gordon, D. M., Richards, R. L., Egan, J. E., Hollingdale, M. R., Gross, M., Silverman, C. and Alving C. R. 1992. Liposomal malaria vaccine in humans: A safe and potent adjuvant strategy. Proc. Natl. Acad. Sci. 89:358-362.

Gabizon, A., Catane, R., Uziely, B, Kaufman, B., Safra, T., Barenholz, Y., and Huang, A. 1992. A pilot study of doxorubicin encapsulated in long-circulating (Stealth^{R}) liposomes (S-DOX) in cancer patients. Proc. Am. Soc. Clin. Oncol. 11:124.

Gabizon, A. 1992. Selective tumor localization and improved therapeutic index of anthracyclines encapsulated in long-circulating liposomes. Cancer Res. 52:891-896.

Gabizon, A. and Papahadjopoulos, D. 1992. The role of surface charge and

hydrophilic groups on liposome clearance *in vivo*. Biochim. Biophys. Acta 1103:94-100.

Gabizon, A. and Papahadjopoulos, D. 1988. Liposome formulations with prolonged circulation times in blood and enhanced uptake by tumors. Proc. Natl. Acad. Sci. 85:6949-6953.

Gabizon, A., Peretz, T., and Ben-Yosef, R. 1986. Phase I study with liposome-associated Adriamycin: preliminary report. Am. Soc. Clin. Oncol. 5:43.

Gabizon, A., Dagan, A., Goren, D., Barenholz, Y., and Fuks, Z. 1982. Liposomes as *in vivo* carriers of adriamycin: Reduced cardiac uptake and preserved antitumor activity in mice. Cancer Res. 42:4734-4739.

Galanos, C., Freudenburg, M. A., Jay, F., Nerkar, D., Veleva, k., Brade, H., and Strittmatter, W. Immunogenic properties of lipid A. Rev. Infect. Dis. 6:546-552.

Garcon, N. M. J. and Six, H. R. 1991. Liposomes that provide T-dependent help to weak antigens. J. Immunol. 146:3697-3702.

Goldman, R. C. and Leive, L. 1980. Heterogeneity of antigenic sidechain length in lipopolysaccharide from *Escherichia coli* 0111 and *Salmonella typhimurium* LT2. Eur. J. Biochem. 107:145-153.

Goren, D., Gabizon, A., and Barenholz, Y. 1990. The influence of physical characteristics of liposomes containing doxorubicin on their pharmacological behaviour. Biochim. Biophys. Acta. 1029:285-294.

Graham, S., and Secombes, C. J. 1990. Do fish lymphocytes secrete interferon-gamma. J. Fish Biol. 36:563-573.

Graham, S., and Secombes, C. J. 1988. The production of macrophage-activating factor from rainbow trout *Salmo gairdneri* leucocytes. Immunology 65:293-297.

Gregoriadis, G., Tan, L., Ben-Ahmeida, E. T. S. and Jennings, R. 1992.

Liposomes enhance the immunogenicity of reconstituted influenza virus A/PR/8 envelopes and the formation of protective antibody by influenza virus A/Sichuan/87 (H3N2) surface antigen. Vaccine 10 (11):747-752.

Gregoriadis, G. 1990. Immunological adjuvants: a role for liposomes. Immunology Today. 11(3):89-97.

Gregoriadis, G. 1973. Drug entrapment in liposomes. FEBS lett. 36:296.

Griffin, B. R. 1983. Opsonic effect of rainbow trout (*Salmo gairdneri*) antibody on phagocytosis of *Yersinia ruckeri* by trout leucocytes. Dev. Comp. Immunol. 7:253-259.

Griffin, P. J., Snieszko, S. F., and Friddle, S. B. 1953. A more comprehensive description of *Bacillus salmonicida*. Transactions of the American Fisheries Society. 82:241-253.

Gruner, S. M., Lenk, R. P., Janoff, A. S., and Ostro, M. J. 1985. Novel multi-layered vesicles: comparison of physical characteristics of

multilamellar liposomes and stable plurilamellar vesicles. Biochemistry. 24:2833-2842.

Gunstone, F. D., Harwood, J. L., and Padley, F. B. 1994. The lipid handbook. Chapman & Hall, London, UK. pp.216-222.

Hahn, H., and Kaufman, S. H. E. 1981. The role of cell- mediated immunity in bacterial infections. Rev. Infect. Dis. 3:1221-1250.

Hakomori, S. 1964. J. Biochem. (Tokyo). 55:205-208.

Harding, C. V., Collins, D. S., Slot, J. W., Geuze, H. J., and Unanue, E. R. 1991. Liposome-encapsulated antigens are processed in lysosomes, recycled and presented to T cells. Cell 64:393-401.

Hasten, T., and Bullock, G. L. 1976. An acute septicaemic disease of brown trout and salmon caused by a *pasturella*-like organism. J. Fish Biol. 8:23-26.

Hastings, T. A. and Ellis, A. E. 1988. The humoral response of rainbow trout, *Salmo gairdneri* Richardson, and rabbits to *Aeromonas salmonicida* extracellular products. J. Fish Dis. 11: 147-160.

Hayden, B. J., and Warr, G. W. 1986. Lymphocyte subpopulations in the teleost, *Salmo gairdneri*. Develop. Comp. Immunol. 10:225.

Heath, T. D. 1987. Covalent attachement of proteins to liposomes. Methods Enzymol. 149:111-118.

Heath, T. D., Fraley, R. T., Montgomery, S. A., Piper, J. R. and Papahadjopoulos, D. 1981. Antibody targeted liposomes: Increase in specific toxicity of methotrexate-8-aspartate. Proc. Natl. Acad. Sci. U.S.A. 80:1377-1381.

Hill, G. J., and Weiss, D. W. 1964. Relationship between susceptibility of mice to heat-killed *Salmonallae* and endotoxin and the affinity of their red blood cells for killed organisms. In "Bacterial Endotoxins." Landy, M. and W. Braun, (Ed's.). Rutgers University. New Bruswick, NJ.

p.422-427.

Hirst, I. D., and Ellis, A. E. 1994. Iron-regulated outer membrane proteins of *Aeromonas salmonicida* are important protective antigens in Atlantic salmon against furunculosis. Fish and Shellfish Immunol. 4:29-45.

Hope, M. J., Bally, M. B., Mayer, L. D., Janoff, A. S., and Cullis, P. R. 1986. Generation of multilamellar and unilamellar phospholipid vesicles. Chem. Phys. Lipids. 40:89-107.

Hope, M. J., Bally, M. B., Webb, G. and Cullis, P. R. 1985. Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. Biochim. Biophys. Acta. 812:55-65.

Hopkins, C. Y. 1961. J. Am. Oil Chemists' Society. 38:664-668.

Huang, S. K., Lee, K-D., Hong, K., Friend, D. S., and Papahadjopoulos, D. 1992. Microscopic localization of sterically stabilized liposomes in colon

carcinoma-bearing mice. Cancer Res. 52:5135-5143.

Ingram, G. A., and Alexander, J. B. 1980. The immune response of the brown trout (*Salmo trutta* L.) to lipopolysaccharide. J. Fish Biol. 16:181-197.

Ishiguro, E. E., Kay, W. W., Ainsworth, T., Chamberlain, J. B., Austen ,R . T., Buckley, J. T., and Trust, T. J. 1981. Loss of virulence during culture of *Aeromonas salmonicida* at high temperatures. J. Bacteriol. 148:333-340.

Janeway, C. A., Jr. 1993. How the immune system recognizes invaders. Scientific American. 269:72-79.

Jay, G. D., Culp, D. J., and Jahnke, M. R. 1990. Silver staining of extensively glycosylated proteins on sodium dodecyl sulfate-polyacrylamide gels: enhancement by carbohydrate-binding dyes. Anal. Biochem. 185:324-330.

Kaastrup, P., Nielsen, B., Horlyck, V., and Simonsen, M. 1988. Mixed

lymphocyte reactions (MLR) in rainbow trout (*Salmo gairdneri*) sibling. Develop. Comp. Immunol. 12:801-808.

Kaattari, S. L. 1994. Development of a piscine paradigm of immunological memory. 1994. Fish and Shellfish Immunol. 4:447-457.

Kaufman, J., Flajnik, M., and DuPasquier, L. 1990. The MHC molecules of ectothermic vertebrates. In "Phylogenesis of immune functions." Warr, G. W. and Cohen, N. (Ed.s). CRC Press, Boca Raton, USA. 125-149.

Kaye, S. D., Boden, J, A., and Ryman, B. E. 1981. The effect of liposome (phospholipid vesicle) entrapment of actinomycin D and methotrexate on the *in vivo* treatment of sensitive and resistant solid murine tumors. Eur. J. Cancer. 17:279-289.

Kennedy-Stoskopf, S. 1993. Immunology. In " Fish Medicine". Stoskopf, M. K. (ed.). W. B. Saunders, Philadelphia, Pennsylvania. p.155.

Keough, K. M. W. and Kariel, N. 1987. Differential scanning calorimetric

studies of aqueous dispersions of phosphatidylcholines containing two polyenoic chains. Biochim. Biophys. Acta. 902:11-18.

Kersten, G. F. A., van de Put, A-M., Teerlink, T., Beuvery, E. C., and Crommelin, D. J. A. 1988. Immunogenicity of liposomes and ISCOMS containing the major outer membrane protein of *Neisseria gonorrhoeae* :influence of protein content and liposomal bilayer composition. Infect. Immun. 56:1661-1664.

Kimball, J. W. The tissues and cells of the immune system. In "Introduction to Immunology. 1986. Macmillan Publishing Co., New York. pp. 131-162.

Kirby, C., and Gregoriadis, G. 1984. Dehydration-rehydration vesicles: a simple method for the high yield drug entrapment in liposomes. Biotechnology November:979-984.

Kobayashi, K., Hara, A., Takano, K. and Hira, H., 1982. Studies on subunit components of immunoglobulin M from a bony fish, the chum salmon (*Oncorhynchus keta*). Mol. Immunol., 19:95-103.

Kuby, J. 1994. Overview of the immune system. In "Immunology", second edition. W. H. Freeman and Co., New York. pp. 1-22.

Kuby, J. 1994. Antigen processing and presentation. In "Immunology", second edition. W. H. Freeman and Co., New York. pp. 239-250.

Kuby, J. 1994. T-cell activation, maturation, and differentiaition. In "Immunology", second edition. W. H. Freeman and Co., New York. pp. 273-296.

Kuhn, H. M., Brade, L., Appelmelk, B. J., Kusumoto, S., Rietschel, E. Th., and Brade, H. 1992. Characterization of the epitope specificity of murine monoclonal antibodies directed against lipid A. Infect. Immun. 60:2201-2210.

Laemmli, U.k. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685.

Laing, G. D., and Threakston, R. D. G. 1993. Immunization against *Echis*

ocellatus (carpet viper) venom using liposomes incorporating immunostimulants: role of lipopolysaccharide in conferring protection in a mouse model. Toxicon. 31:615-626.

Lasic, D. 1992. Liposomes: Synthetic lipid microspheres serve as multipurpose vehicles for the delivery of drugs, genetic material and cosmetics. American Scientist. 80:20-31.

Lasic, D. D., Martin, F. J., Gabizon, A., Huang, S. K. and Papahadjopoulos, D. 1991. Sterically stabilized liposomes: a hypothesis on the molecular origin of the extended circulation times. Biochim. Biophys. Acta. 1070:187-192.

Laude-Sharp, M., Caroff, M., Simard, L., Pusineri, C., Kazatchkine, M. D. and Haeffner-Cavaillon, N. 1990. Induction of IL-1 during hemodialysis: Transmembrane passage of intact endotoxins (LPS). Kidney Intl. 38:1089-1094.

Lee, K. K., and Ellis, A. E. 1991. Susceptibility of salmonid erythrocytes to

lysis by bacterial lipopolysaccharides. J. Fish Dis. 14:461-465.

Lee, K. K., and Ellis, A. E. 1991. The role of extracellular cytolysin of *Aeromonas salmonicida* in the pathology of furunculosis. J. Fish Dis. 14:453-460.

Lee, K. K., and Ellis, A. E. 1990. Glycerophospholipid: cholesterol acyltransferase complexed with lipopolysaccharide (LPS) is a major lethal endotoxin and cytolysin of *Aeromonas salmonicida*: LPS stabilizes and enhances toxicity of the enzymes. J. Bacteriol. 172(9):5382-5393.

Lichtenburg, D., and Barenholz, Y. 1988. Liposomes: preparation, characterization, and preservation. Methods and biochemical analysis. 33:337-461.

Liu, D., and Huang, L. 1992. Size homogeneity of a liposome preparation is crucial for liposome distribution *in vivo*. J. Liposome Res. 2:57-66.

Liu, D., Wada, A. and Huang, L. 1992. Potentiation of the humoral response

of intravenous antigen by splenotropic liposomes. Immunology Letters. 31:177-182.

Lobb, C. J. and Clem, L. W., 1983. Distinctive subpopulations of catfish serum antibody and immunoglobulin. Mol. Immunol. 20:811-818.

Long, E. L. 1976. Selective Aspects of Sample Handling in Liquid Scintillation Counting. Beckman Biomedical Technical Report, 1042-NAC-76-55T.

Luderitz, O., Freudenberg, M. A., Galanos, C., Lehmann, V., Rietschel, E. T. and Shaw, D. H. 1982. Lipopolysaccharides of Gram-negative bacteria. In "Current Topics in Membranes and Transport." F. Bronner and Kleinzeller, A. (Eds.). Academic Press, New York, p.79-151.

Luderitz, O., Staub, A. M. and Westphal, O. 1966a. Immunochemistry of O and R antigens of *Salmonella* and related *Enterobacteriaceae*. Bacteriol. Rev. 30:192.

Luderitz, O., Galanos, C., Risse, H. J., Ruschmann, E., Schlecht, S.,

Schmidt, G., Schulte-Holthausen, H., Wheat, R. and Westphal, O. 1966b. Structural relationships of *Salmonella* O and R antigens. Ann. N.Y. Acad. Sci. 133:349.

Lund, V., Jorgensen, T., Holm, K. O., and Eggset, G. 1991. Humoral immune response in Atlantic salmon, *Salmo salar.*, to cellular and extracellular antigens of *Aeromonas salmonicida.* J. Fish Dis. 14:443-452.

MacIntyre, S., Trust, T. J., and Buckley, J. T. 1980. Identification and characterization of outer membrane fragments released by *Aeromonas* sp. Can. J. Biochem. 58:1018-1025.

Manesis, E. K., Cameron, C. H., and Gregoriadis, G. 1979. Hepatitis B surface antigen-containing liposomes enhance humoral and cell-mediated immunity to the antigen. FEBS lett. 102:107-111.

Mayer, L. D., Tai, L. C. L., Ko, D. S. C., Maisin, D., Ginsberg, R. S., Cullis, P. R. and Bally, M. B. 1989. Influence of vesicle size, lipid

composition, and drug-to-lipid ratio on the biological activity of liposomal doxorubicin in mice. Cancer 49:5922-5930.

Mayer, L. D., Hope, M. J., and Cullis, P. R. 1986. Vesicles of variable sizes produced by a rapid extrusion procedure. Biochim. Biophys. Acta. 858:161-168.

Mayer, L. D., Hope, M. J., Cullis, P. R., and Janoff, A. S. 1985. Solute distributions and trapping efficiencies observed in freeze-thawed multilamellar vesicles. Biochim. Biophys. Acta. 817:193-196.

Mayhew, E., Papahadjopoulos, D., Rustum, Y. M., and Dave, C. 1978. Use of liposomes for the enhancement of the cytotoxic effects of cytosine arabinoside. Ann. N.Y. Acad. Sci. 308:371-386.

Miller, M. W., Sizemore, R. C., and Clem, L. W. 1985. Phylogeny of lymphocyte heterogeneity: the cellular requirements for *in vitro* antibody responses of channel catfish leukocytes. J. Immunol. 134:2884-2888.

Miller, M. W., and Clem, L. W. 1985. Microsystem for *in vitro* primary and secondary responses of channel catfish (*Ictalarus punctatus*) leukocytes with hapten-carrier conjugates. J. Immunol. Methods. 72:367-379.

Minch, M. J. 1989. Experiments in Biochemistry. Projects and Procedures, Prentice Hall, New Jersey, p.264.

Montaraz, J. A., Winter, A. J., Hunter, D. M., Sowa, B. A., Wu, A. M., and Adams, L. G. 1986. Protection against *Brucella abortus* in mice with O-polysaccharide-specific monoclonal antibodies. Infect. Immun. 51:961-963.

Morrison, D. C. and Rudbach, J. A. 1981. In " Contemporary Topics in Molecular Immunology." Inman, P. and Mandy, W.J.(Eds). Plenum, New York, vol.8 p.187.

Morrison, D. C., and Ryan, J. L. 1979. Bacterial endotoxins and host immune responses. Adv. Immunol. 28:293-451.

Morrison, D. C. and Jacobs, D. M. 1976. Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. Immunochemistry 13:813-818.

Nakagawa, Y., Nojima, S., Inoue, K. 1980. Transfer of steroids and α-tocopherol between liposomal membranes. J. Biochem. 87:497-502.

Nakhla, A. N., Szalai, A. J., Banoub, J. H., and Keough, K. M. W. 1994. Uptake and biodistribution of free and liposomally incorporated lipopolysaccharide of *Aeromonas salmonicida* administered via different routes to rainbow trout (*Oncorhynchus mykiss*). J. Liposome Res. 4:1029-1048.

Nakhla, A. N. 1988. Structural elucidation and synthesis of the glycoconjugate of the O-antigen isolated from the lipopolysaccharide of the Gram-Negative bacteria *Yersinia ruckeri.*, M.Sc. thesis, Department of Biochemistry, Memorial University of Newfoundland.

Neter, E. 1956. Bacterial hemagglutination and hemolysis. Bacteriol. Rev.

20:166-188.

Niki, L., Evelyn, T. P. T, and Albright, L. J. 1994. Trials with an orally and immersion-administered B-1,3 glucan as an immunoprophylactic against *Aeromonas salmonicida* in juvenile chinook salmon *Oncorhynchus tshawytscha*. Dis. Aquat. Org. 17:191-196.

Niki, L., Albright, L. J., and Evelyn, T. P. T. 1991. Influence of seven immunostimulants on the immune response of coho salmon to *Aeromonas salmonicida*. Dis. Aquat. Org. 12:7-12.

Nonaka, M., Yamaguchi, N., Natsuume-Sakai, S., and Takashashi, M. 1981. The complement system of rainbow trout (*Salmo gairdneri*). I. Identification of the serum lytic system homologous to mammalian complement. J. Immunol. 126:1489-1494.

Nowotny, A. 1984. Heterogeneity of endotoxins. In "Handbook of Endotoxin. Vol.I. Chemistry of Endotoxin." E. T. Rietschel (Ed.). Elsevier Science Publishers, Amsterdam. pp. 308-338.

Old, L. J. 1988. Tumor necrosis factor. Scientific American. 258:59-75.

Olivier, G., Evelyn, T. P. T. and Lallier, R. 1985. Immunity to *Aeromonas salmonicida (Oncorhynchus Kisutch)* induced by modified Freund's complete adjuvant : its non-specific nature and the probable role of macrophages in the phenomenon. Develop. Comp. Immunol. 9:419-432.

O'Neill, J. G. 1985. An *in vitro* study of polymorphonuclear phagocytosis and the effect of temperature. In " Fish Immunology." M. F. Tatner and M. J. Manning (Ed's). London, Academic Press. pp.47-55.

Ornellas, E. P. Roantree, R. J. and Steward, J. P. 1970. The specificity and importance of humoral antibody in the protection of mice against intraperitoneal challenge with complement-sensitive and complement-resistant *Salmonella*. J. Infect. Dis. 121:113-123.

Ostrowsky, N. 1993. Liposome size measurements by photon correlation spectroscopy. Chem. Phys. Lipids. 64:45-56.

Palva, E. T., and Makela, E. H. 1980. Lipopolysaccharide heterogeneity in *Salmonella typhimurium* analyzed by sodium dodecyl sulfate/ polyacrylamide gel electrophoresis. Eur. J. Biochem. 107:137-143.

Park, Y. S., Maruyama, K., and Huang, L. 1992. Some negatively charged phospholipid derivatives prolong the liposome circulation *in vivo*. Biochim. Biophys. Acta. 1108:257-260.

Papahadjopoulos, D., and Walkins, J. C. 1967. Phospholipid model membrane, II. Permeability properties of hydrated liquid crystals. Biochim. Biophys. Acta. 135:639-652.

Patterson, W. D., and Fryer, J. L. 1974. Effect of temperature and antigen dose on the antibody response of juvenile coho salmon (*Oncorhynchus kisutch)* to *Aeromonas salmonicida* endotoxin. J. Fish. Res. Board Can. 31:1743-1749.

Petrov, A. B., Semenov, B. F., Vartanyan, Y. P., Zakirov, M. M., Torchilin, V. P., Trubetskoy, V. S., Koshinka, N. V., L'Vov, V. L., Verner, I.

K., Lopyrev, I. V. and Dmitriev, B. A. 1992. Toxicity and immunogenicity of *Neisseria meningitidis* lipopolysaccharide incorporated into liposomes. Infection and immunity. 60(9):3897-3903.

Pfeiffer, R. 1892. Z. Hyg. Infectionskrankh. 11:393.

Philips, N. C. Skamene, E. and Tsoukas, C. 1991. Liposomal encapsulation of 3'-azido-3'-deoxythymidine (AZT) results in decreased bone marrow toxicity and enhanced activity against murine AIDS-induced immunosupression. J. Acquired Immune Deficiency Syndrome. 4:959-966.

Porreau, C. N., Evenberg, D., De Raadt, W. M. Van Mechelen, J. A. N. and Van Muiswinkel, W. B. 1986. Does *Aeromonas salmonicida* affect the immune system of carp, *Cyprinus carpio L.* ? Vet. Immunol. Immunopathol., 12:331-338.

Poste, G., Bucana, C., and Raz, A. 1981. Analysis of the fate of systemically administered liposomes and implication for their use in drug delivery.

Cancer Research. 42:1412-1422.

Pouchert, C. J. 1981. The Aldrich Library of NMR Spectra. Edition II, Volume 2. United States of America Library of Congress.

Power, C. A., Markham, R. J. F. and Donald, A. W. 1990. Uptake and tissue distribution after intraperitoneal administration to rainbow trout, *Oncorhynchus mykiss* (Richardson): a preliminary report. J. Fish Dis. 13:329-332.

Poznansky, M. J. and Juliano, R. L. 1984. Biological approaches to the controlled delivery of drugs: A critical review. Pharmacol. Rev. 36:277-336.

Price, N. C., Stevens, L., Duncan, D., and Snodgrass, M. 1989. Proteases secreted by strains of *Aeromonas salmonicida*. J. Fish Dis. 12:223-232.

Proffitt, R. T., Williams, L. E., Presant, C. A., Tin, G. W., Uliana, J. A., Gamble, R. C., and Baldeschweiler, J. D. 1983. Liposomal blockade of

the reticuloendothelial system: Improved tumor imaging with small unilamellar vesicles. Science. 220:502-505.

Qureshi, N., Takayama, K., Mascagni, P., Honovich, Wong, R. and Cotter, R. J. 1988. Complete structural determination of lipopolysaccharide obtained from deep rough mutant of *Escherichia coli*. J. Biol. Chem. 263 (24): 11971-11976.

Racker, E. 1973. A new procedure for the reconstitution of biologically active phospholipid vesicles.

Raz, A., Bucana, C., Folger, W. E., Poste, G, and Fidler, I. J. 1981. Biochemical, morphological, and ultrastructural studies on the uptake of liposomes by murine macrophages. Cancer Res. 41:487-494.

Rietschel, E. Th., Kirikae, T., Schade, F. U., Mamat, U., Schmidt, G., Loppnow, H., Ulmer, A. J., Zahringer, U., Seydel, U., Di Padova, F., Schreir, M., Brade, H. 1994. Bacterial endotoxin: molecular relationships of structure to activity and function. FASEB J. 8:217-225.

Rietschel, E. T., and Brade, H. 1992. Bacterial endotoxins. Scientific American. 267:54-61.

Rietschel, E. T., Schade, U., Luderitz, O., Fischer, H., and Peskar, B. A. 1980. Prostaglandins in endotoxicosis. In " Microbiology-1980 ". D. Schlessinger (Ed.). American Society for Microbiology, Washington D.C. p.66-72.

Rijkers, G. T. 1981. Introduction to fish immunology. Develop. Comp. Immunol. 5:527-534.

Roberts, R. J. 1993. In "Bacterial Diseases of Fish." V. Inglis, R. J. Roberts and N. R. Bromage (ed's). Blackwell Scientific Publications, London. p. xvi-xvii.

Robertson, B., G. Rorstad, R. Engstad, and J. Raa. 1990. Enhancement of non-specific disease resistance in *Salmo salar* L., by a glucan from *Saccharomyces cerevisiae* cell walls. J. Fish Dis. 13:391-400.

Rockey, D. D., Fryer, J. L., and Rohovec, J. S. 1988. Separation and *in vivo* analysis of two extracellular proteases and the T-haemolysin from *Aeromonas salmonicida*. Dis. Aquatic Org. 5:197-205.

Rodgers, C. J. 1990. Immersion vaccination for control of fish furunculosis. Dis. Aquat. Org. 8:69-72.

Sanchez, C., and Dominguez, J., 1991. Trout immunoglobulin populations differing in light chains revealed by monoclonal antibodies. Mol. Immunol. 28:1271-1277.

Sanchez, C., Coll, J. and Dominguez, J., 1989. One-step purification of the major rainbow trout immunoglobulin. Vet. Immunol. Immunopathol. 27:383-392.

Sanchez, C., Dominguez, J., and Coll, J. 1989. Immunoglobulin heterogeneity in the rainbow trout *Salmo gairdneri* Richardson. J. Fish Dis. 12:459-465.

Saukkonen, K., Leinonen, M., Kayhty, H., Abdillahi, H., and Poolman, J. T. 1988. Monoclonal antibodies to rough lipopolysaccharide of *Neisseria meningitidis* protects infant rats from meningococcal infection. J. Infect. Dis. 158:209-212.

Schroit, A. J., Tanaka, Y., Madsen, J., and Fidler, I. J. 1984. Biol. Cell. 51:227-238.

Sculier, J-P., Brassine, C., Laudron, C., Delcroix, C., and Hollaert, C. 1989. Phase I study with pharmacokinetic analysis, of intravenous administration of 6-aminochrysene entrapped in sonicated liposomes in patients with advanced cancer. J. Liposome Res. 1:177-193.

Selser, J. C., Yeh, Y., and Baskin, R. J. 1976. Biophys. J. 16:337.

Senior, J., Delgado, C., Fisher, D., Tilcock, C. and Gregoriadis, G. 1991a. Influence of surface hydrophilicity of liposomes on their interaction with plasma protein and clearance from the circulation: studies with poly(ethylene glycol)-coated vesicles. Biochim. Biophys. Acta 1062:77-

82.

Senior, J. H., Trimble, K. R., and Maskiewicz, R. 1991b. Interaction of positively-charged liposomes with blood: implications for their application *in vivo*. Biochim. Biophys. Acta. 1070:173-179.

Senior, J., and Gregoriadis, G. 1982. Stability of small unilamellar vesicles in serum and clearance from the circulation: the effects of the phospholipid and cholesterol components. Life Sci. 30:2123-2136.

Shaw, D. H., Hart, M. J. and Luderitz, O. 1992. Structure of the core oligosaccharide in the lipopolysaccharide isolated from *Aeromonas salmonicida* ssp. *salmonicida*. Carbohydr. Res. 231:83-91.

Shaw, D. H., Lee, Y., Squires, J. M., and Luderitz, O. 1983. Structural studies on the O-antigen of *Aeromonas salmonicida*. Eur. J. Biochem. 131:633-638.

Shek, P. N. 1984. Applications of liposomes in immunopotentiation. In

"Immunotoxicology". Mullen, P. W. (Ed.). Springer-Verlag, Berlin. pp.103-125.

Snieszko, S. F. 1972. Progress in fish pathology in this century. Symposium of the Zoological Society of London. 30:1-14.

Snieszko, S. F., Griffin, P. J., and Friddle, S. B. 1950. A new bacterium (*Haemophilus piscium* n.sp.) from ulcer disease in trout. J. Bacteriol. 59:699-710.

Snieszko, S. F., and Bullock, G. L. 1957. Treatment of sulfonamide resistant furunculosis in trout and determination of drug sensitivity. U.S. Fish Wildl. Fish. Bull. 57:555-564.

Stedmans Medical Dictionary. Illustrated 23rd Edition. 1977.

Stolen, J. S., Gahn, T., Kaspar, V., and Nagle, J. J. 1984. The effect of environmental temperature on the immune response of a marine teleost (*Paralichthys denatus*). Dev. Comp. Immunol.8:89-98.

Su, D. and Van Rooijen, N. 1989. The role of macrophages in the immunoadjuvant action of liposomes: effects of elimination of splenic macrophages on the immune response against intravenously injected liposome-associated albumin antigen. Immunology. 66:466-470.

Su, S., McNamara Ward, M., Apicella, M. A., and Ward, R. E. 1990. Analysis of the immune response to LPS. J. Immunol. 145:2994-3001.

Suzuki, K. 1984. A light and electron microscope study on the phagocytosis of leucocytes in rockfish and rainbow trout. Bull. Jap. Soc. Sci. Fish. 50:1305-1315.

Szalai, A. J., Banoub, J. H., Nakhla, A. N., and Keough, K. M. W. An alternative procedure for the isolation of rainbow trout, *Oncorhynchus mykiss*, serum immunoglobulin. J. Appl. Aquacullture. In press.

Szoka, F., and Papahadjopoulos, D. 1978. Procedure for preparation of liposomes with large internal aqueous phase and high capture by reverse-phase evaporation. Proc. Natl. Acad. Sci. USA. 75:4194-4198.

Tatner, M. F. 1990. Antigen-induced blastogenesis of salmon, *Salmo salar L.*, head kidney leucocytes to modified *Aeromonas salmonicida* antigens: a preliminary evaluation method for potential vaccine antigens. J. Fish Biol. 36:731-739.

Taylor, L. R. 1961. Aggregation, variance and the mean. Nature 189:732.

Therien, H.-M., Shahum, E., and Fortin, A. 1991. Liposome adjuvanticity: Influence of dose and protein:lipid ratio on the humoral response to encapsulated and surface-linked antigen. Cell. Immunol. 136:402-413.

Thornton, J. C., Garduno, R. C., and Kay, W. W. 1994. The development of live vaccines for furunculosis lacking the A-layer and O-antigen of *Aeromonas salmonicida*. J. Fish Dis. 17:195-204.

Titball, R. W., and Munn, C. B. 1985. The purification and some properties of H-lysin from *Aeromonas salmonicida*. J. Gen. Microbiol. 131:1603-1609.

Titball, R. W., and Munn, C. B. 1983. Partial purification and properties of a haemolytic activity (T-lysin) from *Aeromonas salmonicida*. FEMS Microbiol. Letters. 20:207-210.

Tom, B. H. 1981. Liposomes and immunology: macrophages, liposomes, and tailored immunity. In " Liposomes and Immunobiology." B.H. Tom and H.R. Six (Eds.). Elsevier, North-Holland, Amsterdam, p.3-22.

Towbin, H., Staehelin, T. & Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. Proc. Nat. Acad. Sci. 76:4350-4354.

Treat, J., Rah, J. K., Wooley, P. V., Neefe, J., Schein, P. S., and Rahman, A. 1987. A phase I study: liposome encapsulated doxorubicin (LED). Am. Soc. Clin. Oncol. 7:41.

Trump, G. N., and Hildemann, W. H. 1970. Antibody responses of goldfish to bovine serum albumin: primary and secondary responses. Immunol. 19:621-627.

Tsai, C. and Frasch, C. E. 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. 119:115-119.

Vaage, J., Donovan, D., Mayhew, E., Uster, P., and Woodle, M. 1993. Therapy of mouse mammary carcinomas with vincristine and doxorubicin encapsulated in sterically stabilized liposomes. Int. J. Cancer. 54:959-964.

Vallejo, A. N., Miller, N. W. and Clem, L. W. 1992. Cellular pathway(s) of antigen processing in fish APC: effect of varying in vitro temperatures on antigen catabolism. Dev. Comp. Immunol. 16:367-381.

van Rooijen, N., and Su, D. 1989. Immunoadjuvant action of liposomes: mechanisms. In "Immunological adjuvants and vaccines". Gregoriadis, G., Allison, A. C., and Poste, G. (Ed's.). Plenum Press, New York. pp.95-106.

Van-Seuningen, I., and Davril, M. 1992. A rapid periodic acid-Schiff staining procedure for the detection of glycoproteins using the PhastSystem.

Electrophoresis. 13:97-99.

Vaughan, L. M., Smith, P. R., and Foster, P. J. 1993. An aromatic dependent mutant of the fish pathogen *Aeromonas salmonicida* is attenuated in fish and is effective as a live vaccine against the salmonid disease furunculosis. Inf. Immun. 61:2172-2181.

Velji, M. I., Albright, L. J. and Evelyn, T. P. T. 1990. Protective immunity in juvenile coho salmon *Oncorhynchus kisutch* following immunization with *Vibrio ordali* lipopolysaccharide or from exposure to *V. ordalii* cells. Dis. Aquat. Org. 9:25-29.

Voss, E. W. Jr., Groberg, W. J. and Fryer, J. L., 1980. Metabolism of coho salmon Ig. Catabolic rate of coho salmon tetrameric Ig in serum. Mol. Immunol. 17:445-452.

Weissman, I. L., and Cooper, M. D. 1993. How the immune system develops. Scientific American. 269:64-71.

Westphal, O., and Jann, K. 1965. Bacterial Lipopolysaccharides: Extraction with phenol-water and further applications of the procedure. Methods Carbohydrate Chem. 5:83-91.

Wicken, A. J. and Knox, K. W. 1980. Bacterial cell surface amphiphiles. Biochim. Biophys. Acta. 604:1-26.

Wong, G., Kaattari, S. L. and Christensen, J. M. 1992. Effectiveness of an oral enteric coated Vibrio vaccine for use in salmonid fish. Immunol. Invest. 21(4):353-364.

Wong, J. P., Cherwonogrodzky, J. W., Di Ninno, V. L., Stadnyk, L. L. and Knodel, M.H. 1992. Liposome potentiation of humoral response to lipopolysaccharide and O-polysaccharide antigens of *Brucella abortus*. Immunology. 77:123-128.

Woodle, M. C. and Papahadjopoulos, D. 1989. Liposome preparation and size characterization. Meth. Enzymol. 171:193-217.

Wright, S. D., Ramos, R. A, Hermanowski-Vosatka, A., Rockwell, P., and Detmers, P. A. 1991. Activation of adhesive capacity of CR3 on neutrophils by endotoxin: dependance on lipopolysaccharide binding protein and CD14. J. Exp. Med. 173:1281-1291.

Wright, S. D., Tobias, P. S., Ulevitch, R. J., and Ramos, R. A. 1989. Lipopolysaccharide binding protein opsonizes LPS-bearing particles for recognition by a novel receptor on macrophages. J. Exp. Med. 170:1231-1242.

Yamauchi, H., H. Kikuchi, K. Yachi, M. Sawada, M. Tomikawa, and Hirota, S. 1993. Effects of glycophorin and ganglioside GM_3 on the blood circulation and tissue distribution of liposomes in rats. Intl. J. Pharmaceutics. 90:73-79.

Yano, T., Yatsuyama, H., and Mangindaan, R.E.P. 1991. Polysaccharide-induced protection of carp, *Cyprinus caprio L.*, against bacterial infection. J. Fish Dis. 14: 577-582.

Yasutake, W. T., and Wales, J. H. 1983. Microscopic anatomy of salmonids: An Atlas. U.S. Dept. Interior, Fish and Wildlife Service. Resource Publication 150:190-214.

Zacharius, R. M., Zell, T. E., Morrison, J. H., and Woodlock, J. J. 1969. Glycoprotein staining following electrophoresis on acrylamide gels. Anal. Biochem. 30:148-152.

APPENDIX I

DETAILED METHODS FROM CHAPTER 2

1.1. Growth of Bacterial Cells from *Aeromonas salmonicida*.

The culture of a virulent autoagglutinating strain of *Aeromonas salmonicida* ssp. *salmonicida* was provided by Dr. T. P. T. Evelyn of the Dept. of Fisheries and Oceans, Nanaimo Biological Station, Nanaimo, British Columbia, Canada. The strain was isolated from Sockeye salmon and given the strain number B1-2-399 and was added to the collection at the Northwest Atlantic Fisheries Centre, St. John's, Newfoundland, Canada as SJ-15. The bacterial cells were usually grown in the presence of Antifoam A™ in order to prevent the loss of media due to bubble formation although in some instances it was necessary to grow the cells in the absence of Antifoam A™.

Growth of Bacterial Cells In the Presence of Antifoam A™;

1. 1 ml of a 15% solution of the bacterium in glycerol is inoculated into 24-1 of Trypticase Soy Broth (Baltimore Biological Laboratory). Antifoam A™ (Sigma Chemical Co., St. Louis, MO, USA) emulsion is added in the culture broth at a final concentration of 0.1% (v/v).

2. The cultures are grown for a period ranging from 16 to 18 h with the temperature is maintained at 24°C and aeration at 20-1/min.

3. The culture is killed by adding formaldehyde to a final concentration of 0.3% and the suspension is stirred for 18 hours at room temperature.

4. Cells are harvested by centrifugation at 20,000 rpm in a Sorvall SS-34 rotor (48,200 x g), washed once with 0.15 M NaCl, lyophilized and stored at -15°C until required.

Growth of Bacterial Cells In the Absence of Antifoam A™;

1. 1 ml of a 15% solution of the bacterium in glycerol is inoculated into 24-1 of Trypticase Soy Broth.

2. The culture is separated into 8 4 l Erlenmayer flasks and are incubated on an orbital shaker overnight at 24°C.

3. Prior to harvesting, bacteria are killed by exposure to formalin at a final concentration of 0.3 % by volume, and the broth is stirred for an additional 18 h.

4. Formaldehyde-fixed cells are collected by centrifugation at 20,000 rpm in a Sorvall SS-34 rotor (48,200 x g) washed once with 0.15 M NaCl, lyophilized and stored at -15°C until required.

1.2. Extraction of LPS from *A. salmonicida*.

1. Freeze-dried cells (20-g) are suspended in water (350-ml) and heated to 70°C.

2. An equal amount of 90% phenol, also at 70°C is added and the mixture is stirred for 15 min. with the temperature maintained above 65°C.

3. After cooling to 10°C, the solution is centrifuged at 3500 rpm in a Sorvall SS-34 rotor (1475 x g) and the aqueous layer separated by aspiration.

4. This procedure is repeated twice more and the aqueous layers are combined and

dialyzed against running tap water until no trace of phenol remained.

5. The dialysate is reduced in volume by evaporation under reduced pressure and centrifuged at 39,000 rpm (105,000 x g) for 3 hours.

6. The supernatant is discarded and the LPS pellet is resuspended in distilled water.

7. Centrifugation is repeated twice more and the pellet is resuspended in distilled water and lyophilized.

1.3. Further Purification of LPS using Polymyxin Beads.

1. A slurry containing 3 ml of polymyxin coated Affi-prep beads (Bio-Rad Laboratories, Richmond, CA, USA) is washed twice using a benchtop centrifuge (set at 2500 x g_{max}) with 0.1 M NaOH and once again in distilled H_2O.

2. LPS from *A. salmonicida* is suspended in phosphate-buffered saline (PBS, Ph 7.4) to a final concentration of 50 mg in 15 ml.

3. The LPS solution is mixed with the washed polymyxin beads and the suspension is shaken overnight at room temperature on an orbital shaker (set at 1500 rpm).

4. The suspension is centrifuged at 2500 x g_{max} (benchtop) and the supernatant containing the non-adherent material is decanted.

5. The adherent LPS is recovered from the polymyxin beads by resuspension of the beads in 15 ml of 0.1 M NaOH followed by vortexing and incubation at room temperature for 10 min.

6. The suspension is centrifuged at 2500 x g_{max}.

7. The supernatant is collected and the pellet is washed once more with 5 ml of 0.1 M NaOH and centrifuged as before.

8. The supernatants are combined and the polymyxin-purified LPS is dialysed against 6 changes of distilled H_2O (3.5 L per change) and lyophilized.

1.4. Hydrolysis of LPS with Acetic Acid.

1. 100 mg of LPS from *A. salmonicida* is suspended in 100 ml of 1% Acetic acid in a 250 ml round bottom flask.

2. The solution is refluxed for 2 h at 100°C.

3. Cool the solution to room temperature.

4. Centrifuge the solution at 3000 x g (5000 rpm in a Sorvall SS-34 rotor) for 30 min. at room temperature.

5. The supernatant (polysaccharide) is refrigerated.

6. Resuspend the pellet (lipid A) in distilled H_2O and centrifuge as above.

7. Remove the supernatant and add to the previous supernatant.

8. Repeat 6 and 7. Dialyse combined supernatants and the resuspended pellet against 6 changes of distilled H_2O overnight (3.5 L per change).

9. Lyophilize to obtain powdered lipid A and polysaccharide moieties.

1.5. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE, Laemmili, 1979)

Discontinuous gel electrophoresis is performed using the BIO-RAD Mini-PROTEAN II dual slab cell system (Richmond, CA, USA). Gel preparation and electrophoretic procedures are performed according to the BIO-RAD Mini-PROTEAN II manual. All stock solutions are prepared as described in the BIO-RAD Mini-PROTEAN II manual (p 16-17).

1. The SDS gels are prepared with a 4% acrylamide stacking gel and either a 12.5 % acrylamide separating gel in reducing conditions (sample buffer contains 2% 2-mercaptoethanol) or a 5 % acrylamide separating gel in non-reducing conditions (no 2-mercaptoethanol in sample buffer, see Mini-PROTEAN II manual p 16).

	5% Acrylamide	12.5% Acrylamide
Distilled H_2O	2.84 ml	1.59 ml
1.5 M Tris-Base, Ph 8.8	1.25 ml	1.25 ml
10% (w/v) SDS	50 μl	50 μl
30% Acrylamide/Bis	0.835 ml	2.08 ml
10% ammonium persulfate	25 μl	25 μl
TEMED	2.5 μl	2.5 μl

2. After pouring, the gels are kept at 4°c overnight to polymerize.

3. The samples to be analysed are diluted 1:2 with sample buffer (see Mini-PROTEAN II manual p 16) and heated at 100°C for 5 min.

4. The samples are cooled to room temperature and applied to the gel using a micropipette.

5. The gels are electrophoresed at 200 Mv for approximately 40 min.

6. The gels are stained accordingly as follows;

1.6. Staining of gels with Coomasie Blue

1. The gels are fixed in 7% glacial acetic acid and 40% absolute ethanol for 20 min at room temperature.

2. The gels are stained for 20 min in Coomassie blue R-250 which was in fixative.

3. The gels are destained with 7% glacial acetic acid and 10% ethanol until the background stain disappears (usually overnight).

4. The gels are washed with H_2O and stored in plastic bags at 4°C in H^2O.

1.7. Silver Staining of gels

Gels are stained using the Daiichi Silver Stain-II ammoniacal silver stain kit with each step conducted at room temperature using an orbital shaker set at 1500 rpm as follows;

1. The gels are incubated in fixing solution I (25 ml MeOH, 5 ml AcOH, distilled 20

ml H_2O) for 10 min. Aspirate solution.

2. The gels are incubated in fixing solution II (15 ml MeOH, 5 ml AcOH, 2.5 ml Reagent 1, 27.5 ml distilled H_2O) for 15 min. Aspirate solution.

3. The gels are incubated in pretreatment solution (25 ml MeOH, 2.5 ml Reagent 2, 22.5 ml distilled H_2O) for 10 min. Aspirate solution.

4. The gels are washed with distilled H_2O for 5 min.

5. The gels are incubated in silver staining solution (2.5 ml Reagent 3, 2.5 ml Reagent 4, 45 ml distilled H_2O) for 15 min. Aspirate solution.

6. The gels are washed with distilled H_2O for 2 min (3X).

7. Add developer (2.5 ml Reagent 5, 47.5 ml distilled H_2O) and monitor color development until appropriate intensity is obtained.

8. Add 2.5 ml stopper (Reagent 6). Aspirate solution. Store gels in plastic bags at 4°C. in H^2O.

1.8. Periodic Acid-Schiff (PAS) staining of gels (Van Seuningen and Davril, 1992).

1. Gels are fixed in 25 ml 20% trichloroacetic acid (TCA) for 5 min at room temperature on an orbital shaker set at 1500 rpm. Aspirate.

2. Wash with distilled H_2O for 5 min.

3. Incubate gels with 25 ml of a solution containing 0.7% periodic acid and 5% AcOH for 10 min at room temperature on an orbital shaker. Aspirate.

4. Wash with distilled H_2O for 2 min (2X).

5. Incubate gels in 25 ml Schiff reagent (Sigma Chemical Co.) for 10 min. at room temperature on an orbital shaker. Aspirate.

6. Incubate gels in 25 ml of a solution containing 5% potassium bisulfite and 5% AcOH for 5 min. at room temperature on an orbital shaker. Aspirate.

7. Incubate gels in 25 ml of a solution containing 5% MeOH and 7.5% AcOH for 10 min. at 50°C. Aspirate.

8. Repeat 7. for 5 min. at 50°C. Aspirate.

9. Incubate gels in 25 ml of a solution containing 50% MeOH and 30% AcOH for 10 min. at 50°C. Aspirate.

10. Repeat 9. for 5 min. at 50°C. Aspirate.

11. Repeat 10. for 2 min. at 50°C. Aspirate.

12. Incubate gels in 25 ml of a solution containing 5% glycerol and 10% AcOH for 10 min. at 50°C. Aspirate. Store gels in plastic bags at 4°C in H^2O.

1.9. Methylation of Polysaccharides.

1. Place the sample (1-2 mg), DMSO (0.2-0.4 ml) and a stirrer bar in a 6 ml hypo vial. Cap with a teflon liner.

2. Flush the sample with N_2 for 20 sec.

3. Stir the sample at room temperature (ultrasonicate if necessary) until a clear solution is obtained.

4. Add 0.4 ml of the methylsulfinyl carbanion to the sample (prepared as described below). A gel is formed.

Preparation of Methylsulfinyl Carbanion.

- NaH/oil (50 mg) is washed 3X with dry (Na) benzene (1 ml X 3).
- Add DMSO (1 ml), stirrer bar and flush with N_2 for 20 sec.
- Stir at 50-60°C for 1 hr. with 23 G needle through stopper to facilitate the removal of H_2. Yields a clear green solution.

5. Stir the reaction mixture at room temperature for 1 h with a 23g needle through the liner at which time a clear solution results.

6. Add 1 ml of CH_3I (use a glass syringe) to the reaction mixture and remove the needle from the liner.

7. Stir the reaction mixture at room temperature for 4 h.

8. Pass the solution through a column (1 x 20 cm) which has been packed with Sephadex LH-20 (approximately 5g in $CHCl_3$).

9. Elute with $CHCl_3$ and collect 1 ml fractions. Stop at first yellow band.

10. Spot the fractions on silica gel plate, spray with 5% H_2SO_4 and char.

11. Combine the appropriate material. Evaporate the combined fractions to dryness.

1.10. Preparation of Acetylated Alditols.

1. Hydrolyze the sample to be acetylated (5 mg) with 1M TFA at 100°C overnight.

2. Evaporate the hydrolysate to dryness and co-evaporate with water 3X.

3. Reduce the sample with $NaBH_4$ (about 50 mg) for 1 hr at room temperature.

4. Destroy excess borohydride with CH_3COOH and evaporate to dryness.

5. Evaporate the sample from CH_3COOH/CH_3OH (1 ml/40 ml) 3 times.

6. Acetylate the sample with $C_5H_5N/(CH_3CO)_2O$ at 100°C for 30 min.

7. Evaporate the sample to dryness and evaporate 3 times from CH_3OH followed by 2 times from $CHCl_3$.

8. Filter the sample into a vial and run on a gas chromatograph. Gas chromatography is conducted using a CP-Sil-5CB column (Chrompack, The Netherlands) with the temperature set at 150° for 15 min and is increased at 5°/min until to 250°. The temperature is held for 5 min.

The carrier gas is helium with a flow rate of 1-ml/minute.

1.11. Preparation of Samples for Coupled Gas Chromatography-Electron Impact Mass Spectrometry (GC-EIMS).

1. Samples of LPS (10-mg) are hydrolyzed for 16 h in 1% trifluoroacetic acid (TFA) at 100°C.

2. The hydrolysate is evaporated to dryness, washed 3 times with distilled water and reduced for 1 hour with $NaBH_4$ as described above.

3. The reaction is stopped by the addition of glacial acetic acid and the solution is evaporated to dryness and washed 3 times with a solution of 5% acetic acid in MeOH to facilitate the removal of borate as methyl borate.

4. The product is acetylated by heating at 100°C in a 1:1 mixture of acetic anhydride: pyridine for 30 min.

5. Evaporate to dryness and co-evaporate with $CHCl_3$ until no trace of pyridine can be detected.

6. Filter the product through a sintered glass funnel. Take up in minimal volume of $CHCl_3$ and inject into GC-MS.

GC-EIMS Conditions.

The apparatus consists of a Hewlett-Packard 5970 mass selective detector coupled with a model 5890 gas chromatograph and a model 300 data system from the same manufacturer. The column is a CP-Sil-5 CB, WCOT fused silica with a length of 25-m, inside diameter 0.25-mm, outside diameter 0.39 mm and a film thickness of 0.12-um (ChromPack, The Netherlands). Chromatographic conditions are as follows: initial temperature of 165°C is held for 15 min and increased to 250°C at the rate of 5°C/minute and then held for 10 min. The carrier gas is helium with a flow rate of 1-ml/minute and a split ratio of 50:1. Injector temperature and transfer line are both maintained at 275°C.

1.12. Conditions for ^{1}H-Nuclear Magnetic Resonance (^{1}H-NMR).

Nuclear magnetic resonance analyses are conducted at ambient temperature (25°C) using a Varian Gemini 300 spectrometer in the pulsed Fourier Transform mode at 300 MHz at a pulse width of 90 degrees and a spectral width of 4500 Hz. The acquisition time is 1.778 sec. for 1600-10000 transients. ^{1}H spectra are obtained using 5 mm-thick walled tubes and either D_2O of D_6-DMSO as a solvent (Sigma Chemical Co.). Chemical shifts are reported relative to that of external tetramethylsilane (TMS).

1.13. Enzyme-linked Immunosorbent Assay (ELISA)

The levels of anti-LPS antibody in trout sera are estimated using an ELISA:

1. LPS from *A. salmonicida* is made up to 100 μg/ml in carbonate-bicarbonate coating buffer (Na_2CO_3, 1.59 g; $NaHCO_3$, 2.93 g; NaN_3, 0.2 g; add H_2O to a final volume of 1 L; pH=9.6).

2. 100 μl of LPS are added to each well of a 96 well pollystyrene microtitre plate (Linbro Titertek, Horsham, Pa, USA) and plates are incubated overnight at 4°C.

3. The next morning, excess buffer is discarded and the plates are blocked with 100 μl/well of 0.5 % gelatin in PBS (NaCl, 8.0 g; KH_2PO_4, 0.20 g; Na_2HPO_4, 1.15 g; KCl, 0.2 g; add H_2O to a final volume of 1 L; pH=7.4) for 1 hour at room temperature.

4. The plates are washed five times with PBS containing 0.05% Tween and test

antisera (serially diluted in 0.25% gelatin in PBS with 0.05% Tween [diluent]) are added. Trout sera reactive with LPS from *A. salmonicida* (donated by Dr. Joseph Banoub, Department of Fisheries and Oceans, Canada) and diluent are added to each plate as positive and background controls, respectively. Pre-bleed sera (normal trout sera, NTS) is added to the plates periodically as negative controls. The plates are incubated overnight at 4°C.

5. The next morning, the plates are washed as before and 100 μl of a 1% (v/v in diluent) solution of monoclonal antibody I-14 (mouse (IgG) anti-trout IgM) is added to each well and the plates are incubated at room temperature for 2 h. The hybridoma was kindly donated by Dr. Gregory Warr, University of South Carolina, and the hybridoma cells were cultured and the supernatants were prepared by Dr. Szalai (see Alex's lab book entitled Cell Culture), who was in our laboratory at the time and is presently at the University of Alabama.

6. Plates are washed as before and 100 μl of rabbit anti-mouse immunoglobulin conjugated with horseradish peroxidase (prepared as a 1:2000 solution in diluent) is added to each well and the plates are incubated at room temperature for 90 min.

7. After final washing, 100 μl of hydrogen peroxide substrate solution (0.1% H_2O_2, + 2% ABTS [2,2'-azino-*bis* (ethylbenzthiazoline-6-sulfonic acid)], 15 mg/ml in H_2O) in citrate buffer, pH 4.0, is added to each well and the reaction is allowed to proceed at room temperature for 30 minutes. The OD was read at 405 nm on a ELISA plate reader (BIO-RAD, Richmond CA).

8. The wells which represented the background controls are averaged and subtracted from all other wells. Anti-LPS antibody titres are defined as the highest dilution of antisera giving an optical density at 405 nm greater than 0.02 after the values have been corrected.

APPPENDIX II

DETAILED METHODS FROM CHAPTER 3

2.1. Radiolabelling of LPS from *A. salmonicida* by a modification to the method of Laude-Sharp *et al* (1990).

1. 50 mg of LPS are oxidized with 10 ml of 0.2 M $NaIO_4$ for 20 minutes with stirring at room temperature.

2. Ethylene glycol (2 ml) is added to the reaction mixture and the solution is dialysed against 6 changes of distilled H_2O overnight (3.5 l per change).

3. The partially oxidized LPS is reduced at 4°C overnight with 5mCi (0.01 mmole) NaB^3H_4 in 0.5 M borate buffer, Ph 9.0.

4. Excess NaB^3H_4 is neutralized with 1% acetic acid, and the sample is centrifuged at 290,000 x g_{max}.

5. Pellet is resuspended in distilled H_2O and dialysed against 6 changes of H_2O overnight (3.5 l per change) to remove any non-LPS material.

6. Free residual radiolabel was removed by passage of dialysed ^{3}H-LPS through a PD-10 desalting column (1.5 x 8 cm, Pharmacia LKB Biotechnology, Uppsala, Sweden).

7. Purified ^{3}H-LPS was assayed for carbohydrate by the phenol-sulphuric acid procedure of Dubois *et al.* (1956) (see below) and its radioactivity was determined using a Beckman LS 1801 liquid scintillation counter. Purified ^{3}H-LPS had a final

specific activity of 0.16 μCi/μg LPS.

2.2. Carbohydrate Determination by the Method of Dubois *et al.* (1956)

1. Appropriate volumes of test samples are added to disposable borosilicate glass tubes (16 x 150 mm).

2. Standard solutions used are either LPS or PS from *A. salmonicida* in distilled H_2O at a final concentration of 100 μl/ml. Appropriate volumes of standard solution are added to disposable borosilicate tubes to allow construction of a standard curve ranging from 25-100 μg LPS or PS.

3. The volume of all tubes is brought to 2 ml with distilled H_2O.

4. Under the fumehood, add 1 ml of 5% phenol solution to all tubes. Vortex.

5. Under the fumehood, add 5 ml of conc. H_2SO_4 to all tubes. Vortex.

6. Let tubes cool to room temperature (approx. 45 min.).

7. Optical density is measured with a spectrophotometer (LKB Biochrom 4049 Novaspec) at 490 nm which has been calibrated with water.

Samples are assayed in duplicate or triplicate and the carbohydrate concentration is determined from the average of these values. When liposomal LPS or PS samples are being assayed, O.D. values are corrected for the contribution of lipid to the assay as follows;

1. Determine the amount of phosphorous in each liposomal sample using the modified Bartlett assay described below. Assay are performed in triplicate and values are averaged.

2. The appropriate amount (from modified Bartlett assay) of lipid is added to borosilicate tubes and perform steps 3-7 described above. Determine the contribution of the given amount of lipid to the assay (i.e. O.D. at 490 nm).

3. Subtract that value from the O.D. value at 490 nm for the liposomes containing LPS or PS.

2.3. Phosphorus Determination by a Modification of the Method of Bartlett (1959) as described by Keough and Kariel, (1987).

1. Phosphorus standard (1 and 2 ml of 2 μg/ml solution in H_2O) is added in duplicate to chromerged digestion tubes. Distilled H_2O is added to a final volume of 9 ml and vortexed.

2. Liposome samples (2 - 5 μl) to be analysed are added in triplicate to chromerged digestion tubes.

3. 1 ml of 70% perchloric acid and anti-bumping granules are added to all tubes.

4. The liposome samples are digested by boiling at maximum heat for 12 minutes under the fumehood. Standard samples (prepared in 1.) are left under the fumehood at room temperature.

5. The liposome samples are allowed to cool under the fumehood and distilled H_2O is

added to a final volume of 9 ml. Vortex.

6. 0.5 ml of 5% ammonium molybdate is added to all tubes. Vortex.

7. 0.5 ml ANSA (1-amino-2-naphthol-4-sulfonic acid, 0.25 %) is added to all tubes. Vortex.

8. All the tubes are immersed in boiling water for 12 minutes, removed and allowed to cool to room temperature.

9. Optical density is measured at 815 nm using a spectrophotometer which has been calibrated with water.

Phosphorus concentration is determined from the average of the O.D. values.

The lipid concentrations were determined by multiplying the phosphorus content by 30.18 for PG-containing liposomes and by 32.52 for SA-containing liposomes. These values were determined as follows;

For PC:CH:PG liposomes → 6:3:1 molar ratio,

total weight lipid = 6(770) + 3(387) + 1(770) = 6551

phosphorus/phospholipid = 7(31)/7(770) = 0.04026 and 1/0.04026 = 24.83

phospholipid/total lipid = 7(770)/6551 = 0.8227 and 1/0.8227 = 1.21.

→ total lipid = phosphorus x 24.83 x 1.21 = phosphorous x 30.18

Similarly, for PC:CH:SA liposomes, total lipid = phosphorous x 32.52

2.4. Preparation of Liposomally-Associated LPS and PS.

Dried lipid films composed of either PC: CH: PG or PC: CH: SA in a 6:3:1 molar ratio are prepared as follows;

1. Egg phosphatidylcholine (PC, 100 mg/ml in $CHCl_3$/MeOH, [9:1]), egg phosphatidylglycerol (PG, 10 mg/ml in $CHCl_3$/MeOH, [98:2], cholesterol (CH, 99%) and stearylamine (SA) were purchased from Sigma Chemical Co. Cholesterol and stearylamine solutions are made at concentrations of 50 and 10 mg/ml, respectively, in $CHCl_3$/MeOH, 2:1. Solutions are kept at -20°C until ready for use.
2. A total of 50 μmoles of lipid in a 6:3:1 molar ratio are dispensed into disposable glass vials. For PC:CH:PG, this is equivalent to 23.0 mg PC, 5.8 mg cholesterol, and 3.8 mg PG while for PC:CH:SA, the PG is replaced by 1.35 mg SA.
3. The solvent is evaporated under a continuous stream of nitrogen.
4. Resultant lipid films are held *in vacuo* overnight and stored at -20°C.

^{3}H-LPS and PS from *A. salmonicida* are incorporated into liposomes as follows;

1. The dried lipid films are warmed to room temperature and hydrated at room temperature with 1 ml of a solution of ^{3}H-LPS (1, 2.5 or 5 mg LPS/ml in 0.15 M NaCL containing 1.8×10^5 dpm), or PS (1, 2.5 or 5 mg PS/ml, no radioactive label) with mild agitation on an orbital shaker at 1500 rpm at room temperature for 1 hour.

2. The dispersions are then vigorously vortexed for 3 one-minute intervals resulting in the formation of MLV.

Large unilamellar vesicles are prepared from MLV at room temperature by extrusion through two stacked polycarbonate filters of 200 nm pore size (Nucleopore Corp., Pleasanton, Cal., USA) using an "Extruder" (Lipex Biomembranes, Vancouver, B.C., Canada) under pressures ranging from 250-650 lb/in^2.

1. The Extruder is assembled as directed in THE EXTRUDER manual. Using tweezers, two polycarbonate filters are placed shiny side up onto the drain disc. Polycarbonate filters are packed shiny side down. 2 ml of 0.15 M NaCl are passed through the EXTRUDER in order to wet the membranes.

2. The MLV containing LPS or PS (1-2 ml) is applied to the sample inlet port and extruded under pressure and collected from the sample outlet tube.

3. This procedure is repeated ten times in succession and the resulting vesicles referred to as LUVET$_{200}$ (Hope *et al.*, 1985), which are clearer than their multilamellar analogs.

Sonicated vesicles (SV) are obtained by placing MLV in a bath sonicator (Papahadjopoulos and Walkins, 1967; Liu and Huang, 1992) for two 12-minute periods with intermittent vortexing for 1 minute (Dijkstra *et al.*, 1988a).

Liposomal formulations with initial lipid compositions of either PC: CH: SA or PC: CH: PG are symbolized by + or - respectively, throughout the text.

Liposomes used for immunizations of rainbow trout are prepared by hydrating lipid films with 1 ml of LPS solution per vial (5 mg/ml in 0.15 M NaCL, no radioactive label).

2.5. Separation of Unincorporated LPS and PS from Liposomes.

Free PS is separated from liposomally-incorporated PS as follows;

1. The liposomes are suspended in 0.15 M NaCl and centrifuged for 1 h at 15,000 x g_{max} at 4°C in a Beckman 80 Ti rotor.
2. The pellets (liposomes) are resuspended in 0.15 M NaCl and centrifuged as described above.
3. Repeat 2.
4. The pellets are resuspended to a final volume of 1 ml using 0.15 M NaCl.
5. Total lipid phosphorous is determined by the modified Bartlett assay as described above. Total lipid is estimated from total lipid phosphorous as demonstrated above.
6. The liposomal formulations are assayed for carbohydrate by the method of Dubois *et al.* as described above using PS as a standard.
7. Incorporation ratios are calculated as μg PS/μg lipid in the liposomal formulations.

^{3}H-LPS or LPS is separated from liposomally-incorporated LPS by density gradient centrifugation using a discontinuous metrizamide gradient according to the method of Heath (1987) as follows;

1. 0.5 ml of liposomal dispersion in 0.15 M NaCl is mixed with 1 ml 10% (w/v) metrizamide in 5 ml polyallomer centrifuge tubes. Metrizamide is made in 0.077 M NaCl in order to achieve an osmotic strength of 280 mosm/l as calculated by; 10% Metrizamide → 0.127 M (127 mosm/l) + 0.077 M NaCl (154 mosm/l) → 280 mosm/l.

2. The suspension is carefully layered with 3 ml of 5 % metrizamide (w/v) which has been prepared by diluting the 10% solution 1:1 with 0.077 M NaCl.

3. The gradient is layered with 0.5 ml of 0.077 M NaCl.

4. When MLV are used, the gradients are centrifuged at 4°C for 2h at 150,000 x g_{max} in a Beckman SW 50.1 rotor (40,000 rpm) with no braking at the end of the run and the acceleration set at minimum. For extruded or sonicated vesicles, conditions are similar with the exception that 30% and 15% metrizamide are used for mixing and layering, respectively, and the ultracentrifugation process is for 16 hours.

5. Following centrifugation, the buoyant pellicles (liposomes) are collected from the gradients and the unincorporated LPS is recovered from the infranatant layers and as small pellets.

6. The liposomes and the unincorporated LPS are dialysed against 6 changes of H_2O overnight (3.5 l per change). The LPS is stored at 4°C.

7. The liposomes are diluted in 0.15 M NaCl and residual metrizamide is removed from the liposomes by centrifugation at 50,000 x g_{max} for 1 hour in a 60 Ti rotor.

8. The liposomes are dialyzed overnight against 6 changes of H_2O (3.5 l per change) of the resuspended pellets against 0.15 M NaCl.

9. Radioactivity associated with the liposomes is estimated with a Beckman LS 1801 liquid scintillation counter. The specific activity of the original LPS solution and the radioactivity present in the liposome preparations are used to calculate the concentration of LPS in the preparations. The lipid content in the buoyant pellicles are determined and incorporation ratios are calculated as μg LPS/ μg lipid.

APPENDIX III

DETAILED METHODS FROM CHAPTER 4

3.1. Digestion of Tissues from Rainbow Trout.

Twenty-four h after administration of radiolabelled materials (see the materials and methods section in chapter 4), rainbow trout are killed by lethal anaesthesia with benzocaine. The kidney, spleen, liver and a portion of muscle are removed and weighed. Muscle samples are taken from sites distal to the site of injection. Blood is collected from the caudal sinus and transferred immediately to heparinized vacutainer tubes. Plasma was separated from blood cells by centrifugation at 2000 x g_{max} at room temperature for 5 minutes. The samples are treated as follows in order to digest and bleach;

1. Subsamples of tissue (25-75 mg), packed blood cells (50-100 μl) and plasma (50-100 μl), are weighed and placed in 20 ml glass scintillation vials.
2. Digestion is achieved by adding 1 ml of 5 M NaOH is added to each sample and the samples are placed in a Fischer Isotemp oven at 70°C for 3h after which they are cooled to room temperature.
3. 0.5 ml of 60% $HClO_4$ and 0.5 ml of 30% H_2O_2 are added to each sample in order to achieve bleaching and to prevent foaming.
4. The samples are incubated overnight at room temperature in the dark.
5. 5 drops of freshly prepared 15% ascorbic acid is added to each sample as an antioxidant.

6. 200 μl of glacial AcOH is added to each sample to decrease chemiluminescence.

7. Samples are adjusted to a Ph between 5-6 with the addition of 3 M NaOH.

8. The samples are allowed to incubate 3-4 h at room temperature in the dark.

9. The vials are filled with Scintiverse E and placed at 4°C in the dark for 96 h.

10. DPM attributable to ^{3}H and ^{14}C for each subsample are estimated using a Beckman LS 1801 liquid scintillation counter.

N.B. Some samples of liver, blood cells and muscle cannot be completely freed of coloured material or particulate matter despite bleaching with $Hclo_4$ and H_2O_2 or prolonged digestion. This situation may cause high quenching in some samples which may cause high variability in DPM.

3.2. CALCULATIONS FOR ESTIMATION OF UPTAKE:

1. To account for detection of quenched ^{14}C emmisions in the ^{3}H channel, DPM are corrected using the ratio method for quench correction (Minch, 1989):

Samples containing only ^{14}C are used to obtain the ratio R = channel 1 cpm/channnel 2 cpm in the absence of ^{3}H counts in channel 1. This ratio is used to estimate the ^{14}C counts in channel 1 for the dual-labelled samples. For example, for an independantly measured R=0.400 and 400 cpm in channel 1 and 900 cpm in channel 2, the following calculation is made;

^{14}C cpm = 900 + 0.4(400) = 1060 cpm

^{3}H cpm = 400 - 0.4(400) = 240 cpm

DPM = cpm/efficiency

This calculation is based on the assumption that no ^{3}H counts appear in channel 2. R values and the correction calculations were performed for all samples.

2. DPM determined for each subsample is normalized (DPM^{*}) to account for differences in sample weight and body weight using the formula $DPM^{*}=DPM/Ws/Wb$, where Ws and Wb are sample weight and body weight, respectively.

3. Recovery of each isotope (R) from each rainbow trout is estimated using

$R= DPM_{total}/ D$, where D is the dose of radiolabel administered and

$DPM_{total}= \Sigma [(DPM/Ws) \times Wo]$ over all organs examined and Wo (g) is organ weight determined for the kidney, liver, and spleen. Wo is estimated for the muscle ($Wo=0.6 \times Wb$) and plasma and blood cells ($Wo= 0.025 \times Wb$).

4. DPM^{*}/R is calculated for each isotope in each organ. This value accounts for all sources of error and is referred to as uptake.

5. Statistical analyses are performed on uptake values using the Statistical Analysis System package (SAS, version 6.01). As the data were skewed, uptake values were transformed prior to parametric analysis using Taylor's Power Law (1961) as follows;

- For each set of data, plot $\log_{10}X$ vs. $\log_{10}s^2$.
- Determine the slope (B) for each plot.
- Normalize uptake values by calculating uptake$^{1-B/2}$.

6. Transformed uptake values were examined using the rank correlation procedure of Spearman (1904) and analysis of variance, ANOVA. Samples were considered significantly different from one another when $P < 0.05$. When Anova suggested preferential uptake by organs, the Student-Newman-Keuls test, SNK, was performed to arrange the organs by degree of uptake.

N.B. The data are presented in all figures as untransformed uptake values.

Corrected DPM values are used to determine $^3H/^{14}C$ ratios for each organ and corresponding 95% confidence limits for sample average ratios are established. Sample ratios are considered different from the ratios in the administered materials if the ratios for the administered materials are outside the 95% confidence limits for the sample averages.

APPENDIX IV

INDIVIDUAL DATA POINTS AND MEANS, MEDIANS, AND MODES FOR FIGURES IN CHAPTER 5.

Figure Legends

Figure 5.1.a. The immunization data illustrated in Fig. 5.1. presented as anti-LPS antibody titres for individual rainbow trout serum samples as opposed to mean $\pm$ 1 SD. The numbers to the left of the symbols indicate the frequency of the corresponding antibody titre within each group. All other conditions are as explained in Fig. 5.1. or as in the materials and methods section in chapter 5.

Figure 5.1.b. The immunization data illustrated in Fig. 5.1. plotted as mean (♦), median (■), and mode (●) of the anti-LPS antibody titres for rainbow trout serum samples as opposed to mean $\pm$ 1 SD. All other conditions are as explained in Fig. 5.1. or as in the materials and methods section in chapter 5.

Figure 5.2.a. The immunization data plotted as anti-LPS antibody titres for individual rainbow trout serum samples as opposed to mean $\pm$ 1 SD as illustrated in Fig. 5.2. The numbers to the left of the symbols indicate the frequency of the corresponding antibody titre within each group. All other conditions are as explained in Fig. 5.2. or as in the materials and methods section in chapter 5.

Figure 5.2.b. The immunization data illustrated in Fig. 5.2. plotted as mean (♦), median (■), and mode (●) of the anti-LPS antibody titres for rainbow trout serum samples as opposed to mean ± 1 SD. All other conditions are as explained in Fig. 5.2. or as in the materials and methods section in chapter 5.

Figure 5.3.a. The immunization data plotted as anti-LPS antibody titres for individual rainbow trout serum samples as opposed to mean ± 1 SD as illustrated in Fig. 5.3. The numbers to the left of the symbols indicate the frequency of the corresponding antibody titre within each group. All other conditions are as explained in Fig. 5.3. or as in the materials and methods section in chapter 5.

Figure 5.3.b. The immunization data illustrated in Fig. 5.3. plotted as mean (♦), median (■), and mode (●) of the anti-LPS antibody titres for rainbow trout serum samples as opposed to mean ± 1 SD. All other conditions are as explained in Fig. 5.3. or as in the materials and methods section in chapter 5.

Figure 5.4.a. The immunization data plotted as anti-LPS antibody titres for individual rainbow trout serum samples as opposed to mean ± 1 SD as illustrated in Fig. 5.4. The numbers to the left of the symbols indicate the frequency of the corresponding antibody titre within each group. All other conditions are as explained in Fig. 5.4. or as in the materials and methods section in chapter 5.

Figure 5.4.b. The immunization data illustrated in Fig. 5.4. plotted as mean (♦), median (■), and mode (●) of the anti-LPS antibody titres for rainbow trout serum samples as opposed to mean $\pm$ 1 SD. All other conditions are as explained in Fig. 5.4. or as in the materials and methods section in chapter 5.

Figure 5.5.a. The immunization data plotted as anti-LPS antibody titres for individual rainbow trout serum samples as opposed to mean $\pm$ 1 SD as illustrated in Fig. 5.5. The numbers to the left of the symbols indicate the frequency of the corresponding antibody titre within each group. All other conditions are as explained in Fig. 5.5. or as in the materials and methods section in chapter 5.

Figure 5.5.b. The immunization data illustrated in Fig. 5.5. plotted as mean (♦), median (■), and mode (●) of the anti-LPS antibody titres for rainbow trout serum samples as opposed to mean $\pm$ 1 SD. All other conditions are as explained in Fig. 5.5. or as in the materials and methods section in chapter 5.

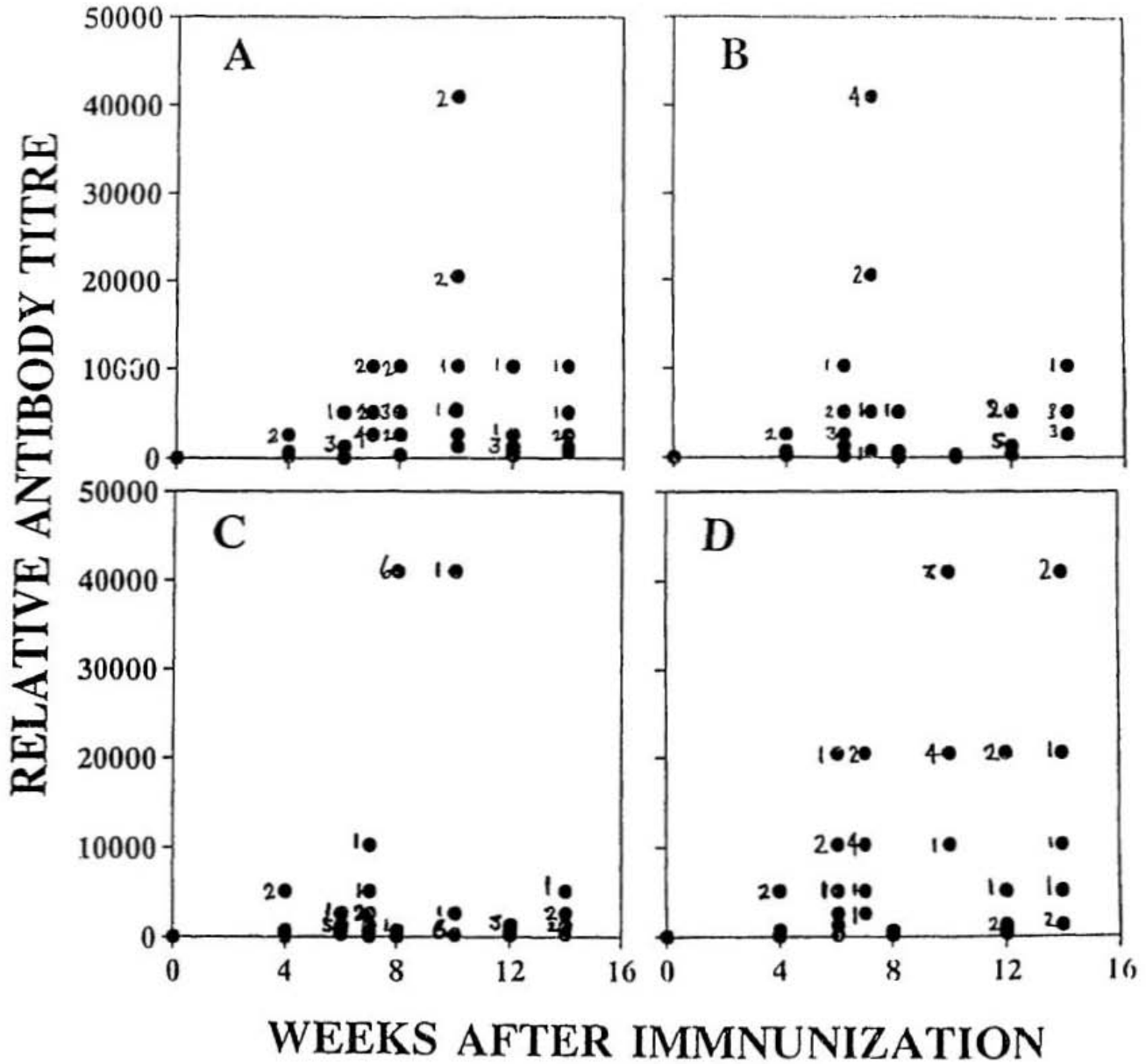

A
B
C
D
RELATIVE ANTIBODY TITRE
50000
40000
30000
20000
10000
0
0
4
8
12
16
WEEKS AFTER IMMNUNIZATION

FIGURE 5.1. a

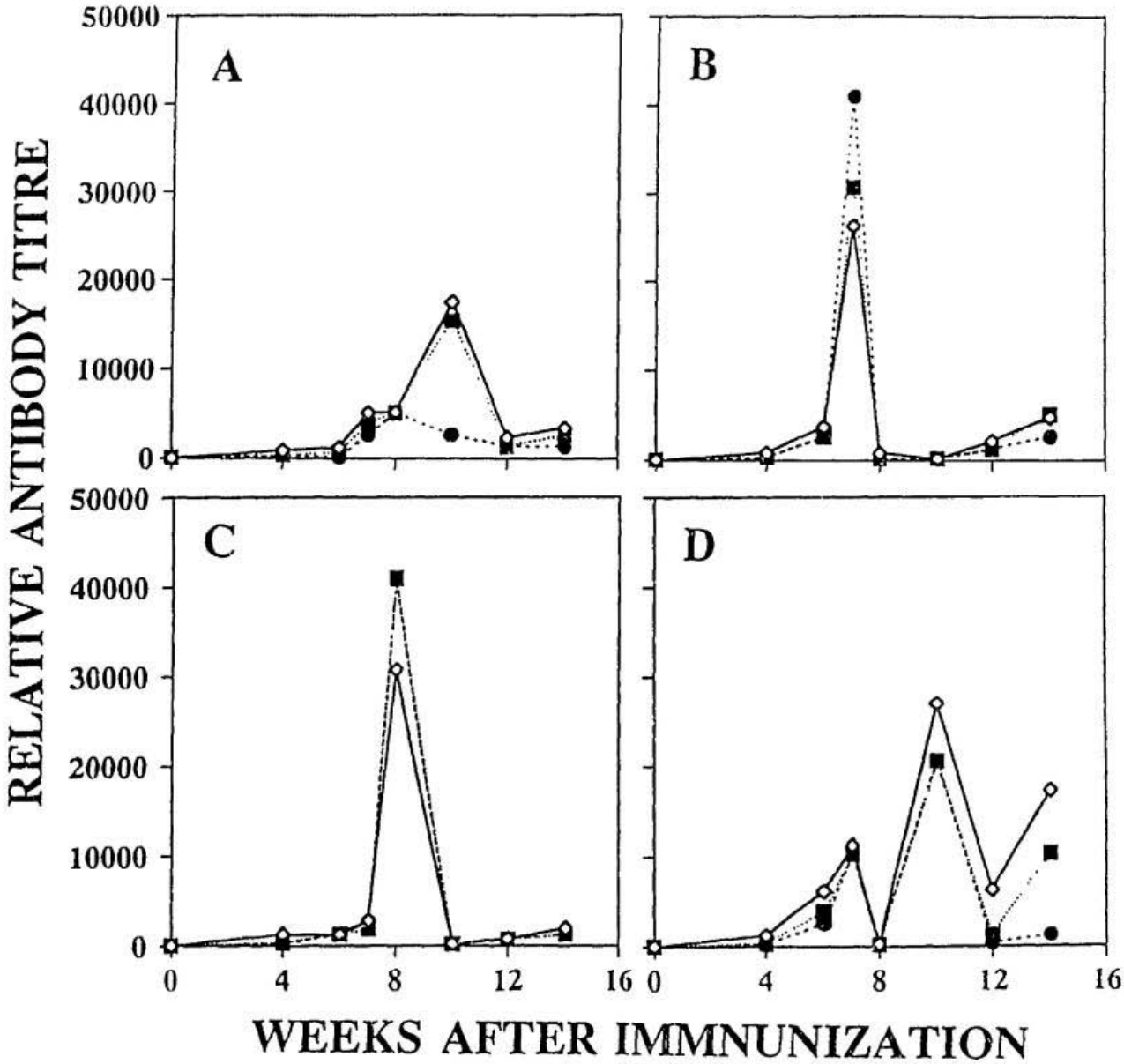

A
B
C
D
50000
40000
30000
20000
10000
0
0
4
8
12
16
RELATIVE ANTIBODY TITRE
WEEKS AFTER IMMNUNIZATION

FIGURE 5.1. b

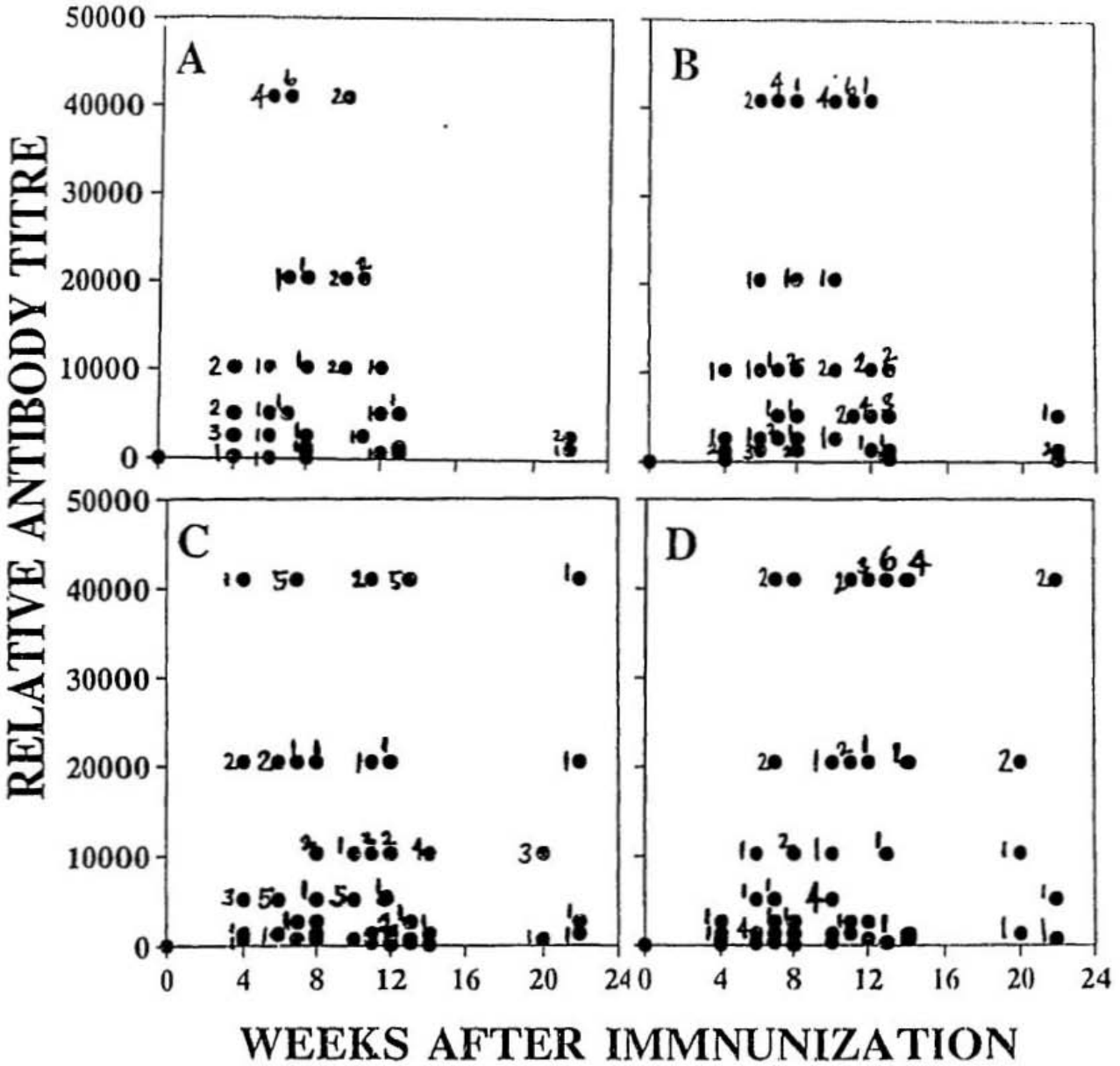

A
B
C
D
RELATIVE ANTIBODY TITRE
50000
40000
30000
20000
10000
0
0
4
8
12
16
20
24
WEEKS AFTER IMMNUNIZATION

FIGURE 5.2. a

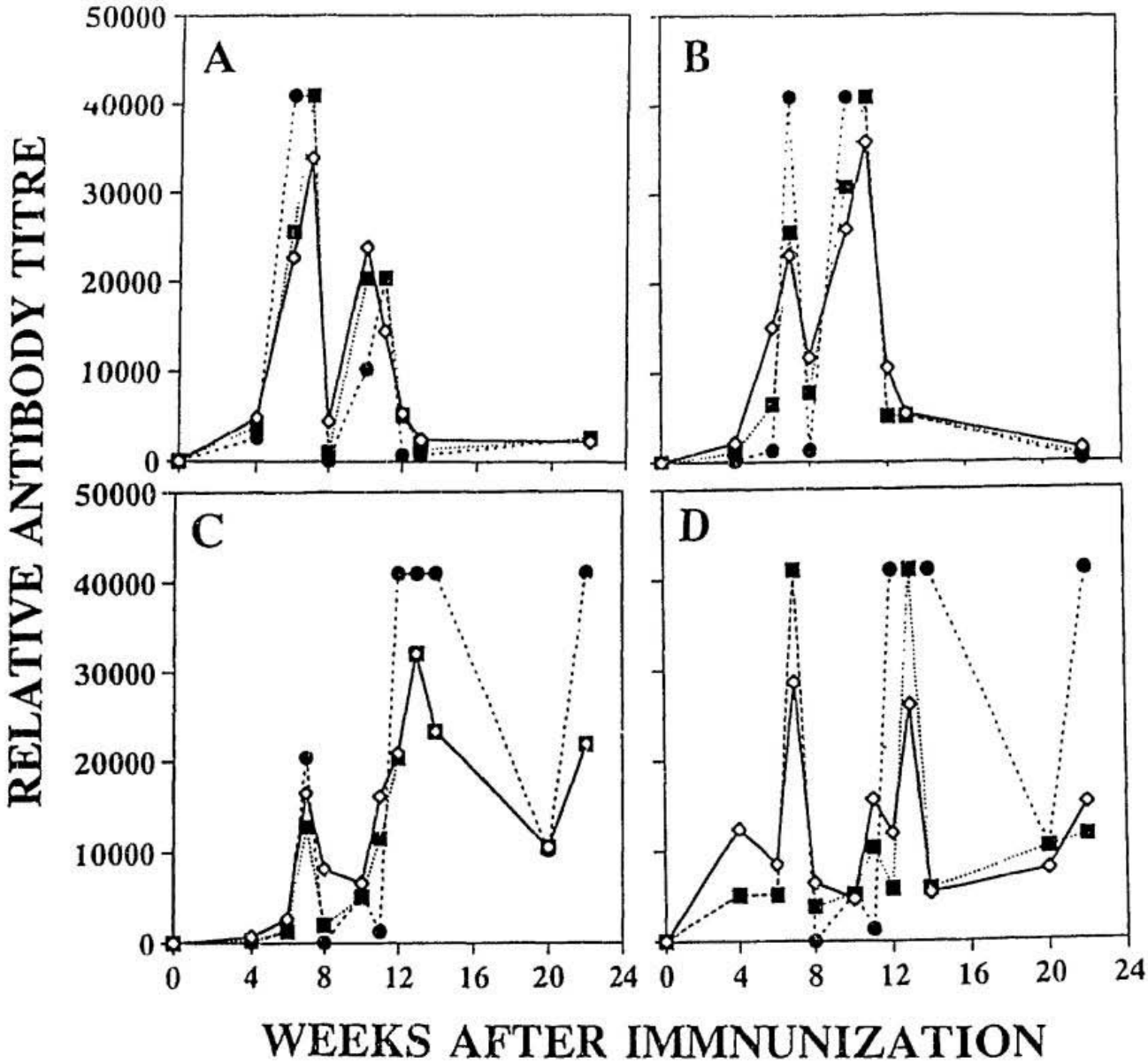

A
B
C
D
50000
40000
30000
20000
10000
0
0
4
8
12
16
20
24
RELATIVE ANTIBODY TITRE
WEEKS AFTER IMMNUNIZATION

FIGURE 5.2. b

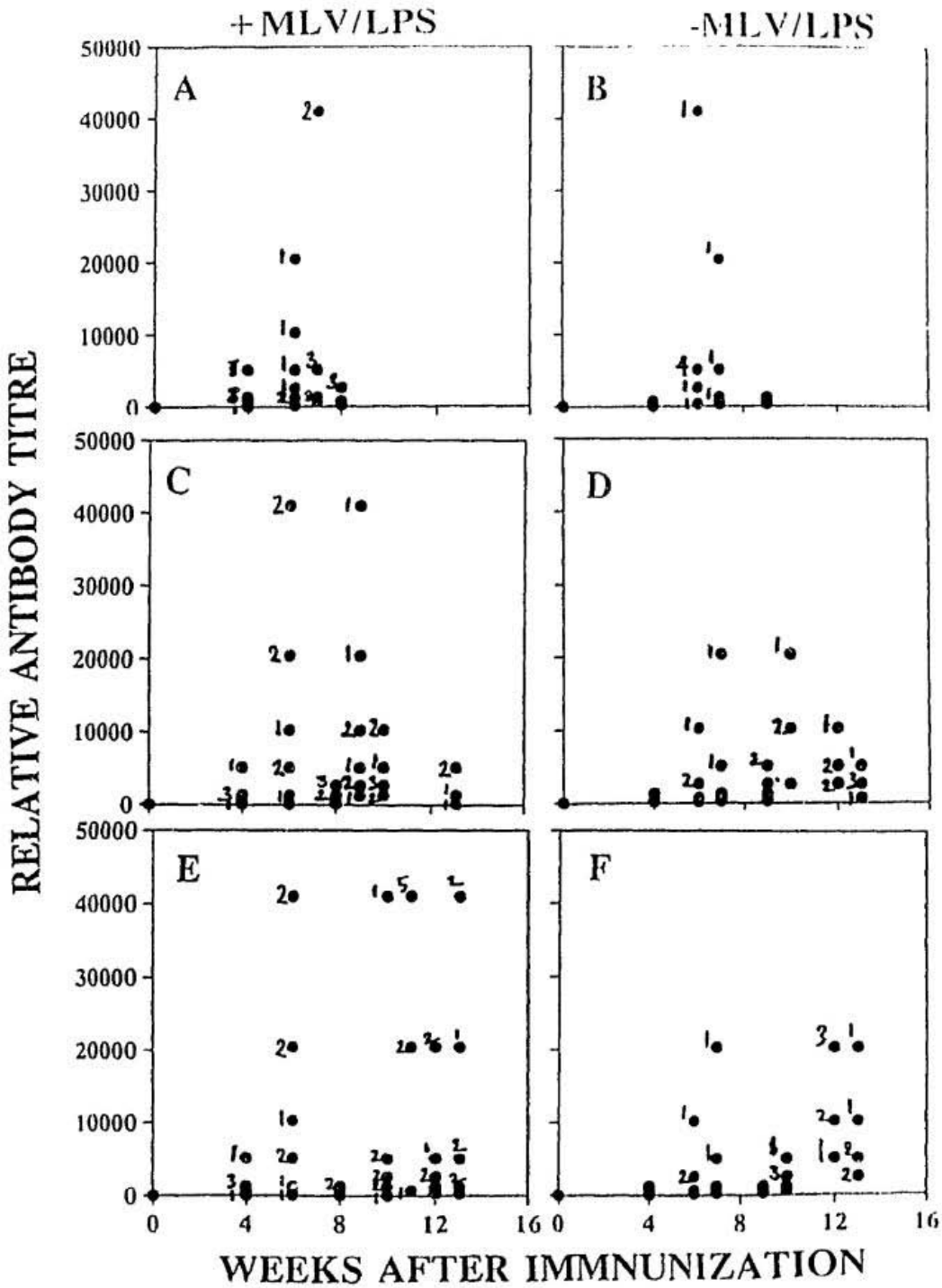

+MLV/LPS
-MLV/LPS
A
B
C
D
E
F
RELATIVE ANTIBODY TITRE
50000
40000
30000
20000
10000
0
0
4
8
12
16
WEEKS AFTER IMMNUNIZATION

FIGURE 5.3. a

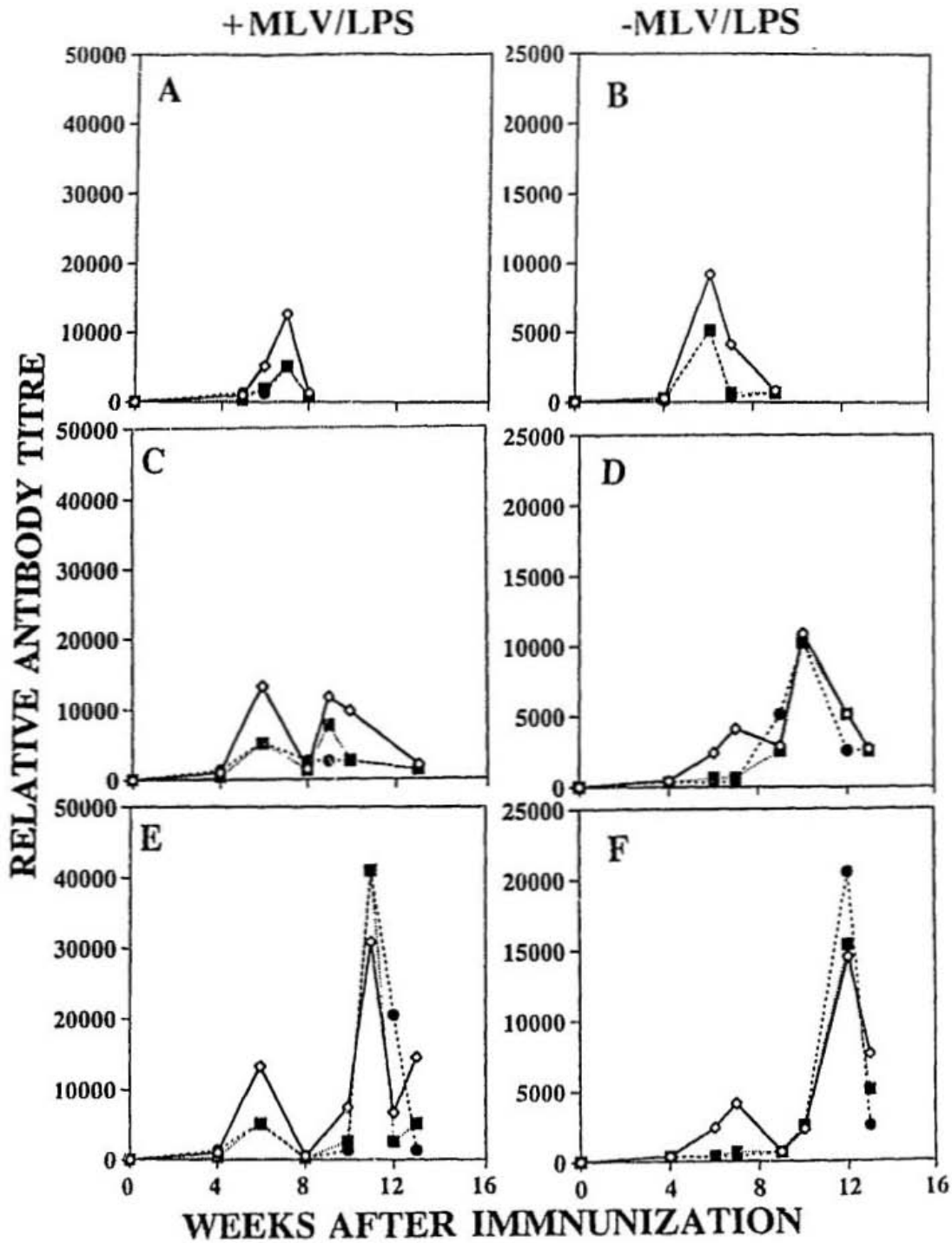

+MLV/LPS
-MLV/LPS
A
B
C
D
E
F
RELATIVE ANTIBODY TITRE
WEEKS AFTER IMMNUNIZATION
50000
40000
30000
20000
10000
0
25000
15000
5000
4
8
12
16

FIGURE 5.3. b

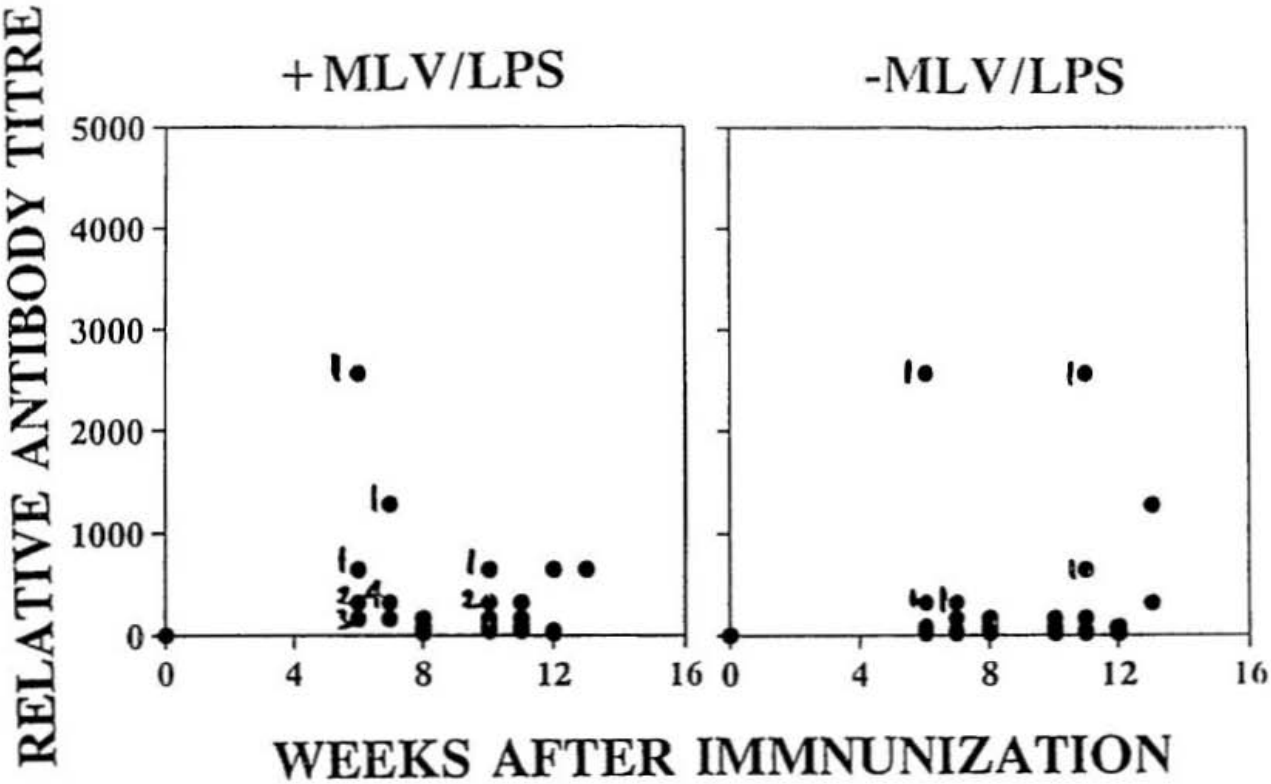

+MLV/LPS
-MLV/LPS
RELATIVE ANTIBODY TITRE
5000
4000
3000
2000
1000
0
0
4
8
12
16
WEEKS AFTER IMMNUNIZATION

FIGURE 5.4. a

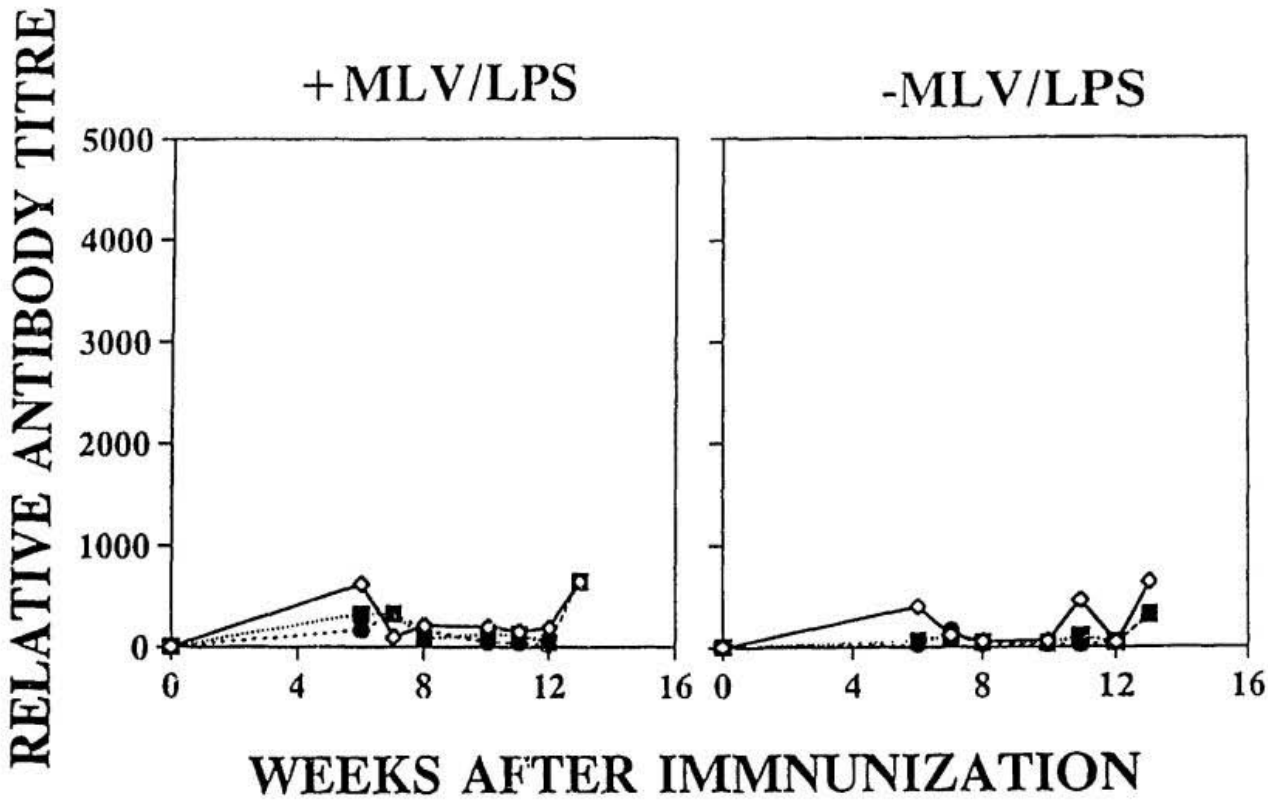

RELATIVE ANTIBODY TITRE
+MLV/LPS
-MLV/LPS
5000
4000
3000
2000
1000
0
0
4
8
12
16
WEEKS AFTER IMMNUNIZATION

FIGURE 5.4. b

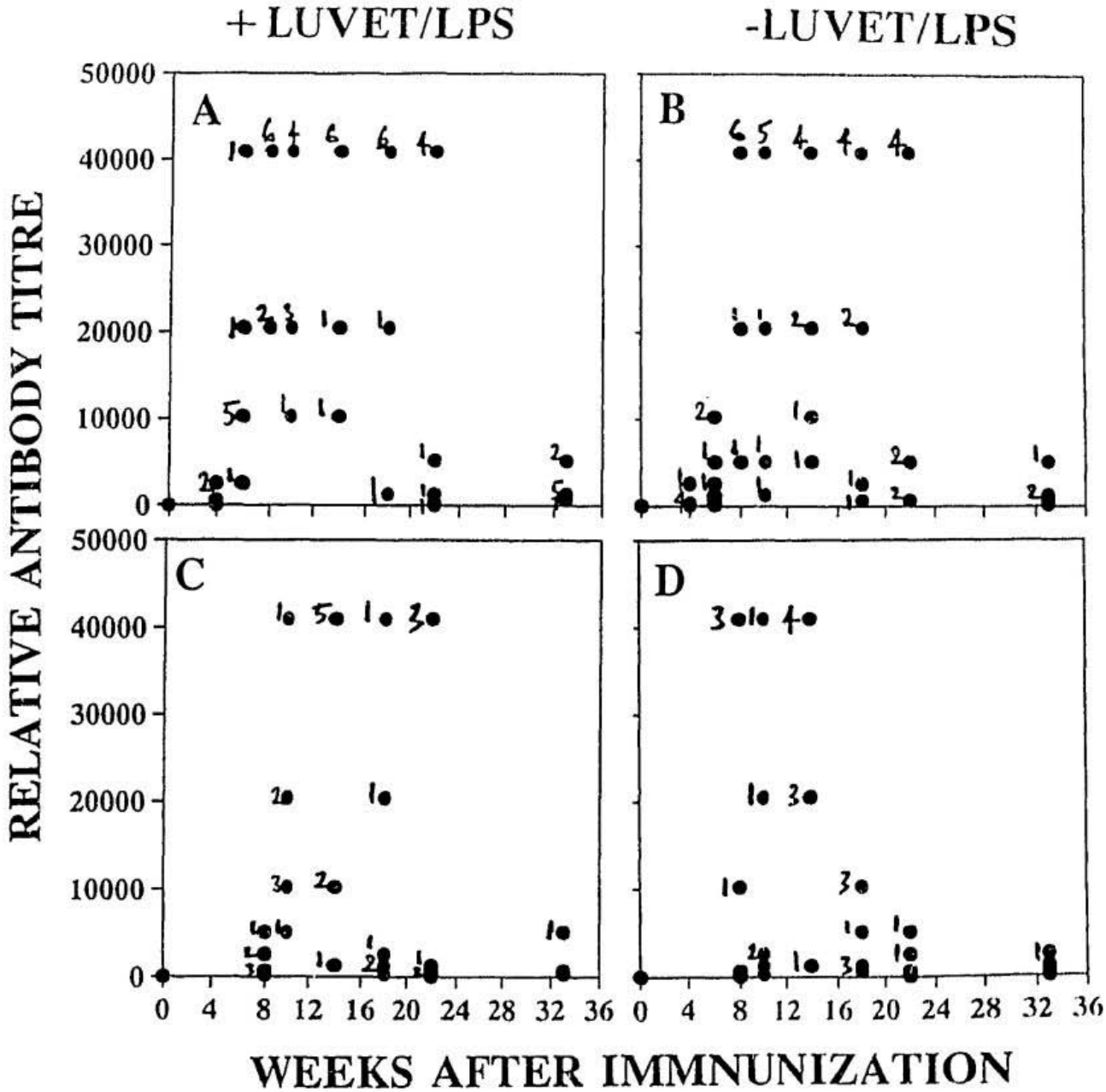

+LUVET/LPS
-LUVET/LPS
A
B
C
D
RELATIVE ANTIBODY TITRE
50000
40000
30000
20000
10000
0
0 4 8 12 16 20 24 28 32 36
WEEKS AFTER IMMNUNIZATION

FIGURE 5.5. a

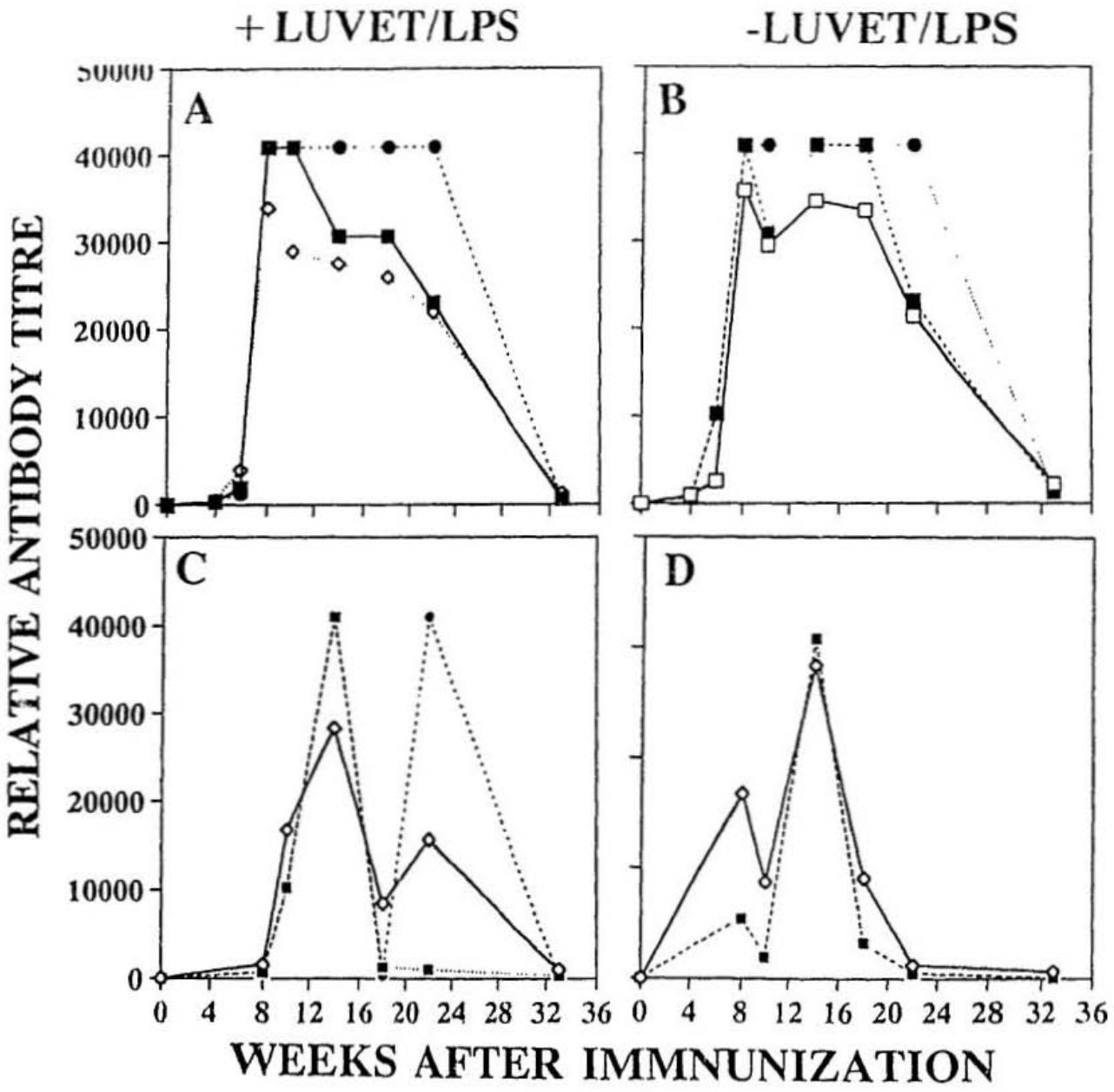

+LUVET/LPS
-LUVET/LPS
A
B
C
D
RELATIVE ANTIBODY TITRE
50000
40000
30000
20000
10000
0
0 4 8 12 16 20 24 28 32 36
WEEKS AFTER IMMNUNIZATION

FIGURE 5.5. b

APPENDIX V

DETAILED METHODS FROM CHAPTER 6

Protein Determination Using BCA Assay.

Protein content in rainbow trout sera is estimated using the bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co., Rockford, Illinois) as follows;

1. Add 1 part of Reagent B to 50 parts of Reagent A and vortex. This solution is referred to as Working Reagent and is stable at room temperature for 24 h.
2. A series of protein standards ranging from 25 - 200 μg/ml are prepared by diluting (with H_2O) a stock protein standard (Bovine Serum Albumin [BSA], 1.0 mg/ml in H_2O).
3. 0.1 ml volumes are added in triplicate to disposable borosilicate glass tubes (13 x 100 mm) in order to construct a standard curve ranging from 2.5 - 20 μg protein. For blanks, use 0.1 ml volumes of H_2O.
4. 1 μl of the serum samples to be assayed are diluted 200-fold with H_2O. 0.1 ml volumes of the diluted sera are added in duplicate to borosilicate glass tubes.
5. Add 2 ml Working Reagent to each tube. Vortex well.
6. Incubate at 37°C for 30 min. Cool to room temperature.
7. Measure the Optical Density at 562 nm.

Precipitation of Serum Proteins with Saturated Ammonium Sulphate.

Serum proteins are concentrated by precipitation with saturated ammonium sulphate according to the method described by Bolag and Edelstein (1991);

1. Prepare solution of saturated $(NH_4)_2SO_4$ (solubility 41.2 g/100 ml at 0°C).
2. 2 ml of pooled, clarified serum from rainbow trout are added drop-wise while stirring to an equal volume of saturated ammonium sulphate.
3. The solution is stirred slowly on ice for 1 h.
4. The solution is incubated overnight at 4°C.
5. The solution is centrifuged at 48,200 x g_{max} (20,000 rpm in a Sorvall SS-34 rotor) for 30 min at 4°C.
6. The supernatant is aspirated and the pellets of precipitated proteins is resuspended in Tris buffered saline (TBS: 10 Mm Tris-Hcl, 140 Mm NaCl, Ph 7.4).
7. Dialyze overnight against 4 changes of TBS (3.0 l per change).
8. Remove residual ammonium sulphate from the protein concentrates by passage through PD-10 desalting columns (Sephadex G-25M, 1.5 x 8 cm) that have previously been equilibrated with TBS.
9. Pool the fractions of effluent containing serum proteins and concentrate by ultrafiltration using Centriprep-10 concentrator as follows;
   - Disassemble device and add sample up to the fill line on the sample container (15 ml)
   - Reassemble device and place in a Sorval SS-34 rotor

- Centrifuge at 5000 x g_{max} at 25°C until equilibrium is reached (30 min)
- Decant filtrate and centrifuge again
- Repeat until the final volume of the retentate is 3.5 mls.

Gel Filtration of Enriched Serum Proteins on Sephacryl S200HR.

1. At room temperature, pack a column (2.6 x 65 cm) of Sephacryl S200-HR and equilibrate with 5 bed volumes (1 bed volume = $\pi r^2 h$ = 345 ml) of TBS (flow rate 1.0 ml/min).

2. The void volume is determined using blue dextran. A 3.5 ml sample of blue dextran (1 mg/ml in TBS containing 5% glycerol) is applied to the column and fifty 3 ml fractions are collected. Void volume (126 ml) is estimated to be at the peak of the absorbance values read at 610 nm.

3. Wash the column with 5 bed volumes of TBS and calibrate the column by applying 3.5 ml a mixture, prepared in 0.5 % glycerol in TBS, of proteins of known molecular masses:

bovine serum albumin (BSA), 66 Kda (1 mg/ml)

alcohol dehydrogenase, 150 Kda (1 mg/ml)

β-amylase, 200 Kda (1 mg/ml)

4. Collect 80 3 ml fractions of effluent and monitor for the presence of protein based on absorbance at 280 nm. The elution volumes of the proteins are approximately 142, 160, and 176 ml for β-amylase, alcohol dehydrogenase, and BSA, respectively.

5. Wash the column with 5 bed volumes of TBS and apply 3.5 ml enriched rainbow trout serum. Collect 85 fractions (2.5 ml) of effluent (flow rate = 100 drops/5 min/fraction) and monitor for the presence of protein based on absorbance at 280 nm.

6. The fractions comprising the major protein peaks are pooled and concentrated by ultrafiltration using Centriprep-10 concentrator devices to a final volume of 2 ml.

7. The samples were diluted to 15 ml with 10 Mm MES (2[N-morpholino]-ethanesulfonic acid, Ph 5.6) and concentrate to a final volume of 2 ml.

8. Repeat 6. two more times and concentrate to a final volume of 2 ml.

Isolation of Trout Serum IgM on Bakerbond Abx.

1. A column (1.5 x 12 cm) of Bakerbond Abx antibody exchanging resin (J.T. Baker Canada, Toronto, Ontario, Canada) is packed (bed volume = 21.2 ml) and then washed with 100 mls of distilled H_2O.

2. The column is washed with 100 ml of m sodium acetate followed by washing with 100 ml of distilled H_2O and then 100 ml 1% acetic acid.

3. The column is equilibrated with the starting buffer (10 Mm MES, Ph 5.6) until the Ph of the effluent reaches 5.6.

4. Gel-filtered, IgM-enriched concentrates in 100 Mm MES, Ph 5.6 are applied to the equilibrated Abx column and 2.0 ml fractions (flow rate = 70 drops/ 1.38 min/ fraction) are collected until the absorbance of the effluent measured at 280 nm was < 0.02. At this point (approx. 40 fractions), the void volume proteins have eluted.

5. Proteins adherent to the column are eluted by subjecting the column to a linear gradient formed by mixing starting buffer with limiting buffer (10 Mm sodium acetate, 200 Mm ammonium sulphate, Ph 5.6). This is achieved by placing 50 ml of starting buffer in one vessel of a two-vessel gradient maker and 50 ml of limiting buffer in the other and allowing the solutions to mix as they pour onto the column. Fractions are collected again as the gradient forms between 0 - 100% $(NH_4)_2SO_4$ at which point adherent proteins are eluted.

6. Measure O.D. at 280 nm for all fractions.

7. Elution profile displays void volume and retained proteins. The fractions representing the void volume proteins and adherent proteins are pooled and concentrated by ultrafiltration using Centriprep-10 concentrators to a final volume of 2.0 ml. Store temporarily at 4°C.

SDS-PAGE and Corresponding Western Blot Analysis (Towbin 1979)

1. 12.5 % reducing or 5% non-reducing, discontinuous, SDS-PAGE is performed according the procedure outlined in appendix I.

2. Gels are soaked in cold elution buffer (Tris Base 9.1 g; glycine 43.2 g; CH_3OH 400 ml, add H_2O for a final volume of 1 l, Ph=8.3).

3. While gels are soaking, nitrocellulose membranes (0.2 μm, Schleicher and Schuell, Keene, NH, USA) is cut to the same size as the gel and are soaked for 20 min. in the elution buffer with the gels.

4. The nitrocellulose membranes are placed on top of the gels and any trapped air-bubbles are removed by rolling a glass tube over the membranes.

5. The gels and membranes are arranged in the transfer cassette (BIO-RAD Mini Trans-Blot Electrophoretic Transfer Cell) so that the current is running through the gel into the nitrocellulose.

6. Fibrous pads are placed on both sides of the gel\membrane sandwich and are attached with clips.

7. The cassette is placed in the BIO-RAD transfer tank and the entire cassette is submerged with elution buffer.

8. The tank is placed in a styrofoam container filled with ice and the proteins are transferred for 4h at 0.2 A.

9. Following the transfer, the cassette is removed and the gel is separated from the nitrocellulose membrane.

10. The gel is stained with Coomassie blue as described in appendix I.

11. The membrane is soaked in 25 ml PONSEAU S solution ((0.1% Ponceau S w/v in 5% acetic acid v/v) for 1-2 min. and washed twice with distilled H_2O. The protein bands on the membranes are marked lightly with a pencil.

12. Unspecific binding sites are blocked by immersion of the membranes in 25 ml of a solution of 3% (w/v) gelatin in TBST. Incubate the membranes for 1 h at room temperature using an orbital shaker.

13. Wash the membranes three times with TBST with agitation for 2 min. per wash.

14. Incubate the membranes with 25 ml of a solution of monoclonal antibody 1.14 (3% (v/v) in diluent [1.5% gelatin in TBST]) with agitation for 1.5 h at room temperature.

15. The membranes are washed as in 13.

16. The membranes are incubated with a 25 ml of a solution of goat anti-mouse IgG conjugated with alkaline phosphatase (1/2000 (v/v) in diluent) for 1 h at room temperature.

17. The membranes are washed as in 13.

18. The membranes are incubated at room temperature with agitation in 25 ml of substrate buffer that is prepared as follows;

- Dissolve 30 mg nitro blue tetrazolium, NBT, in 1 ml of 70 % dimethylformamide (DMF) → solution A.

- Dissolve 15 mg 5-bromo-4-chloro-3-indonyl phosphate (*p*-toluidine salt), BCIP, in DMF → solution B.

- Add 1 ml of A and 1 ml of B to 100 ml of carbonate buffer, Ph 9.6, prepared as in appendix I.

19. After sufficient colour development (approximately 5 minutes), the reaction is terminated by repeated washing of the membrane with distilled water.

20. The membranes are allowed to dry overnight at room temperature.