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Title: B-CELL HETEROGENEITY IN SALMONIDS

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Salmonid B lymphocytes were stimulated both in vivo and in vitro and shown to produce antibody specific for the immunogen. A passive hemolytic plaque assay was employed to demonstrate B cells producing specific antibody. Characterization of plaque forming cells (PFCs) involved sequential inhibition with a series of graded concentrations of free antigen. Splenic and posterior kidney derived PFCs exhibited greater heterogeneity in the population of responding B cells compared to anterior kidney derived PFCs. Anterior kidney PFCs were restricted in heterogeneity and lacked high affinity antibody, however, the splenic and posterior kidney PFCs possessed high affinity antibody producing B cells. Thus, the anterior kidney possessed immature B cell populations, characteristic of a lymphopoietic organ. Splenic and posterior kidney B cell populations were demonstrated to be functionally mature.

The in vitro antibody production system facilitated investigations into B cell activation signals. T cell independent (TI) B cell activation by trinitrophenylated lipopolysaccharide (TNP-

LPS) was shown to occur via anti-TNP specific B cell receptors. The ability of salmonid B cells to discern different molecular forms of antigen (protein and lipopolysaccharide) was investigated indicating that the same subpopulation of B cells responds to both forms of antigen. Low dose gamma irradiation effects on B lymphocyte responses illustrated differential sensitivity of responses to this cellular insult, indicating the presence of a radiosensitive regulatory cell.

Fine specificity analyses assessed the ability of in vitro generated antibody to recognize analogs of TNP. The effect of different molecular substituents on antibody recognition of the hapten analog indicated that a carboxyl group present on the substituent molecule proximal to the hapten analog hindered the antibody's ability to recognize the hapten. The simplest analog which revealed the heterogeneity of the antibody population was the dinitrophenol analog bearing two nitrite groups.

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INTRODUCTION

The immune system can be traced back to its origins in the fishes (McKinney, 1976). Fish are the first vertebrates to arise in the evolutionary scheme and also the first organisms to possess a true immune system. The piscine immune system is compartmentalized with distinct immune organs. B lymphocytes are integral components of the fish immune system and are defined by the presence of surface immunoglobulin and the ability to respond to foreign antigens by producing specific antibody. Fish possess a true immunoglobulin molecule, most commonly observed as a tetrameric IgM molecule consisting of four monomeric units. Two heavy (H) and light (L) chain pairs compose the monomeric subunits in the tetramer.

The study of the fish immune system is interesting from a comparative viewpoint by virtue of its evolutionary position. Since these animals were the first to possess a true immune system, any similarities or differences found compared to mammalian immune systems would be informative as to the changes or conservation in the system over evolutionary time. If components of mammalian and piscine immune systems were essentially identical, this would indicate conservation of a particular immune function over a long evolutionary time period and thus a uniquely important function. Immune functions similar in nature might indicate how a particular immune function had to adapt in a different organism or to a different environment.

The objectives of this study were to characterize B cell populations and the antibody they produced. A plaque forming cell assay was developed and adapted to demonstrate organ dependent functional B cell heterogeneity between splenic and anterior kidney lymphocyte populations. This study represents the first functional characterization of antibody producing cell populations in a fish. In this study an in vitro antibody production system was developed, being the first of its kind demonstrated in salmonids. Fine specificity analyses were also employed, which characterized in vitro, anti-TNP antibody, the first such analysis in salmonids. These data lend functional support to the histolgical evidence that the salmonid kidney is the lymphopoietic center in fish (Zapata, 1979).

LITERATURE REVIEW

Impact of B Cell Activation Studies

The study of the immune system has led to the expansion of many frontiers in biological disciplines. Investigations using B cell activation, maturation, and diversification as general biological models have contributed to the advancement of our knowledge of the fields of cellular biology, biochemistry, medicine, and immunology. B cell studies have created this broad scientific impact as a consequence of the inherent complexity of B cell development, activation and function. These B cell features are dependent upon a highly integrated network of cells and molecules. Although important signals responsible for B cell activation and the subsequent passage through developmental pathways have yet to be elaborated, certain B cell activation signals have been described (Click, 1972; Gronowicz, 1975; Andersson, 1977). Activation can be mediated by cellular contact (Mond, 1982), and subsequent cooperation, with other cells of the immune system (Asano, 1982). Activation by cellular contact can be abridged if the appropriate biochemical factors have been produced (Asano, 1982; Zlotnik, 1983) thereby defining potential mechanisms of this cellular cooperation. B cell activation also depends heavily on the repertoire of cell surface receptors for the activation signals (Asano, 1982) and moreover for specific activation by a foreign antigen (Watson, 1974; Marchalonis, 1975). B cell activation can lead to a number of phenotypically distinct developmental stages (Gronowicz, 1974; Gronowicz, 1975). These developmental stages can

be functionally differentiated at the cellular level (Gronowicz, 1974; Coffman, 1982) and biochemically by observation of the responses to defined stimuli.

Introduction to B Cells and Antibody

The specificity and adaptive nature of the immune system is primarily due to the B cell product, the antibody molecule. Antibody production is strictly a B cell function as well as being its primary function (Wigzell, 1974; Honjo, 1985). The antibody, or immunoglobulin (Ig) molecule, is a remarkable molecule considering the estimated 10^7 different specificities that can be produced by an individual (Sherman, 1983). The antibody molecule may be secreted by a plasma cell, defining this developmental stage of a B cell, or it can be found as a B cell surface receptor (Marchalonis, 1975). When a B cell surface Ig binds the antigen for which it is specific, that particular B cell may become activated (Sell, 1965; Feldmann, 1972). Upon specific activation the B cell undergoes proliferation, expanding that particular B cell into a clone of cells, and subsequently produces and secretes exact replicas of the surface antibody molecule present on that B cell (Greaves, 1974; Watson, 1974). Thus B cell funtion and the antibody these cells produce are mutually dependent on one another.

B Cell Immune Organs

Cells of the immune system develop and reside in the various immune organs. The major organs of a typical mammalian immune system (the murine system) include the fetal liver, bone marrow, spleen, lymph nodes and thymus (Paul, 1984). Immune cells can also be found in circulating blood. B lymphocytes reside in all previously mentioned immune organs, however only small numbers have been demonstrated to reside in the thymus of the rabbit (Jentz, 1979) and the mouse (Herzenberg, 1974; Bosma, 1974). Primary immune organs are so designated because lymphocytes (B and T cells) and accessory cells (macrophages and monocytes) develop in these organs from hematopoietic stem cells (Abramson, 1977). Mammalian primary immune organs are the fetal liver, bone marrow and the thymus. The peripheral or secondary immune organs, which harbor mature lymphocytes and play no major role in lymphocyte development, are the spleen and lymph nodes (Owen, 1972).

B Cell Ontogeny

Differentiation of the three main blood cell types (erythroid, myeloid and lymphoid) is first observed in the fetal liver and bone marrow, however the majority of stem cells reside in the bone marrow from neonatal life onward. In the bone marrow, stem cells undergo further differentiation into B and T cell lineages (LePault, 1983). At this point of development pre-T cells migrate from the bone marrow to the primary site of T cell development and maturation, the thymus

(Owen, 1972). The pre-B cell stage, those lymphocytes possessing demonstrable cytoplasmic mu heavy chains, and the small B lymphocyte stage which possesses surface IgM develop in the bone marrow (Osmond, 1974). The small B lymphocytes may then migrate to the secondary immune organs such as the spleen or lymph nodes to further differentiate, should they be exposed to antigen or another activation signal occur.

Mechanisms of B Cell Activation

Specific activation of B cells by antigen occur via a number of pathways depending primarily on the molecular form of the antigen. Two general B cell activation pathways, the T cell independent (TI) and the T cell dependent (TD), have been described (Miller 1969; Fung, 1980; Mond, 1982). Polysaccharide or lipopolysaccharide (LPS) antigens activate B cells in a TI manner (Mond, 1982) whereas protein antigens activate B cells in a TD manner (Fung, 1980). Not only do the antigens determine the particular activation pathway but separate subpopulations of B cells exist, which will only respond to one or the other antigenic form (Gorczynski, 1975; Jennings, 1976; Lewis, 1976; Fung, 1980).

T Independent B Cell Activation

Historically, TI B cell activation was defined as that activation which occurs in the absence of T cells such as is seen in

a T cell deficient (nude) mouse or *in vitro* cultures depleted of T lymphocytes (Mond, 1982). TI B cell activation can be further distinguished as that being triggered by type 1 antigens or type 2 antigens. Evidence for two distinct types of TI B cell activation came from experiments using a genetically abnormal strain of mice. The CBA/N strain of mice possesses x-linked immunodeficiency (xid) which prevents it from responding to polysaccharide antigens (TI-2 antigen), but leaves it responsive to LPS antigens (Mosier, 1976; Mosier, 1977). Examples of TI-2 antigens are ficoll, dextran sulfate and type III *pneumococcal* polysaccharide, whereas TI-1 antigens are LPS, *Brucella abortus* capsular antigen and *Mycobacterium* purified protein derivative (PPD; Mond, 1982; Singer, 1982). Certain TI-1 and TI-2 antigen responses were studied in a controlled manner by the attachment of a common, small antigenic unit (hapten) to TI-1 or TI-2 carrier molecules. Modulation of this anti-hapten response could then be fully ascribed to the form of the carrier (Mosier, 1974; Jacobs, 1975). The physical properties, that made haptens particularly advantageous for such studies was that their molecular structure was known and their chemical structure allowed easy attachment to a wide variety of carriers. The trinitrophenyl group is one example of a hapten that could be easily linked to various type 1 and type 2 carriers. Since a CBA/N mouse could respond to trinitrophenylated lipopolysaccharide (TNP-LPS) with specific anti-TNP antibody but not to trinitrophenylated ficoll (TNP-ficoll), it indicated that this mouse does not possess B cells responsive to TI-2 antigens or carrier molecules. The various TI carriers could be broadly grouped as those eliciting a response (type 1) or those

unable to elicit a response (type 2) in a CBA/N mouse (Mond, 1982; Mosier, 1976).

TI-1 B cell activation is thought to involve two receptors, the antigen specific surface Ig molecule and a mitogen receptor (Coutinho, 1973). A mitogen receptor, while being specific for its particular ligand, exists on most if not all B cells. Interaction of ligand with this receptor activates that B cell to proliferate and/or go on to the production of the antibody for which that B cell is specific (Click, 1972; Andersson, 1976 and 1977). Antigenic specificity of B cell activation by these nonspecific B cell activators is provided by the hapten attached to this mitogen/carrier (i.e. TNP in the previous example). The mitogen is then thought to be focused onto anti-TNP B cells through the anti-TNP immunoglobulin receptor (Andersson, 1972), thus providing a signal for an anti-TNP antibody response (Jacobs, 1975; Singer 1980).

Polysaccharide molecules are thought to activate B cells as a result of their repetitive, antigenic saccharide units (Feldmann, 1971; Feldmann, 1972). The multivalency of these antigenic units are presumed to activate a B cell via the cross-linking of antigen specific surface Ig receptors. This cross-linking serves as the activation signal (Feldmann, 1971; Feldmann, 1972). In the example of the trinitrophenylated-polysaccharide, TNP attaches to each saccharide unit and therefore becomes the multivalent antigenic structure. B cells bearing anti-TNP receptors will have these receptors cross linked and therefore a specific anti-TNP antibody response is produced (Gronowicz, 1975; Chused, 1976; Persson, 1977).

T Dependent B Cell Activation

T cell dependent (TD) B cell activation is the second major pathway for the stimulation of antibody production. This form of activation is the most complex and interactive pathway. Utilization of macrophages, T cells, factors derived from these cells, and the specific protein antigen are required before the B cell can be activated by this mechanism (Leibson, 1984).

The pathway begins with the processing of antigen, by the macrophage, for presentation to the T cell (Thomas, 1976; Swierkosz, 1978) Recognition and binding of macrophage surface bound antigen (Pierce, 1974) is accomplished via the T cell antigen receptor (Schwartz, 1978). The T cell must also recognize a "self" molecule, closely associated with macrophage bound antigen, which is also a function of the T cell receptor (Schwartz, 1978). The "self" molecule on the macrophage surface is referred to as a class II restriction molecule, a product of the major histocompatibility gene complex (Swierkosz, 1978). The class II restriction molecule is also represented on the B cell surface (Asano, 1982). This complex recognition of antigen by the T cell receptor is required for macrophage activation and leads to macrophage production of interleukin-1 (IL-1; Watson, 1979; Larsson, 1980; Smith, 1980). IL-1 is essential and a primary requisite as activation of T cells can occur with IL-1 alone, even in the absence of macrophages (Farrar, 1980). The IL-1 activated, antigen bound T cells then proliferate and produce interleukin-2 (IL-2; Larsson, 1980; Smith, 1980). T cells can then act in two ways to help antigen specific B cells

respond to antigen (Asano, 1982). Antigen specific T cells may link with B cells through the surface B cell class II restriction element and antigen bound on the B cell surface, or T cells (through the elaboration of IL-2) can directly and nonspecifically activate antigen bound B cells (Asano, 1982). When T cells are rigorously depleted from a pool of lymphocytes they then require IL-2, however IL-2 alone is insufficient for activation of an antigen primed B cell population (Leibson, 1981). The addition of interferon gamma (IFN-gamma) in conjunction with IL-2 however, can activate rigorously T cell depleted, antigen primed B cell populations (Leibson, 1981; Leibson, 1982; Zlotnik, 1983). The ability of factors alone to activate antigen primed B cell populations has been demonstrated (Leibson, 1984). Antigen primed B cells, stringently depleted of macrophages and T cells can be activated by the addition of IL-1 from a cloned macrophage line, IL-2 from a cloned T cell line, and recombinant IFN-gamma. This suggests the importance of all of these factors as playing a role in activation. B cells thus activated can proliferate, differentiate and ultimately produce antibody for secretion.

Redefining Our Views of B Cell Activation

Recently our views on TI and TD activation have been altered by more sophisticated cell culture and separation techniques. It has been demonstrated that macrophages (Chused, 1976; Persson, 1977) as well as T cells (Mond, 1979; Mond, 1980; Letvin, 1981) are absolutely required in some type 2 TI responses. Further, it has been shown

that after applying rigorous techniques to deplete B cell populations of T cells and macrophages that all TI responses (type 1 and type 2) were significantly reduced and the response could be restored by the addition of low numbers of T cells and macrophages. With the discovery of the effect of T cell and macrophage elaborated factors, their role in B cell activation is now being more precisely defined. As stated previously, when T cells and macrophages are rigorously depleted, type 2 TI responses are found to be absolutely dependent on the presence of IL-2 and IFN-gamma. In this same T cell/macrophage depleted system type 1, TI antigens can stimulate reduced but still significant responses in vitro, however this activity is greatly enhanced by the addition of IL-2 (Endres, 1983; Mond, 1983). Considering this evidence and a report that a polyclonal LPS response was shown to be dependent on T cell help (Zubler, 1982), a strong case is being built for the hypothesis that all B cell responses are T cell dependent at least to some degree. Letvin (1981) has suggested that a continuum of the degree of T cell dependency of B cell responses may exist. The proponents of the hypothesis that all B cell responses are T dependent, feel that if methods are developed, to more rigorously deplete T cells, all responses will be found to be T cell or IL-2 dependent (Mond, 1983). Objections to the thought of a truly TI population of B cells are fueled by the fact that in the nude mouse, where TI responses were defined, low numbers of T cells have been demonstrated. In the opinion of these investigators IL-2 enhanced type 1 TI responses illustrate that maybe a residual contaminating T cell population is effecting the enhancement.

However, a small subpopulation of B cells may truly be T cell independent and the enhancement by IL-2 would then indicate a second B cell subset being recruited for this response (Endres, 1983; Roehm, 1986).

Investigation of the Antibody Molecule

The antibody molecule is a key component of the immune system. The monomeric structural unit of all antibody molecules is composed of two heavy (H) and two light (L) chain polypeptides. Mammalian immune systems possess five different classes of antibody molecules or isotypes which differ functionally, physically and antigenically. Antibody isotypes are differentiated on the basis of H chain polypeptide differences. These heavy chain isotypes are classified as mu, delta, gamma, alpha, and epsilon. Immunoglobulin M (IgM; composed of mu H chains), and IgG (composed of gamma H chains) dominate in concentration, the serum antibody response. IgM, a pentameric molecule, is associated with the first or primary antibody response to antigen. IgM levels in the secondary response remain nearly the same but dramatic increases in IgG occur in secondary, subsequent immune responses to the same antigen. IgG can further be differentiated into four more isotypes, or subclasses (IgG_1, IgG_{2a}, IgG_{2b} and IgG_3, in the murine system). IgE (epsilon), another monomeric Ig molecule is strictly seen in allergic or hypersensitivity reactions. IgA (alpha) is a monomer in sera but is dimeric or trimeric when isolated from secretions. IgA is usually associated with mucosal surfaces within the body. Each H chain

isotype produced by a particular B cell will first manifest this polypeptide as a lymphocyte cell surface receptor before it is able to secrete that isotype of antibody. IgD (delta), a monomeric Ig, functions as a surface associated receptor and is studied primarily as a B cell differentiation marker, however low levels of serum IgD have been reported (Abney, 1976; Neuberger, 1981). The function of IgD as a free antibody has not yet been discovered.

Regardless of the antibody class, antibody molecules produced by individual B cells or their clonal progeny are specific for distinct antigenic determinants. The specificity of an antibody molecule has been likened to a lock (antibody combining site) fitting a specific key (antigenic determinant). The specificity of an antibody molecule is determined by the cleft (combining site) resulting from joining variable regions of both heavy and light chain polypeptides. Complimentarity determining regions (CDR) makeup the particular antigen binding site in the variable regions of the heavy and light chain polypeptides (Tonegawa, 1983). Each of these three CDR is flanked on either side by framework regions (FR) which have been shown to play an important role in the proper orientation of the CDR composing the antigen combining site (Tonegawa, 1983). A particular B cell produces a single and distinct antibody of a particular antigenic specificity, however the constant region of the heavy chain (isotype) may switch during B cell differentiation for a particular cell. Therefore, the theory of one cell-one antibody, holds for the antibody binding site but not the isotype of the antibody.

Generation of Antibody Diversity

The mechanism by which tremendous antibody diversity is generated has been an important area of study in the field of immunology, receiving attention for more than eighty years. The central paradox lies in the ability of the individual, with limited germline genetic material (immunoglobulin genes), to encode enough information necessary for the production of an estimated 10^7 antibody specificities (Sherman, 1983, Owen, 1982). Ehrlich proposed his somatic theory in 1906, noting that the immune system seemed competent to deal with all antigens encountered. Pauling proposed the template theory in the 1940's and Burnet's clonal selection theory was proposed in 1955 each attempting to explain this enormous capacity for antibody diversification (reviewed by Silverstein, 1984).

The immunoglobulin molecule is made up of heavy and light chains encoded on three different chromosomes (Tonegawa, 1983). On chromosome 12 of the mouse are the structural genetic units which rearrange and are processed to ultimately encode a functional H chain coding unit (Sakano, 1980; Maki, 1981). The functional H chain variable region coding unit is composed of the products of V_H, D and J_H genes. The H chain subscript designation is included to distinguish these from light (L) chain V and J genes. One V_H gene is selected from a pool of an estimated 100-1000 V_H subunits to be coupled with one of 20 possible D genes and one of 4 possible J_H genes (Honjo, 1983). Subunit coupling and splicing generates a new single contiguous gene. This subunit coupling process includes

deletion of intervening DNA, followed by a slightly imprecise joining which is the basis for creating combinatorial diversity (Alt, 1982; Weigert, 1982). When this V_HDJ_H recombination has been completed the variable region coding unit is now juxtaposed to one of eight constant region gene subunits (Alt, 1981; Maki, 1980). Again, the coupling process involves deletion and processing of DNA. The completed H chain gene may now be transcribed and further processed to ultimately produce an H chain polypeptide.

Murine chromosomes 6 and 16 contain genetic subunits which are similarly rearranged to produce the only two light chains known, kappa and lambda (Tonegawa, 1983). Here the K and L subscript refer to kappa and lambda light chains respectively. In the case of kappa light chains one V_K gene segment is selected from a possible 90-320 such genes (Honjo, 1983; Cory, 1981; Honjo, 1985) to be joined through intervening DNA deletion to one of 4 J_K genes (Max, 1979; Sakano, 1979). The completed variable light chain coding unit is then juxtaposed, again by deletion of intron DNA, to the only constant kappa gene known, thus completing the kappa light chain coding unit. A single cell will produce only one light chain type. If the kappa light chain rearrangement has been non-productive then the lambda light chain genes will rearrange in an attempt to produce a usable light chain (Early, 1981). If kappa light chain rearrangement is non-productive, then V_L has 2 possible gene segments and 3 functional J_L coding units which are selected from and linked to one of three C_L coding units by deletion of DNA and processing (Honjo, 1983). A further amplification of the germline and

combinatorial diversity is provided by the association of a particular H chain polypeptide with a light chain polypeptide. Still more diversity may arise through somatic point mutations that can occur in the selected coding sequence (Bothwell, 1981; Gearhart, 1981; Kaartinen, 1986). These point mutations can affect the amino acid sequence of the immunoglobulin and thus have dramatic effects on antibody specificity (Kaartinen, 1986).

Analysis of Antibody Affinity and Heterogeneity

Antibody repertoires, the range of antibody specificities or heterogeneity have been effectively studied by the analysis of the affinity of serum antibody. Studies of the affinity of serum antibody responses to a particular antigen have revealed that during the time course of the antibody response, the average affinity of the antibody population increased. Furthermore, secondary immune responses demonstrate still greater increases in affinity over that seen in the late primary response (Farr, 1958; Stupp, 1969). This increase in affinity has been referred to as affinity maturation.

Studies of antibody affinity on the level of the individual B cell have been undertaken employing the hemolytic plaque forming cell assay. Through sequential inhibition of plaque forming B cells with increasing concentration of free antigen (Andersson, 1970), inhibition profiles were generated which could be used to determine the number of B cells producing high or low affinity antibody as well as the relative heterogeneity of the responding B cell population (Davie, 1972; Claflin, 1973; Goidl, 1974). Since one B cell can

produce only one antibody of a distinctive affinity, then the spread or heterogeneity with respect to concentration of antigen needed for the inhibition of all B cells (the inhibition profile) is an indication of the heterogeneity of the total antibody response. The development of the PFC inhibition system was important because individual B cells producing a particular antibody could be quantitatively determined. Furthermore, this was important because the affinity of antibody from a B cell population could be determined at a precise time point during the response, whereas serum pools of antibody represented the cumulative antibody produced from the onset of the immune response until the time of serum sample collection (Anderson, 1970). Since the lifespan of an antibody molecule in serum is longer than the length of time that a PFC is able to secrete antibody, the low avidity serum antibody produced early in the response might mask the high avidity antibody produced at later times. However, PFCs responding early would certainly die or stop secreting antibody by day 30 thus the higher avidity antibody produced later in the immune response is more easily demonstrated.

Quantitative assessment of antibody affinity from a particular B cell population corroborated the qualitative evidence obtained from the analysis of serum antibody pools. Andersson (1970) was able to show that during the primary immune response in the murine system, the avidity of antibody produced from PFCs steadily increased from day 7 through day 30.

Inhibition analyses of PFCs have been employed to elegantly demonstrate antibody avidity changes during ontological development

(Goidl, 1974). Inhibition profiles were found to be different for B cell populations in primary and secondary immune organs at different times during ontogeny. Goidl (1974) generated histogram plots to depict the inhibition profile where the inhibition produced at a particular inhibitor concentration was plotted as a single histogram bar. Inhibition profiles of primary immune organs, fetal liver and bone marrow, demonstrated that there was limited heterogeneity in their antibody specificities early in life. Furthermore, fetal liver and bone marrow lymphocytes showed an absence of high avidity antibody. However, PFCs from secondary immune organs (spleen and lymph nodes) yielded heterogeneous inhibition profiles demonstrating antibody of high and low affinities (Goidl, 1974). Later in ontogeny (by 3 weeks of neonatal life) fetal liver and bone marrow inhibition profiles demonstrated a heterogeneous population of both high and low affinity antibody, presumably due to recirculation of mature B cells back through the primary immune organs (Goidl, 1974).

Spectrotype Analysis of Antibody Diversity

A more direct approach to demonstrate antibody heterogeneity differences between different lymphocyte populations is by the use of isoelectric focusing (IEF) of antibody populations (Montgomery, 1972; Perlmutter, 1977; Briles, 1980; LeJeune, 1982; Manheimer, 1984). IEF separates proteins due to their mobility in an electric field applied across a pH gradient. A protein will migrate in this electric field through the pH gradient until it reaches its isoelectric point. The molecule will then cease movement because the charge placed on the

gel has no effect on the mobility of a molecule at its isoelectric point. This technique has been shown to be sufficiently sensitive to separate proteins differing by only a single amino acid. Since the specificity of an antibody molecule is directly dependent on its variable region amino acid sequence, then IEF demonstrates each antibody specificity by separating them to their unique isoelectric point.

IEF has been used to compare the antibody repertoires from neonatal and adult animals. A spectrotype analysis was performed on antibody populations from neonatal and adult rabbits primed to respond to DNP (Montgomery, 1972). A uniform, restricted spectrotype was seen in neonates, however adults responded to this same antigen with a heterogeneous spectrotype. Spectrotype analysis thus demonstrated the inability of a neonatal rabbit to generate a heterogeneous antibody response.

Spectrotype analysis has also demonstrated restricted IEF patterns when T cells were limiting in a TD response (Doenhoff, 1979). When T cell pools were allowed to regenerate, a heterogeneous spectrum of antibodies would then develop.

Monoclonal Antibodies and Gene Probing to Study the Origin of Diversity

Recently, hybridoma technology has been employed in the study of the generation of antibody diversity (Karjalainen, 1980; Gearhart, 1981). During an immune response hybridomas of B cells producing

specific antibody can be generated allowing for expansion of a single, specific B cell clone to numbers which can facilitate mRNA analysis of that particular clone. Subsequent analysis of the cDNA has led to estimations as to the extent of the B cell antibody gene repertoire Owen, 1982). Employing various immunoglobulin gene probes and hybridizing these with DNA preparations from different hybridomas has yielded information on the usage of particular genes and whether rearrangement or somatic mutation has been employed in producing that particular antibody molecule (Cumano, 1985). The above techniques can be applied to hybridomas made early and late in the primary response and in the secondary response in attempts to correlate genetic changes with the affinity maturation of an immune response. This technique allows one to determine the genetic causes of antibody specificity changes during an immune response.

Hybridoma technology has been used to investigate the maturation of the immune response to 2-phenyloxazolone (Kaartinen, 1983; Griffiths, 1984; Berek, 1985). Hybridomas were generated from B cells responding to 2-phenyloxazolone (phOx) at three different times during the primary response. In this study a number of different clones were generated. Analysis of the mRNA of these cells indicated the use of a single set of unmutated V_H and V_K genes in the generation of the phOx repertoire. At later time points in the primary immune response, a more heterogeneous hybridoma population was generated, producing antibodies of higher affinity. Analysis of the mRNA from these hybridomas led to the correlation of the production of higher affinity antibodies with somatic mutations in the germline gene sequence (Kaartinen, 1983; Griffiths, 1984). In

the secondary response to phOx, hybridomas with high affinity were observed that were employing new germline genes and subsequent somatic mutation of these genes (Berek, 1985). The studies implicated somatic mutation, combinatorial diversity and germline diversity as all playing a role in the generation of antibody diversity and affinity maturation. Rittenberg (1986) employed similar techniques to look at the DNA of different anti-phosphorylcholine (PC) hybridomas. This work demonstrated that the germline gene repertoire contributes greatly to the generation of antibody diversity. The primary response to PC is characterized as being very restricted, using predominantly the gene coding for the T15 idiotype. However, secondary responses to PC are not so restricted, employing genes coding for many non-T15 idiotypes. PC hybridomas from a secondary response were found that used the same V_L that is found in the T15 idiotype, but a new V_H gene was used. The combination of a transcript of a new V_H gene with a transcript of V_L from the T15 idiotype generated a new idiotype of antibody specific for PC but lacking T15 idiotype character (Rittenberg, 1986).

Salmonid Immune Organs

B cells can be found in all fish immune organs. The major salmonid organs containing B cells are the spleen and the kidney. The thymus of the salmonid is also a lymphoid organ but possesses only minimal B cell function. The existence of lymphoid cells and function in the salmonid kidney is not seen the mammalian immune

system (Chiller, 1969). Salmonids lack bone marrow and significant numbers of lymphocytes have yet to be demonstrated in the liver, thus the salmonid hematopoietic tissue must be present elsewhere. Teleosts, the family to which salmonids belong, possess a kidney which has been demonstrated to be their hematopoietic center (Zapata, 1979; Yasutake, 1983). The anterior region of the kidney has thus been identified as an analog of mammalian bone marrow, possessing primary lymphoid functions. The lymphoid cell population dominates other tissue types in the anterior kidney. Lymphoid cells can be observed in many stages of differentiation and many blast cells are present, which is indicative of a lymphopoietic tissue (Zapata, 1979; Yasutake, 1983). In the more posterior regions of the kidney, nephrons and renal function are more fully represented, however there is also a large complement of lymphocytes. Zapata found that the posterior kidney lymphocyte population possessed more mature cell types, unlike the anterior kidney, and thus had the appearance of a secondary immune organ. The salmonid spleen also has the appearance of a secondary immune organ sharing features with its mammalian counterpart. The secondary immune organs of salmonids possess lymphoid cells in terminal stages of differentiation suggesting that, as in mammalian secondary immune organs, this is the final area where mature lymphocytes primarily reside.

The salmonid possesses two distinct thymi. Significant but low numbers of antibody producing B cells have been demonstrated in the teleost (Tilapia mossambica) thymus (Sailendri, 1975). The numbers of surface immunoglobulin positive lymphocytes in the thymus have also been reported to be very high, when screened with a polyclonal

reagent (Warr, 1979; Feibig, 1983). However, DeLuca (1983) reported only 5% of thymocytes were surface Ig positive using a monoclonal anti-fish Ig antibody. The ultrastructure of the thymus appears to be a diffuse collection of lymphocytes (Yasutake, 1983), suggesting that the salmonid thymus is polyfunctional, containing B and T cells thus appearing more like a mammalian lymph node than a primary developmental center for T cells.

Salmonid lymphoid cells are first detected in the thymus and the anterior kidney (Grace, 1980). Subsequently, lymphoid cells can then be detected in the spleen (Grace, 1980). The thymus increases in size with body weight, however, the numbers of thymic lymphocytes, or thymocytes, decrease with age (Tatner, 1985). Thus, thymocytes must leave the thymus. Thymocytes have been radiolabelled and tracked *in vivo* and found to lodge in the spleen at twofold higher levels than the anterior kidney (Tatner, 1985). The anterior kidney and the thymus demonstrate the earliest lymphoid infiltration, however no migration of immature lymphoid cells occurs between these two organs. Therefore the kidney must acquire its hematopoietic stem cells independently. Furthermore, Tatner states that early thymectomy does not affect the kidney lymphoid population but, does affect the splenic lymphoid composition (Tatner, 1985), indicating that the lymphoid population of the kidney is self generating whereas the splenic lymphoid population is dependent on other lymphopoietic sources.

B Cell Activation in Salmonids

B cell activation in salmonids has been investigated, but this phenomenon requires further experimental attention before models with the sophistication as seen with mammals can be developed. Salmonid B cells have demonstrated surface Ig (Warr, 1979), therefore, they are equipped with at least one essential receptor that is required in mammalian B cell activation. Salmonid B cells have been demonstrated to respond to mitogens (LPS, PPD and PWM) by proliferation and antibody production (Etlinger, 1976; Faulman, 1983; Kaattari, 1987). Thus, it is inferred that salmonids possess the necessary receptors and signals for a TI form of B cell activation, however no distinction as to type 1 or type 2 responses can be made in salmonids as yet. In vitro study of B cell activation has recently begun in fish (Miller, 1985). T-independent B cell responses by channel catfish lymphocytes were demonstrated with TNP-LPS. This response could also be reduced if macrophages were removed from the cultures (Miller, 1985). A culture supernatant generated by in vitro stimulation of catfish macrophages could functionally replace the macrophage and restore ability to produce a TI response (Miller, 1985).

T Dependent B Cell Activation

In fish, a classical T-dependent B cell response has been difficult to describe on the cellular level until the recent work of Miller (1985). However, the ability of fish to respond to protein

antigens (TD antigens) has been demonstrated in earlier investigations (Yocum, 1975; Ruben, 1977; Blazer, 1984). Unlike the murine system, no strains of fish are known to be T cell deficient and fish T cell markers have yet to be defined. Therefore, isolation of a cellular population cannot be accomplished in fish, which would allow for the demonstration of T cell dependence to a particular antigen.

Functional evidence for the presence of T cells does however exist in fish systems. The effects of carrier antigen priming on an antibody response to a protein antigen have illustrated a requirement similar to that which is required of mammalian TD responses (Ruben, 1977; Yocum, 1975; Weiss, 1977; Stolen, 1975). Temperature has also been effectively used to cause differential effects on putative T cell functions in fish (Weiss, 1977; Miller 1984). Weiss has shown that carrier priming is necessary for a response to TD antigens, however if the animals were switched to a temperature colder than the optimal for these animals, then no T cell priming was observed. Miller (1985) has also demonstrated the necessity of a surface Ig negative lymphocyte for <u>in vitro</u> B cell responses to the haptenated protein antigen, trinitrophenylated-keyhole limpet hemocyanin (TNP-KLH). If this Ig negative population was restored to a purified Ig positive lymphocyte culture, then a response would occur. Also, in agreement with the work of Avtalion, if the incubation temperature was lowered from the optimal temperature prior to cell addition then T cell help did not occur. Since mammalian T cells are Ig surface negative and also possess this same helper function, by analogy it

would seem that this Ig negative fish lymphocyte is a T cell.

The work of Miller (1985) demonstrated that both the macrophage and the Ig negative lymphocyte were necessary to generate a B cell response to TNP-KLH. Again, this TD response could be restored by addition of catfish macrophage populations and more importantly by a macrophage culture supernatant. The effects of this supernatant on restoring a macrophage function is reminscent of the role of mammalian IL-1.

Antibody Molecules in Fish

Two isotypes of immunoglobulin molecules have been demonstrated in different fish species (Acton, 1971; Marchalonis, 1977; Kobayashi 1984). In various fish species immunoglobulin studies have revealed tetrameric, pentameric, and hexameric IgM. This multimeric IgM molecule is made up of pairs of two heavy and two light chains completing the monomeric unit. The different numbers of monomers are held together by a J chain much like mammalian IgM. IgM antibody in fish has not been demonstrated to isotype switch although a distinct IgA-like secretory isotype has been demonstrated by Lobb (1981) in the sheepshead and by Kobayashi (1984) in the skate. Affinity maturation or other characteristics of an anamnestic response have not been conclusively demonstrated in fish.

IgA antibody, as mentioned previously, has been shown in a few fish that have been studied. The secretory nature of this IgA molecule is demonstrated by its isolation from bile fluid and surface secretions. Kobayashi (1984) has demonstrated that this IgA antibody

has H chains of different molecular weight compared to IgM H chains from the same animals. As with mammalian IgA, the fish IgA possesses a secretory piece and can be found as a trimeric molecule, however dimeric and monomeric forms of IgA have not been demonstrated in fish as is the case in mammals.

Attempts to study the diversity of piscine antibody responses have employed fine specificity, spectrotype and germline gene analyses. A spectrotype analysis study employed the H and L chains of natural (non-immunized) serum antibodies (Wetzel, 1985). As far as reducing the antibody molecule to H and L chains, Wetzel notes this could reduce apparent Ig polymorphism. Polymorphism would be reduced because the diversity of antibody is largely dependent upon the combination of variable heavy and light chain polypeptides. Reduction of the large IgM fish antibody was necessary, however, because of molecular size limitations of the isoelectric focusing in polyacrylamide gels.

The work of Litman on antibody heterogeneity in the shark first employed N terminal amino acid sequencing of shark H chains and found only one difference in 45 H chain preparations (Litman, 1982). Fine specificity analyses of shark anti-DNP antibodies indicated lower antibody affinities to all DNP analogs as when compared to rat IgM antibodies (Makela, 1980). Litman concluded, on the basis of these findings that the shark antibody repertoire was relatively restricted. However, using a murine variable region probe to isolate germline shark V region genes, Litman was able to isolate 6 or 7 components of a closely related sequence. Restriction endonuclease

mapping has shown 17 different isolated genes showing homology with the V region gene of the mouse (Litman, 1985). This data indicated a heterogeneous germline repertoire for antibody.

The study of piscine antibody diversity has recently been investigated by different researchers employing various strategies. These different approaches have yielded conflicting evidence as to whether the fish antibody repertoire is restricted or diverse. The success of the plaque forming cell inhibition analysis system in the elucidation of murine antibody diversity demonstrates its utility in the study of fish antibody diversity.

MATERIALS AND METHODS

Animals

Yearling (50-100 g) coho salmon (Oncorhynchus kisutch) and 2-3 year coho (150-300 g) were obtained from hatcheries located in the state of Oregon, primarily Fall Creek Hatchery. These coho salmon were used in the assessment of the in vivo, anti-Vibrio plaque forming cell responses. Two to three year rainbow trout (Salmo gairdneri), from Oak Springs Hatchery, were used in the characterization of the in vitro antibody responses to TNP and in the lymphocyte irradiation assays. One to two year coho from Fall Creek Hatchery were used in the characterization of the in vitro anti-trinitrophenyl (TNP) response, antibody repertoire analysis and for mitogen assays.

All fish were held in free-flowing (12°C) pathogen-free well water in 220 gallon circular tanks at the Oregon State University Fish Disease Laboratory. Fish were fed the Oregon Moist Pellet diet.

Adult female New Zealand White (NZW) rabbits and adult female BALB/c mice were maintained by the Laboratory Animal Resource Center at Oregon State University.

Antigens

An 0-antigen extract of Vibrio anguillarum (LS-174) was prepared as described by Anderson (1979). Briefly, bacterial cultures were grown to turbidity in BHI broth cultures which were

agitated at room temperature (22-24°C). BHI broth was prepared by rehydration of dehydrated Brain Heart Infusion (37 g/l) with distilled water. The broth was autoclaved and cooled before inoculation with bacteria. The bacteria were killed by the addition of formalin to a final concentration of 0.4% (v/v). The killed Vibrio cells were washed three times with phosphate buffered saline (PBS; see appendix IV) by centrifugation for 10 minutes at 6000 x g, followed by resuspension in PBS. Fifty ml of wet-packed bacterial cells were suspended in 10 volumes of 2% saline. The cells were then heated in a boiling water bath for 2 hr, then washed in 2% saline by centrifugation for 10 minutes at 6000 x g. The pellet was resuspended in 95% ethanol and incubated for 48 hr at 37°C. Insoluble material was pelleted again by centrifugation for 10 minutes at 6000 x g and washed twice in acetone by centrifugation for 10 minutes at 6000 x g. The pellet was then dried overnight at 37°C. Dried antigen was ground to a fine powder with a mortar and pestle, yielding approximately 6 g of powdered antigen per 50 ml initial volume of wet-packed cells.

Trinitrophenylated keyhole-limpet hemocyanin (TNP-KLH) was prepared by a modification of the method described by Rittenberg (1966). Picrylsulfonic acid (15.1 mg) was dissolved in 5 ml cacodylate buffer (see appendix IV) and added dropwise to a constantly stirred solution of KLH (72 mg) in 0.85% saline (5 ml at 14.4 mg/ml) contained in a foil-wrapped tube. Keyhole-limpet hemocyanin was a generous gift from Dr. Marvin Rittenberg (Department of Microbiology and Immunology, Oregon Health Sciences University,

Portland, OR). The picrylsulfonic acid, KLH mixture was stirred at room temperature for 1 hr. TNP-KLH was dialyzed against 4 (1 l) changes of PBS and a final change of 500 ml RPMI 1640 (Gibco). The dialyzed TNP-KLH was filter sterilized (0.45 um) and assayed for protein content by the method of Lowry (1951). The TNP-KLH stock solution was stored at 4°C. Stock TNP-KLH was diluted appropriately with sterile Mishell-Dutton media (MDM; see In vitro cell culture section) prior to culture initiation. Trinitrophenylated lipopolysaccharide (TNP-LPS) was prepared by the method of Jacobs and Morrison (1975). Lipopolysaccharide W (161 mg, LPS-W), from E. coli (055:B5, Difco, Detroit, MI) was suspended in cacodylate buffer (8.05 ml) with constant stirring. The pH of the solution was adjusted with 10 N sodium hydroxide to 11.5. The tube was foil wrapped and picrylsulfonic acid (96 mg), also dissolved in cacodylate buffer (8 ml), was added dropwise with constant stirring. The solution was stirred for 2 hr at room temperature. This solution of TNP-LPS was then dialyzed against 4 (1 l) changes of saline (0.85%) and finally against RPMI 1640 (1 l). TNP-LPS was pasteurized for 30 min at 70°C and stored at 4°C. Stock TNP-LPS was diluted with sterile MDM just prior to addition to cultures.

Mitogens

A stock solution of Lipopolysaccharide W (20 mg/ml) was prepared by addition of an appropriate volume of RPMI 1640. The LPS stock was pasteurized by incubation for 30 min in a 70°C water bath and stored frozen at -20°C. The LPS stock was thawed and diluted

appropriately in MDM prior to addition to mitogen cultures.

Phytohemagglutinin (#L9132, Sigma, St. Louis, MO) was prepared at a stock concentration (1 mg/ml) by addition of RPMI 1640 to the lyophilized powder. The stock solution was sterile filtered (0.45 um) and stored at 4°C. Phytohemagglutinin (PHA) was diluted in MDM prior to addition to cultures.

Antigen Inhibitors

Bacterial soluble antigen inhibitor was prepared by heating a suspension of the powdered antigen (30 mg/ml) in PBS, in a boiling water bath for 1 hr (Anderson, 1979). Particulate matter was centrifuged at 4°C for 10 min at 6000 x g. The supernatant fluid was removed from the particulate material, filter sterilized (0.45 um), and stored at 4°C. Vibrio ordalii was grown and an O-antigen extract prepared exactly as described above for the Vibrio anguillarum O-antigen preparation. Renibacterium salmoninarum was grown to turbidity in agitated KDM-2 broth (see appendix IV) at 15°C. Whole bacterial cells were washed in PBS by centrifugation for 10 min at 8000 x g. The bacterial pellet was resuspended with a volume of PBS yielding a reading of 10 O.D. units at a wavelength of 500 nm. The cell suspension was heated 1 hr in a boiling water bath. The resulting particulate material was removed by centrifugation at 8000 x g for 10 min. The supernatant fluid was collected, filter sterilized (0.45 um), and stored at 4°C. A Lowry (1951) protein assay was used to determine the protein quantity in each soluble

antigen extract. Carbohydrate content determinations were made for the soluble antigen extracts of Vibrio anguillarum and Vibrio ordalii by the phenol-concentrated sulfuric acid method (Nowotny, 1979).

Analogs of the TNP hapten, which were used as inhibitors, were purchased in solid form and then were dissolved in RPMI 1640 holding media to concentrations which approached their solubility limits. Stock epsilon-TNP-L-lysine monohydrochloride, epsilon-(2,4)-DNP-L-lysine monohydrochloride (ICN Biochemical, Cleveland, OH) were dissolved to a concentration of 10^{-3}M. Stock solutions of paranitrophenol, DNP-L-phenylalanine and DNP-gamma-amino-N-butyric acid (ICN Biochemical, Cleveland, OH) were brought to a concentration of 10^{-2}M. Stock solutions of 2,4-Dinitrophenyl- acetic acid, 2,4-Dinitrophenol and 4-Nitrophenylacetic acid (Aldrich, Milwaukee, WI) were dissolved at a concentration of 10^{-2}M.

Immunizations

Coho salmon were injected intraperitoneally with 0.1 ml (10 ug) of a 100 ug/ml suspension of powdered Vibrio anguillarum antigen in PBS. The fish were anesthetized in a benzocaine bath (2 ml/3.8 l H_2O). Benzocaine solution consisted of ethyl-p-aminobenzoate (10 g) in 100 ml of 95% ethanol. Fish were sacrificed for the assessment of plaque forming cell responses on days 7, 12, 16, and 21 post immunization.

Complement

Sera from spawned adult steelhead trout Salmo gairdneri, gairdneri, were used as the source of complement. Fish were bled by severing the caudal peduncle. Blood was collected in 25 ml pools using 50 ml Corning centrifuge tubes (Corning, Corning, NY) held on ice and allowed to clot overnight at 4°C. The sera were removed and centrifuged in a Beckman microfuge for 5 min to remove the remaining red blood cells. The cleared sera were pooled, then divided into 0.5 ml aliquots, and stored at -60°C until used. Each batch of sera were periodically titrated, by dilution in modified barbital buffer MBB (see appendix IV), for optimal activity in a plaque forming cell assay. Complement titration was necessary because activity varied between batches and over time.

Anti-Coho Immunoglobulin

Rabbit anti-coho immunoglobulin (Ig) was a generous gift from Dr. W. Groberg (Oregon Dept. of Fisheries and Wildlife, Oregon State Univ., Corvallis, OR). Briefly, antiserum was obtained from a rabbit injected with affinity purified coho anti-TNP Ig. The specificity of the reagent for fish immunoglobulin was confirmed by appearance of a single precipitin band in an immunoelectrophoretic analysis against whole coho serum (Groberg, 1982).

Preparation of Antigen Targeted Sheep Red Blood Cells (SRBC)

Vibrio anguillarum coated target SRBC were prepared as described by Anderson (1979). Antigen used to coat SRBC was a soluble extract of the O-antigen powder. Powdered antigen (1 mg/ml) was suspended in PBS and heated with periodic mixing in a boiling water bath for 1 hr. Particulate matter was centrifuged at 6000 x g for 10 min at 4°C and the supernatant was collected. Sheep red blood cells were washed 3 times in PBS by centrifugation at 500 x g for 5 min. Washed SRBC were resuspended in PBS (20% v/v). Equal volumes of supernatant fluid and washed 20% SRBC were incubated 1 hr in a 37°C water bath. The Vibrio coated SRBC (VSRBC) were then incubated overnight at 4°C. Immediately prior to use in the plaque assay, the VSRBC were washed 5 times by centrifugation in PBS followed by a final wash in MBB and resuspended in MBB to (12% v/v).

Trinitrophenylated SRBC target cells were prepared using a modification of the method described by Rittenberg (1969). Sheep red blood cells were washed 3 times in MBB by centrifugation at 1400 x g for 10 min at 4°C. Wet packed (1 ml) SRBC were added dropwise to a foil-wrapped screw-cap test tube containing 20 mg of picrylsulfonic acid (Sigma, St. Louis, MO) in 7 ml cacodylate buffer. This suspension was briefly mixed by aspiration with a pasteur pipet, the tube was capped and slowly tumbled on a rotating tumbler for 15 min at room temperature. Seven ml of cold MBB (4°C) was added to the suspension and mixed by aspiration. The TNP coated SRBC (TSRBC) were centrifuged for 5 min, 1400 x g at 4°C. The supernatant was removed and the TSRBC pellet was resuspended in MBB (11.7 ml) containing

glycylglycine (7.4 mg). The TSRBC were again pelleted by centrifugation as above and washed by centrifugation 3 more times in MBB (4°C). The washed TSRBC pellet was resuspended in MBB (10% v/v).

In Vitro Cell Culture Media

The Mishell-Dutton tissue culture medium (MDM), used for in vitro cell cultures for both mitogen assays and antibody production, is modified from that originally described by Mishell (1967). The MDM consists of sterile filtered RPMI 1640 (Gibco Laboratories, Grand Island, NY.) supplemented with sterile filtered sodium bicarbonate (2 g/l), (10% v/v) sterile filtered fetal calf serum (hybridoma screened, M.A. Bioproducts, Walkersville, MD), (1 ml/l) gentamicin sulfate (sterile, 50 mg/ml, M.A. Bioproducts), (50 uM) sterile filtered 2-mercaptoethanol (MCB, Cincinnati, OH), 2 mM sterile filtered L-glutamine (200 mM, Gibco, Chagrin Falls, OH.) and sterile filtered nucleosides (10 ug/l); adenosine, uracil, cytosine, and guanosine (Sigma, St. Louis, MO).

A nutritional cocktail for culture supplementation was also prepared as previously described by Tittle (1978) and fed every other day to the cell cultures as described below. Stock nutritional cocktail was prepared by adding 20 ml of essential amino acids (sterile, 50X, M.A. Bioproducts), 10 ml of non-essential amino acids (sterile, 100 mM, M.A. Bioproducts) 10 ml of sterile filtered dextrose (200 mg/ml), 10 ml of sterile filtered L-glutamine (200 mM) and gentamicin (0.2 ml) to sterile filtered RPMI 1640 (140 ml)

containing bicarbonate (2 g/l). The stock was mixed and pH adjusted to 7.2 with sterile 1 N sodium hydroxide. The stock cocktail was sterile filtered (0.22 um) and divided into 15 ml aliquots. The stock cocktail was held at -20°C. Complete feeding cocktail was prepared by adding sterile filtered fetal calf serum (7.5 ml), (1 ml) sterile filtered nucleosides (AUC, 1 g each/l and G, 1 g/l) to a tube containing 15 ml stock solution. The complete cocktail was mixed and stored at 4°C.

Holding medium consisted of sterile filtered RPMI 1640 supplemented with fetal calf serum (10%), bicarbonate (2 g/l) and gentamicin (1 ml/l).

Preparation of Coho Lymphocytes for In Vivo PFC Assay

Lymphocytes were obtained by preparing single cell suspensions of the spleen, anterior and posterior kidney, and thymus on ice. Single cell suspensions were produced by gentle aspiration of organ fragments in holding medium (1 ml) employing a 1 ml syringe. Lymphocytes were washed twice by centrifugation at 100 x g for 10 min at 4°C. Washed cell pellets were finally resuspended in holding medium to an appropriate dilution for plaque enumeration (200 to 400 PFC per slide chamber).

Lymphocyte Separation by Ficoll Hypaque Density Centrifugation

Lymphocytes were separated from heparin treated whole blood by density dependent centrifugation over a ficoll hypaque gradient. One

ml of heparin treated whole blood was diluted with 4 ml of holding media and gently layered on top of the ficoll with a pasteur pipet. The gradient was centrifuged at 100 x g for 30 min at 4°C. A layer of lymphocytes at the media/ficoll interface was removed by gentle aspiration with a pasteur pipet. Collected lymphocytes were washed twice in holding media by centrifugation (400 x g, 10 min, at 4°C). The resulting cell pellet was resuspended to the original volume of one ml in holding media and was determined to contain less than 5% red blood cells.

Trypan Blue Exclusion Test for Cell Viability

The trypan blue exclusion test is a method where viable cells will not be stained by the dye suspension however, nonviable cells will stain blue. Trypan blue stain is prepared by suspending trypan blue (0.4 g/100 ml) in saline (0.85%). This suspension is passed through a funnel containing filter paper (Whatman #1) and stored at room temperature. An aliquot of 0.1 ml trypan blue stain is added per 0.5 ml of a lymphocyte suspension and allowed to stain the cells for at least 10 min.

Cell Irradiation

Lymphocytes were suspended to 2 x 10^7 cells/ml in holding medium in 5 ml plastic tubes and held on ice. The tubes were placed, on ice, in the low flux region of a Cobalt 60 gamma irradiator at the

OSU Radiation Center for the calculated amount of time necessary for each radiation dose. The cells were then washed by centrifugation (5 min, 4°C at 400 x g) and resuspended in MDM. Irradiated lymphocytes were counted, resuspended to 2 x 10^7 cells/ml, and plated as described below.

In Vitro Cell Culture Initiation and Maintenance

A single cell suspension was prepared from the spleen and anterior kidney of each test animal by aspiration of organ samples with a 1 ml syringe. The single cell suspensions were washed two times in holding medium by centrifugation at 500 x g for 10 minutes at 4°C. The final washed cell pellet was resuspended in MDM. Lymphocytes were enumerated by the use of a hemocytometer. Cell viability was determined by the trypan blue exclusion method. Triplicate cultures of 4 x 10^6 lymphocytes in 200 ul MDM were added to the wells of a 24-well, flat-bottomed, tissue culture plate (Corning, Corning, NY) containing antigen. Mitogen assays employed triplicate cultures of 5 x 10^4 lymphocytes in 50 ul MDM added to individual wells of a 96-well, flat-bottom tissue culture plate (Corning, Corning, NY) containing mitogen. The tissue culture plates were then incubated in plastic culture boxes (C.B.S. Scientific, Del Mar, CA) in an atmosphere of 10% CO_2 at 16°C. The cultures were maintained by adding approximately 50 ul of complete cocktail every other day and placing back in the culture boxes in a CO_2 atmosphere. Mitogen cultures were not supplemented with complete cocktail.

Mitogen Assay

Twenty-four hours prior to culture well harvest, each well was pulsed with 1 uCi of tritiated thymidine (methyl-^{3}H, ICN Biomedicals, Irvine, CA) prepared in 50 ul MDM. Cells were harvested with distilled water onto glass fiber filters, with a Skatron cell harvester (Sterling, VA). The filters were then dried, placed in scintillation vials with cocktail (10 ml) and counted on a Beckman liquid scintillation counter (EC 3800). Scintillation cocktail consisted of 6g PPO (Sigma, St. Louis. MO) and 5 mg POPOP (Amersham, Arlington Heights, IL) added to 1 l toluene.

ELISA Assay for Quantitation of Total Salmonid Immunoglobulin

A hybridoma cell line producing monoclonal antibodies (McAb, 1-14) to fish immunoglobulin was obtained from Dr. G. Warr (Dept. of Microbiology, Univ. of S. Carolina). The hybridoma cells were injected intraperitoneally into a BALB/c mouse. Approximately two weeks later, the ascites fluid was aspirated with a needle and syringe, and centrifuged. The pelleted cells were injected into another mouse and the supernatant collected for partial purification of the antibody (1-14). The ascites fluid was cut three times with 50% saturated ammonium sulfate (SAS), then extensively dialyzed against PBS, sterile filtered and assayed for anti-fish immunoglobulin activity.

Biotinylated 1-14 was prepared by dialyzing 3 mg of the SAS cut 1-14 against 0.1 M carbonate buffer and then adding 20 ul of 0.1 M

biotinyl-n-hydroxysuccimide ester (Cal-Biochem, La Jolla, CA) in dimethyl formamide. The mixture was agitated at room temperature for one hour, then dialyzed against PBS. The biotinylated 1-14 stock was mixed 1:1 with glycerol and stored at -20°C.

Total fish immunoglobulin in serum and tissue culture medium supernatants was quantitated in the following assay system. Flat-bottomed, 96 well ELISA plates (Costar, Cambridge, MA) were used for all assays. Tris buffered saline (TBS; 6.07 g Tris Base, 8.7 g NaCl, and 0.409 g EDTA-$2H_2O$ in one liter of distilled water, pH 8.0) with 0.1% Tween 20 added (TTBS) was used throughout except as indicated. Between each step, the wells were rinsed 4 times with TTBS and then 4 times with TBS.

Wells were coated by overnight incubation with 5 ug/ml of 1-14 in coating buffer in (0.159 g Na_2CO_3 and 0.293 g $NaHCO_3$ in 100 ml distilled water, pH 9.6) at 17°C in a covered plate. Wells were then blocked with 1% bovine serum albumin-TBS for one hour at room temperature followed by the addition of dilutions of fish immunoglobulin standards and unknowns for three hours. After rinsing, a 1/500 dilution of biotinylated 1-14 in TTBS was added and incubated for two hours, followed by a 1/100 dilution of streptavidin-horseradish peroxidase for 20 min. The substrate (75 ul ABTS, 5 ul H_2O_2, and 10 ml 0.2% (w/v) citrate buffer, pH 4.0) was then added. Optical densities were read at 10 min intervals at a wavelength of 405 nm on a Biotek EL310 ELISA reader (Biotek, Burlington, VT).

Plaque Forming Cell (PFC) Assay

Cells secreting anti-*Vibrio* antibody were detected by a modification of the Cunningham plaque assay (Cunningham, 1968). Lymphocyte suspensions (75 ul) were mixed in individual wells of a 96-well microtiter plate (Linbro, McLean, VA) with VSRBC or SRBC (25 ul), complement (25 ul of an appropriate dilution of salmonid sera in MBB), and holding media (25 ul). This mixture was aspirated with a pasteur pipet and deposited in a microscope slide chamber, sealed with melted paraffin and incubated for 1-3 hr at 15-17°C. Slide chambers were constructed from pre-cleaned, 75x25 mm microscope slides (American Scientific Products, # M6155-1, McGraw Park, IL) and flat stock liner, double coated tape (# 410), 6.35 mm width (Scotch 3M, St. Paul, MN). Plaques were then enumerated with the aid of a dissecting microscope.

In vitro stimulated anti-TNP antibody producing cells were detected in a PFC assay similar to that described above. Briefly, cells were harvested from individual culture wells by addition of an aliquot of holding media and gentle aspiration with a pasteur pipet. Lymphocytes were washed in holding media as described above, and resuspended in an appropriate volume of holding media (ideally 200-400 PFC per 75 ul aliquot). The assay was performed using 75 ul of the lymphocyte suspension, holding media or hapten inhibitor (25 ul), 25 ul of a suspension of SRBC or TSRBC (10% v/v in MBB) and complement (25 ul). The resulting suspension was mixed by gentle aspiration and placed in slide chambers with a pasteur pipet. The Cunningham chambers were incubated for 1-3 h at 15-17°C. Plaques

were enumerated with the aid of a dissecting microscope.

Antigen/Hapten Specific Inhibition of the PFC Response

The anti-Vibrio PFC response generated in vivo was analyzed by inhibition of the PFC response with a series of graded concentrations of soluble Vibrio antigen. Serial 3 fold dilutions of Vibrio antigen were prepared containing 64, 21, 7, 2.4, 0.8, 0.26, and 0.09 ug/ml carbohydrate. Each of these inhibitor concentrations (25ul) were added to the PFC assay mixture replacing the holding media (25ul) to compete with red blood cell bound Vibrio antigens for the antibody produced. Inhibitor concentrations were tested in triplicate allowing for percent inhibition calculations and subsequent generation of inhibition profiles.

The specificity for hapten of in vitro generated antibody was tested by employing TNP-lysine (10^{-3}M) as a competitive inhibitor of plaque formation and nonhaptenated lysine as a negative control. TNP-LYS or lysine (25 ul at 10^{-3}M) replaced MDM (25 ul) in the PFC assay mixture to specifically compete with red blood cell bound TNP for hapten specific antibody. Repertoire and fine specificity analyses were accomplished employing various analogs of the TNP-lysine inhibitor. These analogs included dinitrophenylacetic acid, dinitrophenyl-lysine, dinitrophenylalanine, dinitrophenylbutyric acid, dinitrophenol, paranitrophenylacetic acid and paranitrophenol. Serial dilutions, ranging in concentration from 10^{-2}M to 4 x 10^{-6}M, of the various inhibitors, were employed in triplicate allowing for

the percent inhibition to be calculated. This data was used to generate inhibition curves or histogram plots. Inhibition by free hapten was shown to be due to competition with red blood cell bound TNP and not to possible cytotoxicity of inhibitor molecules against the lymphocytes. This was demonstrated either by preincubation of lymphocytes with the hapten inhibitor or by assessing the amount of inhibition due to substituents attached to the hapten. Assessment of lymphocytotoxicity by preincubation of cells with paranitrophenol was accomplished by incubating lymphocytes for two hours at 15°C with this inhibitor and then washing in MDM by centrifugation (twice for 5 min, 400 x g at 4°C). Control cells preincubated in MDM, and inhibitor treated cells were then assayed and compared for their ability to produce plaques, now in the absence of free hapten inhibitor. Nonhaptenated butyric acid, phenylalanine, acetic acid and lysine were added to PFC assay mixtures in triplicate to assess the amount of inhibition they cause.

RESULTS

Major Salmonid Immune Organs

B cell responses in the salmonid immune system were investigated in this study and comparisons were made between B cells derived from the different immune organs of individual animals. Therefore a schematic representation of the major salmonid immune organs has been provided (Figure 1). Illustrated are two major B cell organs of the salmonid, the spleen and kidney. Two different regions of the kidney (anterior and posterior) are designated because, as will be discussed later, they appear to contain functionally distinct B cell subpopulations. The thymus is also represented by one of a pair of distinct thymi.

Organ Distribution and Kinetics of the Plaque Forming Cell Response

Production of specific antibody is a direct indicator of an immune response. The Cunningham modification of the Jerne hemolytic plaque assay (Cunningham, 1968) enables one to precisely enumerate the lymphocytes producing specific antibody, thereby allowing a quantitative assessment of a particular immune response. The *in vivo* response of salmonid lymphocytes to *Vibrio anguillarum* antigen was assessed at 4 or 5 day intervals from day 7 through day 21 post immunization. A single hemolytic plaque is shown represented by a zone of lysis in a lawn of indicator, *Vibrio*-coated, sheep red blood cells (VSRBC; Figure 2). Plaque forming cells (PFCs) were shown

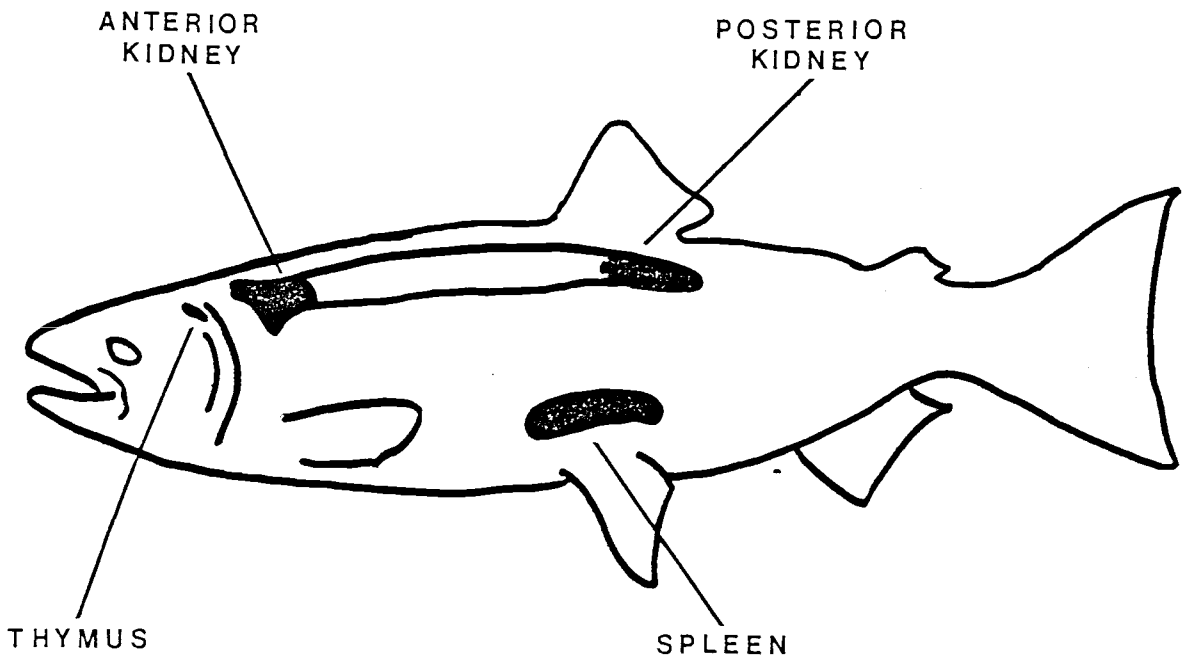


Figure 1. Schematic representation of the major salmonid immune organs. The spleen and kidney are both internal organs and their approximate locations within the animal are depicted. Anterior and posterior kidney regions are designated as functional differences between these tissue regions have been found. The thymus lies on the outer body of the animal, separated from the external environment by a single layer of epithelium. The thymus is located beneath the operculum where the gill arches join the dorsal side of the head.

Figure 2. Illustration of a hemolytic plaque. Formation of circular zone of hemolysis results from an antibody secreting lymphocyte (see arrow) present within a surrounding lawn of indicator red blood cells.

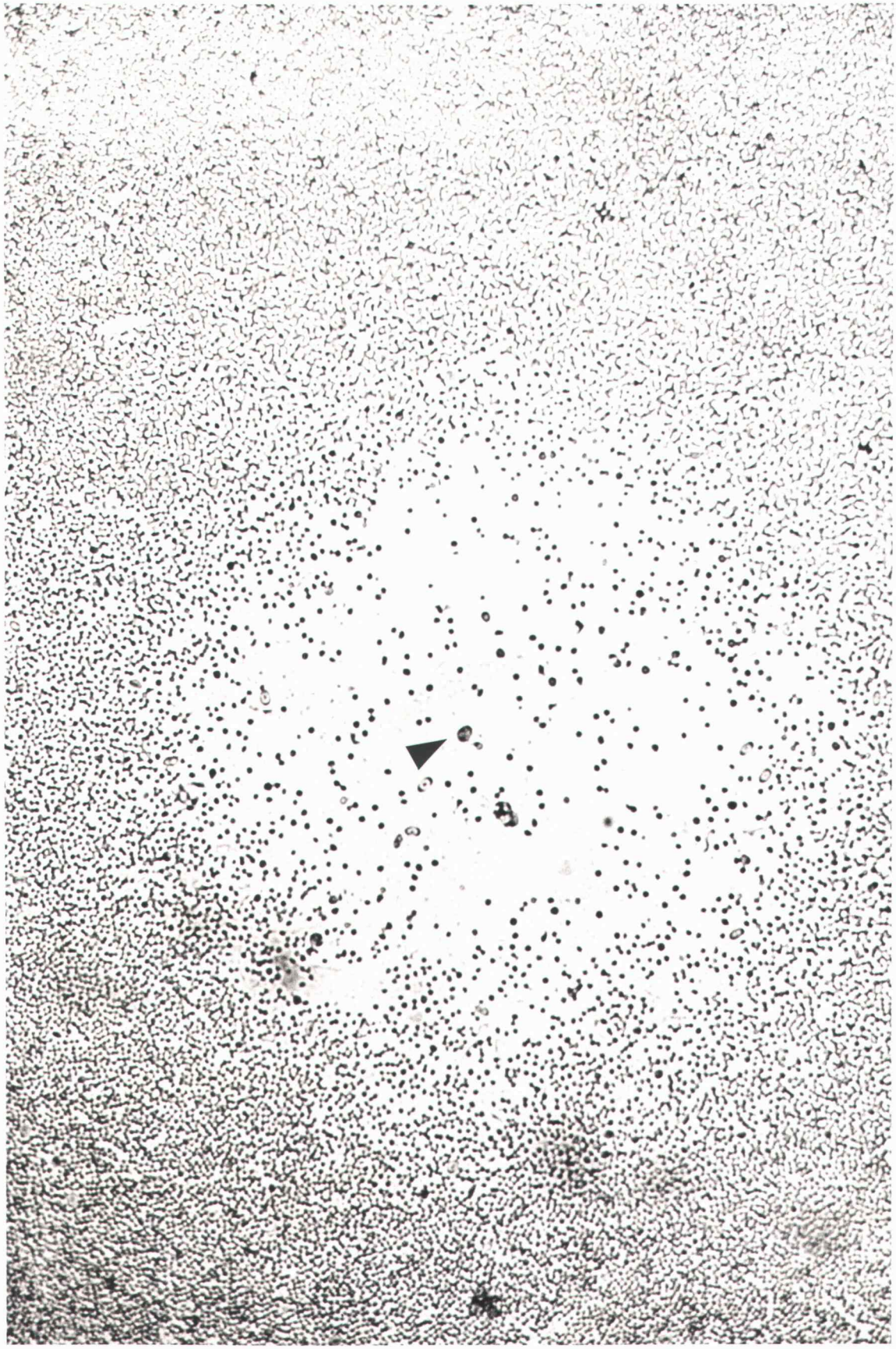


Figure 2

detectable by day 7 post immunization in anterior kidney and splenic tissue preparations and by day 16 for thymocytes (Figure 3). Non-immunized control animals exhibited anti-Vibrio PFCs far below the reponses of immunized animals, ranging from 0 to 50 PFCs per organ throughout the assay period (Figure 3). The peak of the plaque forming cell (PFC) response in the spleen and anterior kidney is observed on day 16 and by day 21 the plaque forming cell numbers begin to decrease. Similar kinetics were observed for both the spleen and the anterior kidney in other kinetic studies. The number of plaque forming cells on day 16, calculated on a per organ basis, were 16,767 and 6,275 for the anterior kidney region and the spleen respectively. The PFC response from the thymus was still rising at day 21 but later time points were not assessed. The thymus contained 180 plaque forming cells per organ on day 21. In both immune and control cell suspensions from all tested organs, less than 20 PFCs/organ were detected employing uncoated SRBC.

Since the thymus is known as being exclusively a T cell organ in mammalian immune systems, it was necessary to investigate if the PFCs seen in the thymus were actually introduced from the blood lymphocytes into the tissue sample. A one ml volume of peripheral blood was collected from immunized animals whose thymi yielded PFCs. Lymphocytes were separated from red blood cells and resuspended to 1.0 ml. Although this peripheral blood lymphocyte preparation was at least in a 100-fold excess of the amount of blood which could have inadvertantly been introduced into the thymocyte preparation, only 4 PFCs/10^6 lymphocytes resulted compared to 30 PFCs/10^6 lymphocytes for

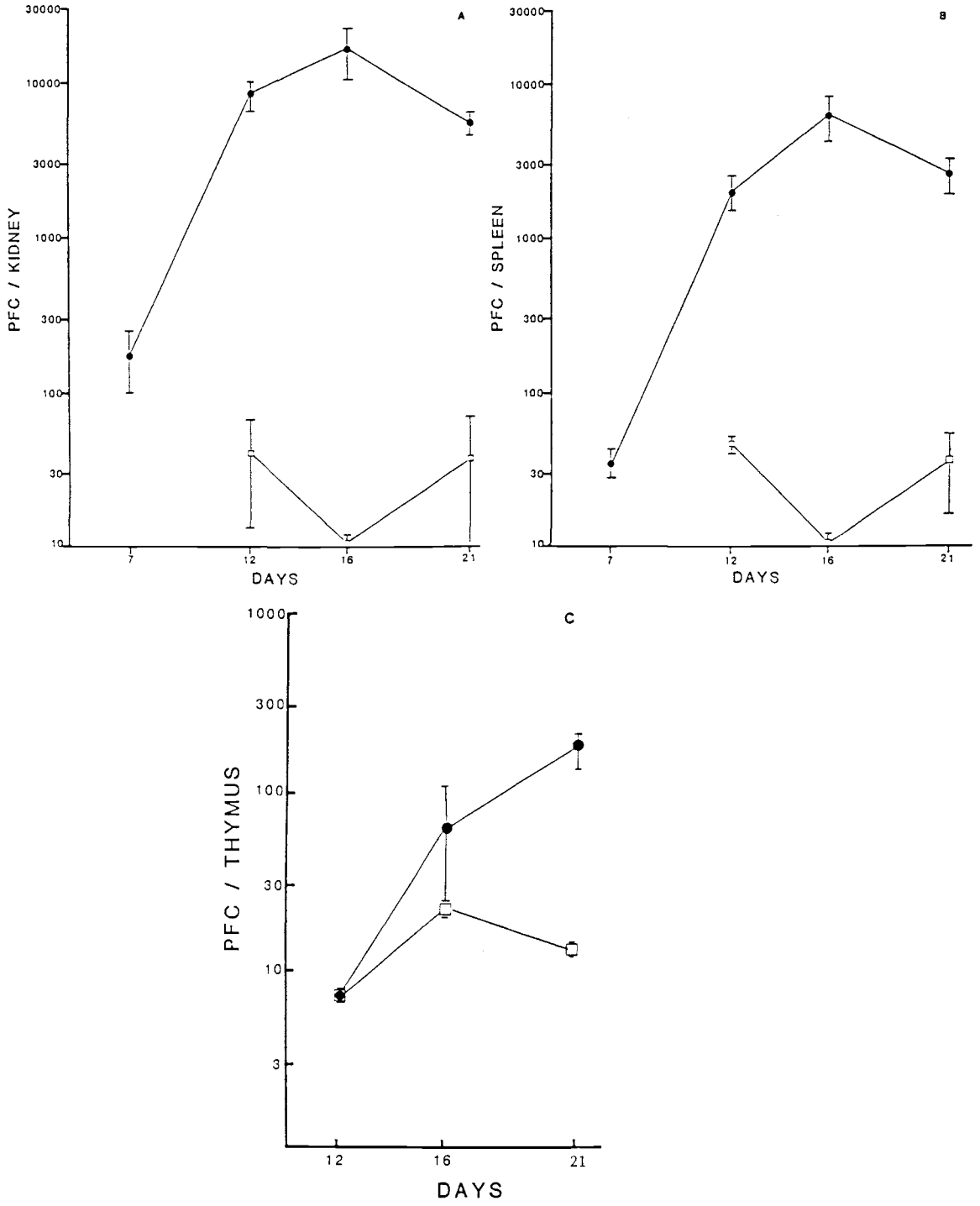


Figure 3. Kinetics of the in vivo, anti-Vibrio anguillarum PFC response. The plaque forming cell response was assessed on days 7, 12, 16 and 21 post-immunization for lymphocytes obtained from the salmonid spleen (A), anterior kidney (B) and thymus (C). The number of PFCs are reported on a semilog scale as PFCs per organ. Closed circles represent responses from immunized animals (●), open boxes represent responses from non-immunized control animals (□). Vertical bars represent two standard errors about the mean of five animals. No more than 20 PFC/organ were detected against uncoated SRBC.

the thymus sample.

Specificity of the In Vivo Plaque Forming Cell Assay

The PFC assay was shown to be immunoglobulin mediated and specific for Vibrio anguillarum antigen. The antibody dependence of this PFC response was demonstrated by the inhibition of the response by rabbit antiserum specific for coho immunoglobulin (figure 4). Complete inhibition (100%) of the control PFC response (223 $\pm$ 30) was observed. However in the control experiment, employing non-immune rabbit serum, a significant amount of inhibition of the response did not occur (11% of the control).

Bacterial antigen extracts were added to the PFC assay mixture to assess the antigenic specificity of the response. A soluble extract of Vibrio anguillarum antigen (64 ug/ml) completely inhibited (100%) the plaque forming cell response (Figure 5A). A soluble antigen extract (64 ug/ml) from Vibrio ordalii, a fish pathogen from the same genus as Vibrio anguillarum provided evidence for cross-reactivity in the antibody response as 20% inhibition was observed (Figure 5B). An antigenic dose of 120 ug/ml of soluble Renibacterium salmoninarum antigen, a salmonid pathogen unrelated to the Vibrios caused no inhibition (0%) of the response (Figure 5C).

Inhibition by free antigen was not due to toxicity of the Vibrio antigens toward salmonid lymphocytes. This was demonstrated by preincubating lymphocytes with Vibrio anguillarum antigen. After a two hour incubation, the duration of a PFC assay, lymphocytes were washed free of antigen and plated in the absence of this inhibitor.

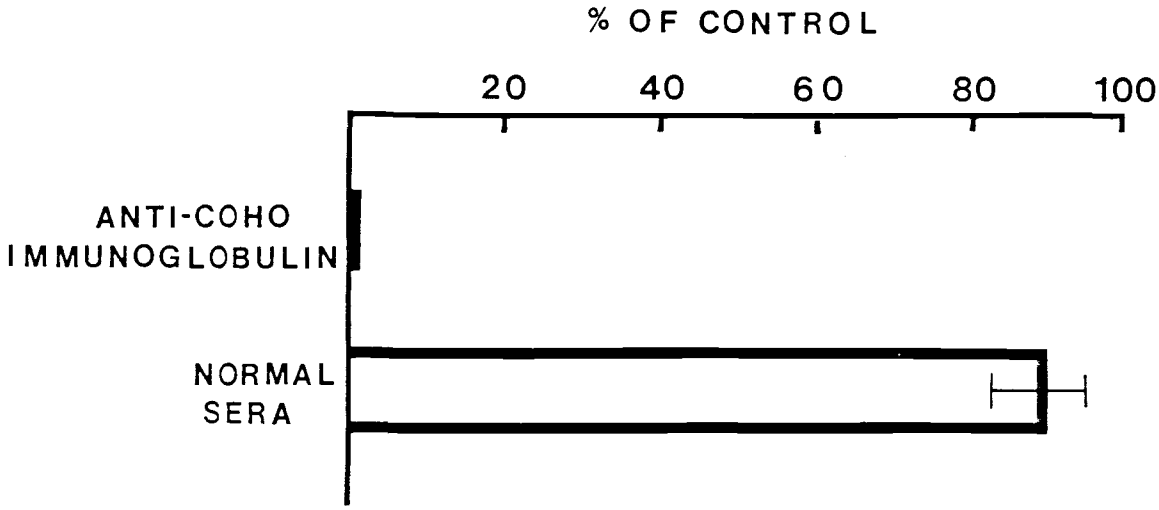


Figure 4. Demonstration of the immunoglobulin dependency of the PFC assay. Rabbit antiserum specific for coho immunoglobulin was added to the plaque assay mixture and was observed to abolish salmonid plaque formation to 0% of the control response. Serum from a non-immunized rabbit did not significantly inhibit the PFC response (89% of the control response). Anterior kidney lymphocytes are shown. Splenic lymphocytes yielded similar results.

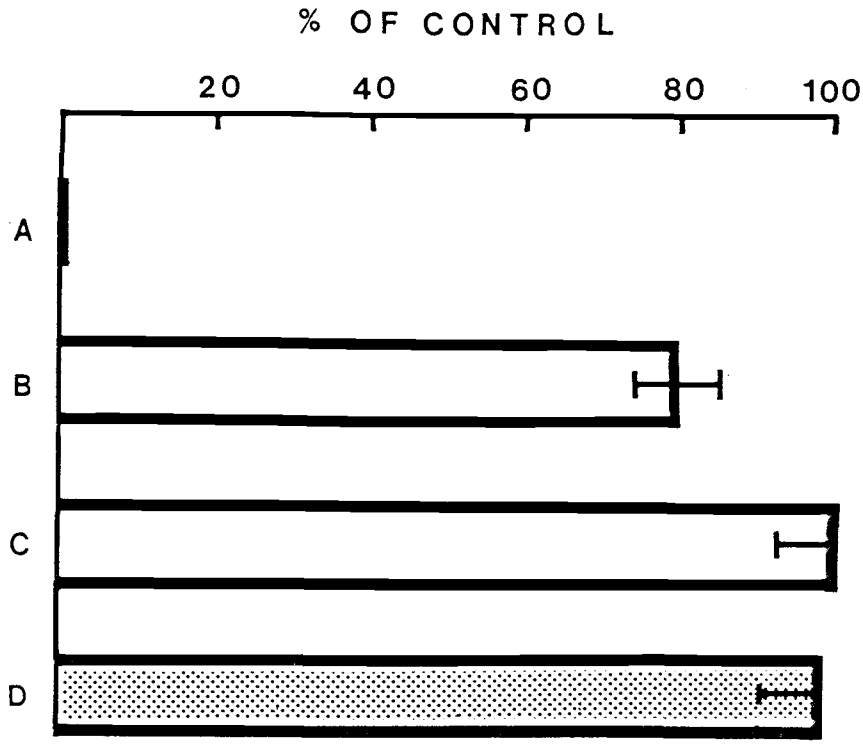
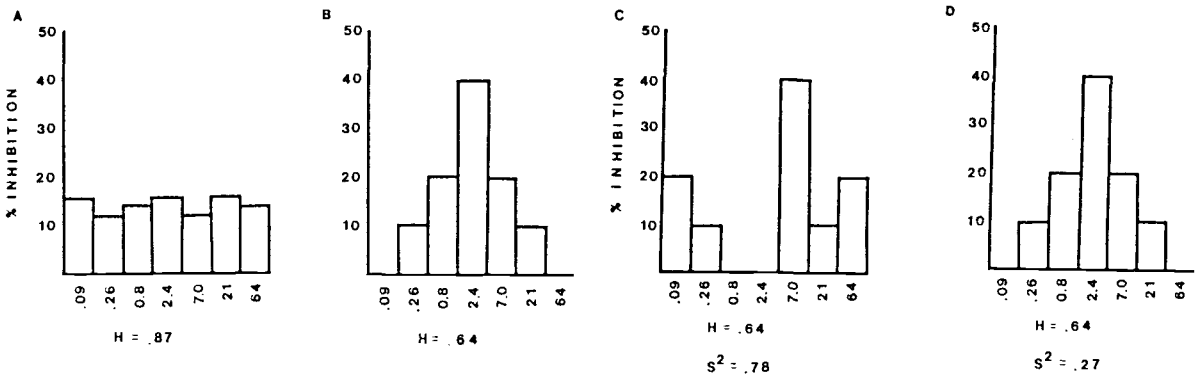


Figure 5. Antigenic specificity of the in vivo PFC assay. Soluble bacterial antigen extracts were used (A, B and C) as free antigen inhibitors of the Vibrio anguillarum generated PFC response in salmonids. Vibrio anguillarum (A) and Vibrio ordalii (B) were added to plaque mixtures (64 ug/ml carbohydrate content) and assayed for their ability to inhibit PFC formation. Renibacterium salmoninarum (C) soluble antigen (120 ug/ml carbohydrate content) was also tested. Inhibitor effects are depicted as the % of the control response of; 159 $\pm$ 4 PFC (A), 114 $\pm$ 12 PFC (B) and 159 $\pm$ 4 PFC (C). Vibrio anguillarum antigen was tested for possible lymphocytotoxicity by incubation with lymphocyte preparations (D). After two hours, the length of a typical PFC assay, lymphocytes were washed of the free antigen and placed in a standard assay mixture. The possible lymphocytotoxic effects are depicted as the % of the control PFC response (D; 226 $\pm$ 16).

A response equivalent to the control response was observed (Figure 5D).

Functional Heterogeneity of In Vivo Generated Plaque Forming Cells From Different Immune Organs

Antigen inhibition analysis of PFC responses is a quantitative means of assessing the numbers of antibody producing cells which secrete high or low affinity antibody. Relative heterogeneity of antibody responses can also be assessed through this method of analysis (see appendix I and Figure 6). Inhibition analyses employing graded concentrations of free Vibrio anguillarum antigen to generate histogram plots, or inhibition profiles, of lymphocytes from the anterior kidney, spleen and posterior kidney are shown in figure 7 (A, B and C respectively). The histogram plots for the anterior kidney (Figure 7A) exhibit restricted heterogeneity in comparison to those for the spleen and the posterior kidney. The anterior kidney plots demonstrate that most of the plaque forming cells are only inhibited by high concentrations of free antigen. In addition there is a relative absence of inhibition observed at low antigen concentrations. Both the spleen (Figure 7B) and posterior kidney (Figure 7C) plots show a more heterogeneous inhibition profile, or more equal distribution of PFCs inhibited by both high and low antigen inhibitor concentrations. The variance from the mean of log transformed data was calculated to mathematically describe heterogeneity in the plaque forming cell responses (refer to appendix



$$\underline{\text{Shannon}} \quad H = -\sum_{i=1}^{k} p_i \log p_i$$

where: k = the number of inhibitor categories
p_i = the proportion of observations found in category i

$$\underline{\text{Variance}} \text{ (heterogeneity index)} \quad s^2 = \frac{\sum_{i=1}^{k} f_i(\log x_i - \log \bar{x})^2}{n}$$

where: $$\log \bar{x} = \frac{\sum_{i=1}^{k} f_i(\log x_i)}{n}$$

and where:
k = the number of inhibitor categories
n = the total possible number of PFC
f_i = the number of PFC inhibited with concentration x_i
x_i = the concontration of inhibitor in category i

Figure 6. Example inhibition profile data. The Sannon heterogeneity and variance formulae are shown. Inhibition profiles A and B depict that when heterogeneity differences are maximal the Shannon heterogeneity index can demonstrate differences between tests. However, inhibition profiles C and D depict examples where the Shannon heterogeneity index fails to correctly reflect heterogeneity differences. Shannon and variance heterogeneity indices have both been calculated for profiles C and D.

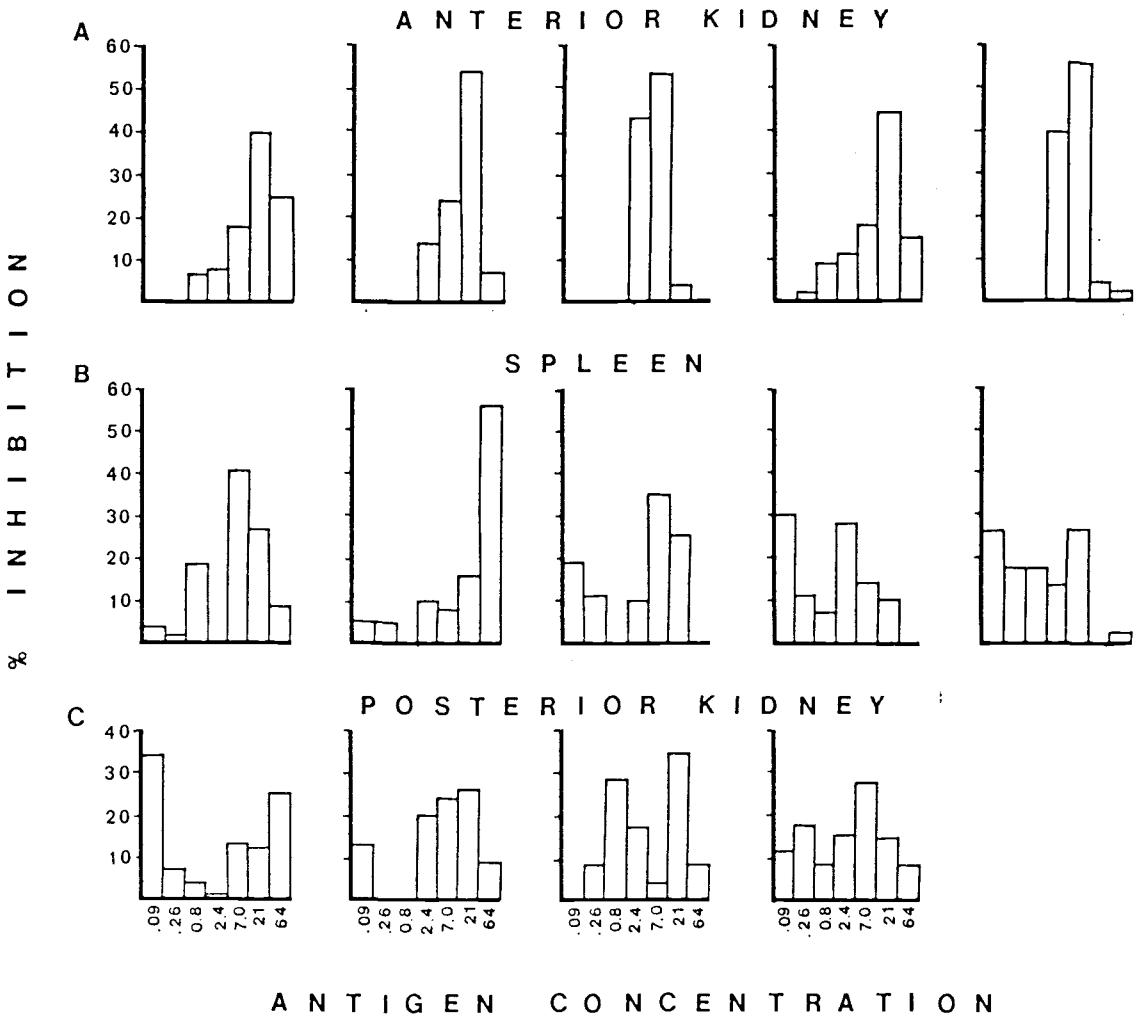


Figure 7. Inhibition profiles generated by Vibrio antigen. Inhibition profiles are shown for the anterior kidney (A), spleen (B), and posterior kidney (C) PFC. Each plot represents in the inhibition profiles resulting from 2 - 3 fish pools of the designated lymphocyte source. The carbohydrate concentration (ug/ml) of Vibrio antigen inhibitor is reported on the abscissa. Histogram bars represent the amount of inhibition contributed to the total inhibition by a particular antigen concentration starting from the lowest antigen concentration. A more thorough explanation of inhibition profile generation and theory is provided in Appendix 1.

I). The variance of a particular histogram plot will be referred to henceforth as the heterogeneity index. More heterogeneous inhibition profiles are reflected by greater heterogeneity indices. Table I lists heterogeneity indices for the histogram plots in figure 7.

Data shown in figure 7, were averaged for each organ and shown in figure 8. The anterior kidney plot is more restricted than the spleen or posterior kidney, with no significant inhibition occuring at the lowest free antigen concentrations. Splenic and posterior kidney plots illustrate greater heterogeneity accompanied by inhibition of plaques at the lowest free antigen inhibitor concentrations. The average variance of the compiled anterior kidney data is $0.20 \pm .12$, for splenic and posterior kidney data it is $0.64 \pm .12$ and $0.86 \pm .40$ respectively.

Dose Response of TNP-LPS and TNP-KLH for the In Vitro Production of Plaque Forming Cells

The generation of specific antibody to a particular antigen is a complex phenomenon influenced by a multitude of environmental and biological factors. In vitro generation of antibody production aids in the study of B cell activation and production of specific immunoglobulin by enabling greater control over many of these environmental and biological variables. The need for replicated cell cultures and the desire to carry out more involved in vitro experiments required a large number of lymphocytes per assay. The spleen and the anterior kidney met these cellular requirements, however the posterior kidney did not. On the basis of in vivo PFC

Table I. *Vibrio anguillarum* inhibition profile variances. The variance values or heterogeneity indices were calculated from the *Vibrio* generated inhibition profiles and are shown.

	Anterior Kidney	Spleen	Posterior Kidney
[a]Heterogeneity Index	0.30	0.61	1.46
	0.35	0.60	0.69
	0.10	0.49	0.74
	0.08	0.80	0.56
	0.15	0.69	
	x = 0.20 + 0.12	x = 0.64 + 0.12	x = 0.86 + 0.40

[a]The variance from the mean of log transformed histogram data has been designated the Heterogeneity Index since it is a reflection of the relative heterogeneity of the total PFC response.

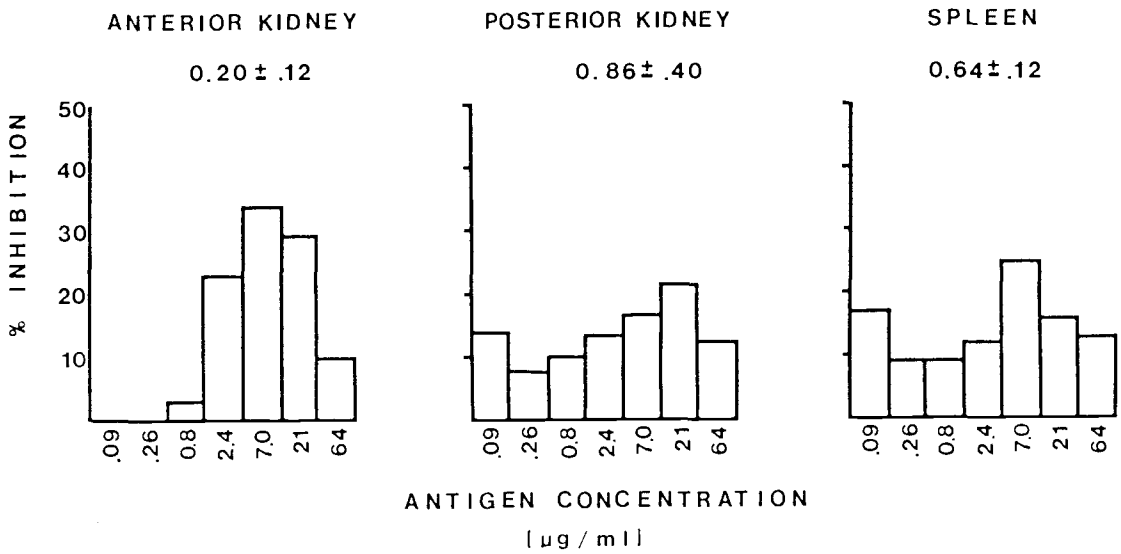


Figure 8. Composite inhibition profiles from the anterior kidney, posterior kidney and spleen. Average histogram plots were generated by taking the average of the inhibition resulting at a particular antigen concentration for net data, for each individual organ. For example, the amount of inhibition contributed by 2.4 ug/ml to the total inhibition in each of four inhibition profiles for the posterior kidney was averaged to generate a value of 13% inhibition. The average of each inhibition profiles variance was calculated along with the standard deviation about the mean and shown for each organ. The average variance for the anterior kidney is 0.20 $\pm$ .12, for the posterior kidney and spleen it is 0.86 $\pm$ .40 and 0.64 $\pm$ .12 respectively.

inhibition analysis, the splenic lymphocyte population was like that of the posterior kidney, but functionally different from the anterior kidney. Thus, assessment of B cell functional heterogeneity with in vitro methods did not include the use of posterior kidney lymphocytes.

The dose response for trinitrophenylated-lipopolysaccharide (TNP-LPS) was performed on in vitro cultures of splenic and anterior kidney lymphocyte preparations (Figure 9A and B) respectively. The peak anti-TNP plaque forming cell response occured at 0.5 ug/ml TNP-LPS for the spleen (2523 $\pm$ 177 PFCs/10^6 lymphocytes) and the anterior kidney (10,814 $\pm$ 1955 PFCs/10^6 lymphocytes). Dose responses for both cell sources decreased sharply to less than 40% of the maximum response at antigen concentrations 100 fold higher or lower than the optimal dose. Control responses (cultures containing no TNP-LPS) were insignificant yielding 10% (247 $\pm$ 107 PFCs) and 6% (628 $\pm$ 67 PFCs) of maximum antigen stimulated responses for the spleen and anterior kidney respectively. The dose response of the T-dependent antigen, trinitrophenylated-keyhole limpet hemocyanin (TNP-KLH) was performed on in vitro cultures of splenic lymphocytes only (Figure 10). The hapten specific response to TNP-KLH was greatest at 100 ug/ml protein where 153 $\pm$ 36 PFCs per 10^6 lymphocytes resulted (Figure 10). An insignificant number of anti-TNP plaques was observed at all unhaptenated KLH concentrations tested (less than 18 $\pm$ 12 per 10^6 lymphocytes). This T-dependent response occurred in the absence of previous carrier priming with KLH.

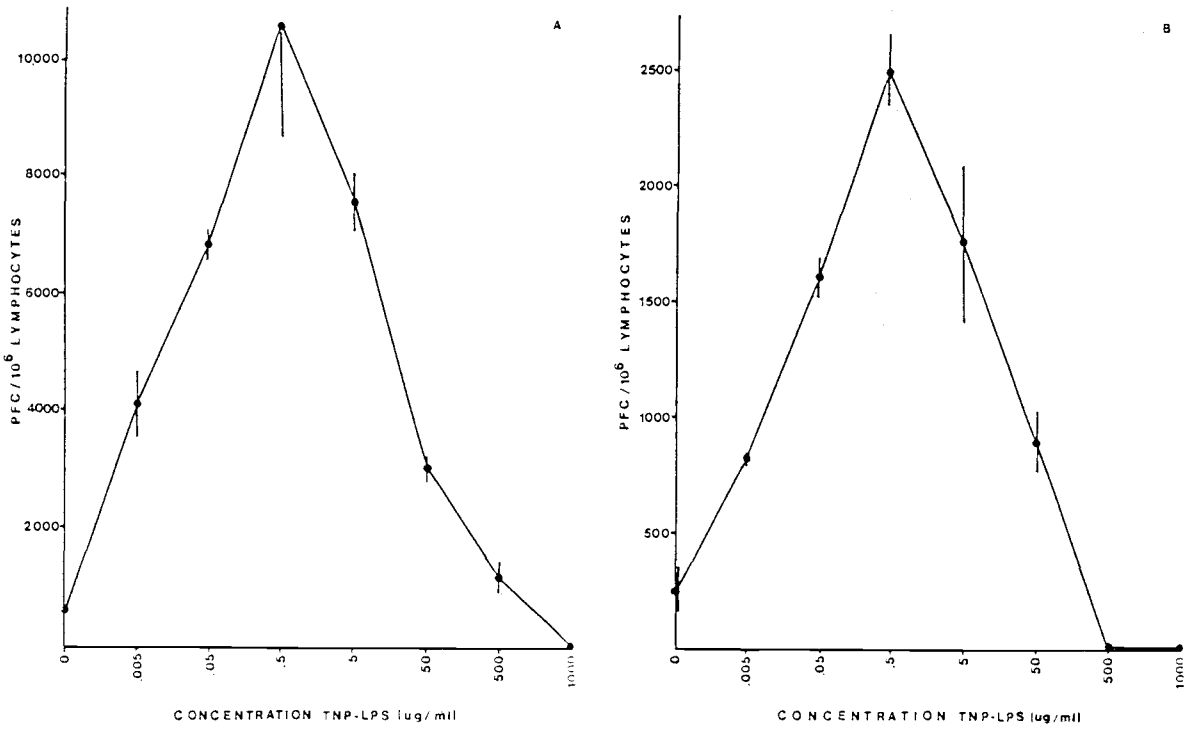


Figure 9. In vitro dose response of TNP-LPS on splenic and anterior kidney lymphocytes. The dose response stimulated by in vitro exposure of salmonid anterior kidney (A) and splenic (B) lymphocytes to TNP-LPS. The number of PFC per 10^6 lymphocytes generated by various concentrations of TNP-LPS are plotted. Vertical bars represent two standard deviations about the mean response of three culture wells. Responses were assessed nine days after culture initiation.

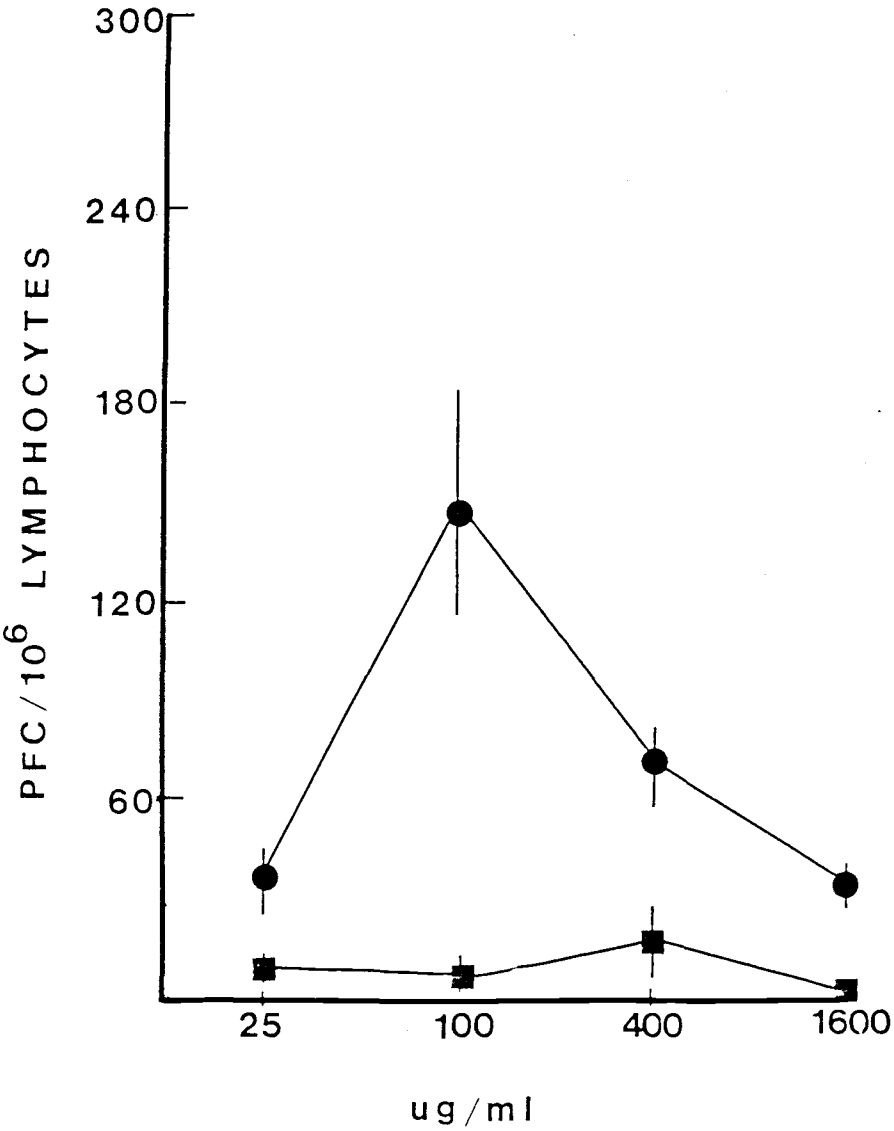


Figure 10. In vitro dose response of TNP-KLH on splenic lymphocytes. Responses for TNP-KLH (●) and KLH (■) were reported as the number of anti-TNP PFC per 10^6 lymphocytes for a range of antigen concentrations. Responses were assessed nine days after culture initiation.

Kinetics of the Anti-TNP Plaque Forming Cell Response Generated by TNP-LPS In Vitro

The kinetics of the in vitro anti-hapten response to TNP-LPS are depicted in figure 11. The optimal in vitro incubation period for anti-TNP responses to the T-independent form of the antigen (TNP-LPS) for both splenic and anterior kidney lymphocyte cultures was day 6 in this experiment. An average of eight culture wells for both the spleen and the anterior kidney generated 8106 $\pm$ 525 and 8320 $\pm$ 560 PFCs per culture well, respectively. A certain degree of variability in the kinetics to reach the peak response of in vitro PFCs generation however existed. In a number of other in vitro kinetics experiments, optimal responses have occurred on days 6 through day 9 (Table II).

Antigenic Specificity of TNP-LPS Induced B Cell Activation

The T-independent antigen, which was used throughout all of the in vitro studies, was a haptenated lipopolysaccharide molecule. Lipopolysaccharide is a well documented polyclonal B cell activator and mitogen for both mammalian (Andersson, 1972) and fish systems (Etlinger, 1976). Since the primary focus of these studies was B cell activation and antibody production, the ability of TNP-LPS to generate PFCs by polyclonal or antigen specific means was investigated. A dose response for both TNP-LPS and nonhaptenated lipopolysaccharide in splenic lymphocyte cultures is shown (Figure

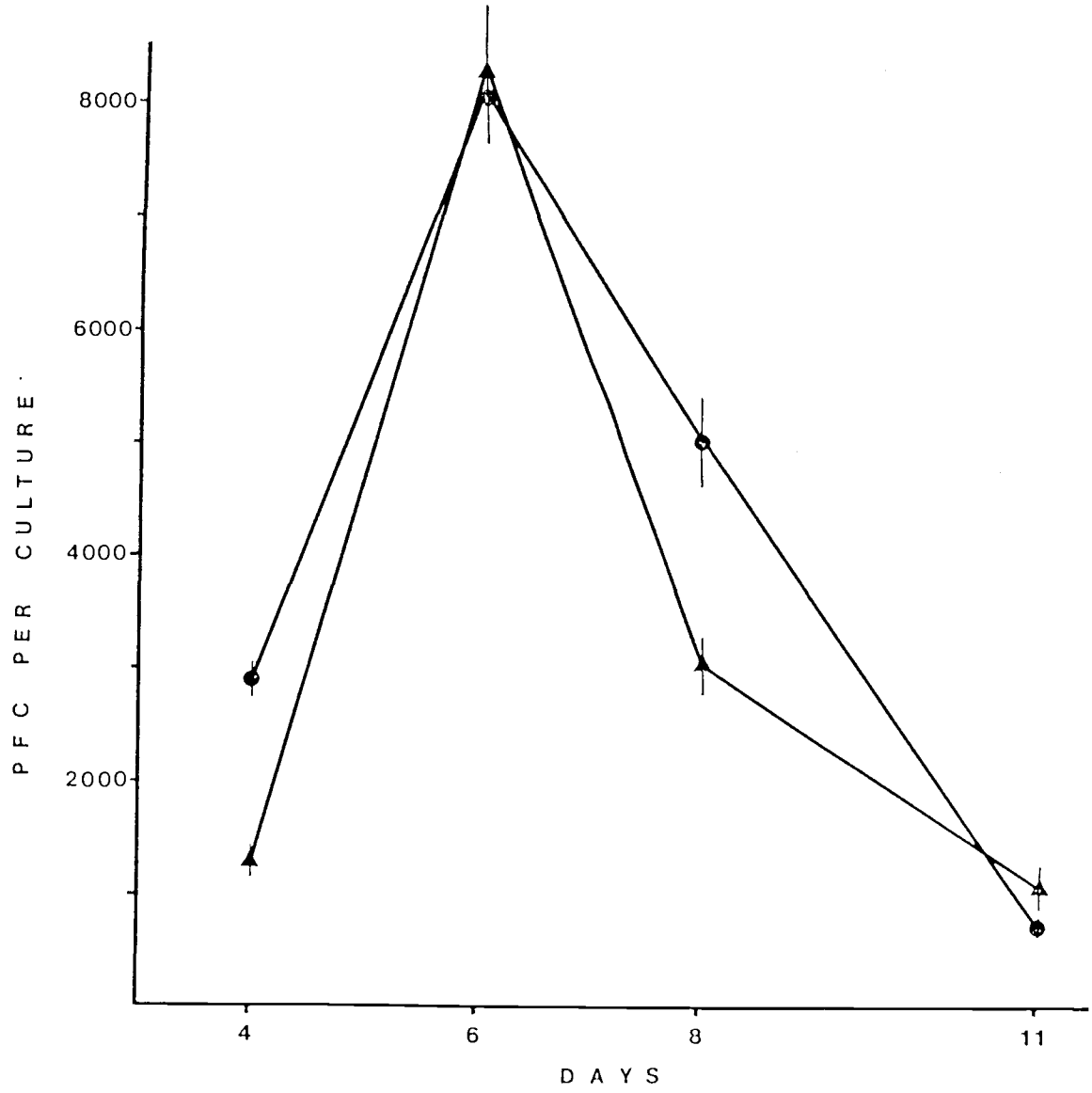


Figure 11. Kinetics of the in vitro PFC response generated by TNP-LPS. The number of PFC per cell culture well, occurring on the indicated day, are reported for salmonid splenic (●) and anterior kidney in vitro lymphocyte cultures (▲). Responses were generated to TNP-LPS (0.5 ug/ml) and represent the average of at least 8 culture wells. The vertical bars represent two standard deviations about the mean.

Table II. *In vitro* response kinetics to TNP-LPS. This table illustrates the anti-TNP PFC response per culture well incubated for the designated number of days.

Days of Incubation	Lymphocyte Source		
	Spleen		Anterior Kidney
4	2893 ± 147	10150 ± 240	1307 ± 147
5	3291 ± 3		3002 ± 460
6	8106 ± 525	12440 ± 1120	8320 ± 560
7	4780 ± 240		6836 ± 1061
8	5067 ± 426	3213 ± 200	3040 ± 267
9	516		7004 ± 818
10			
11	739 ± 76		1102 ± 284
12			2231 ± 151

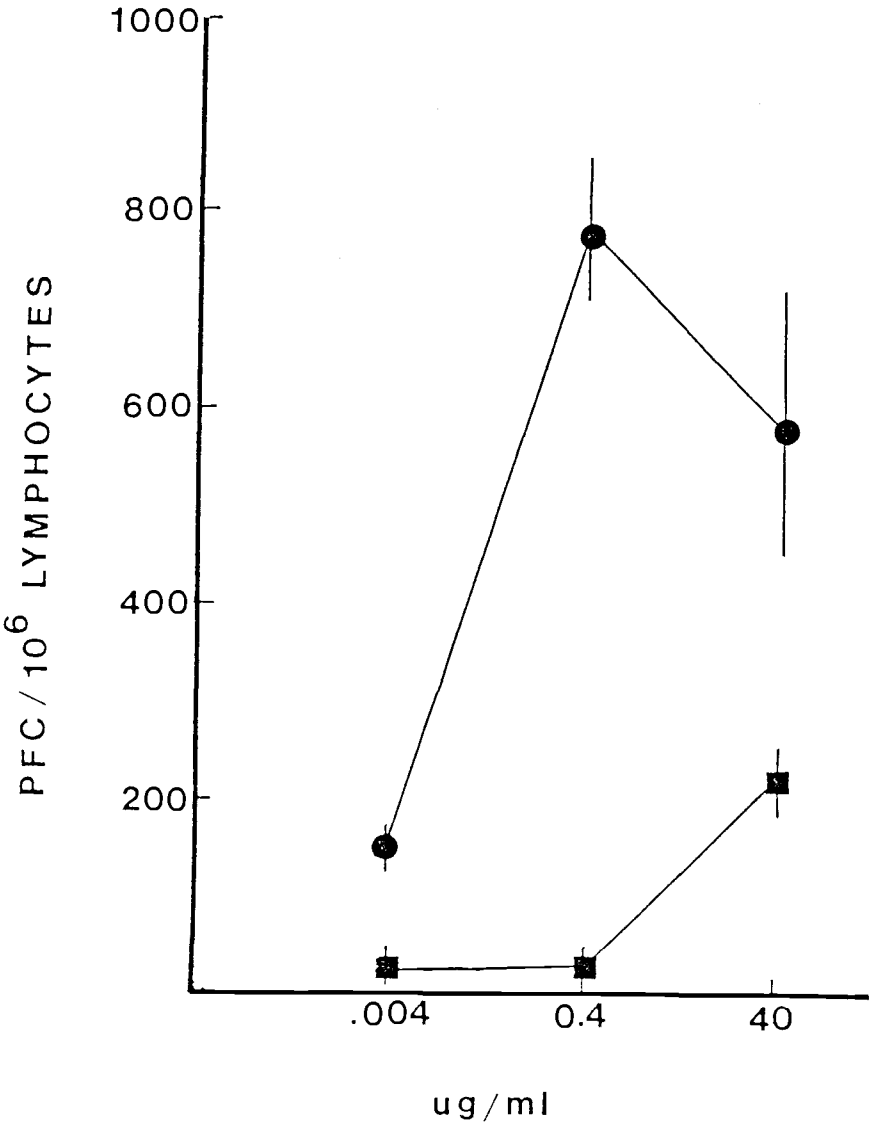


Figure 12. LPS and TNP-LPS dose response curves for the generation of anti-TNP PFCs. Salmonid splenic PFC per 10^6 lymphocytes are reported resulting from three different concentrations of LPS (■) and three concentrations of TNP-LPS (●). Cultures were assessed for TNP-specific PFC after nine days of incubation. Vertical bars represent two standard deviations about the mean of three culture wells.

12). The peak response elicited by TNP-LPS occurred at a dose of 0.4 ug/ml (777 ± 73 PFCs per 10^6 lymphocytes), however lipopolysaccharide demonstrated no ability to produce TNP specific PFCs at this concentration (32 ± 20 PFCs/10^6 lymphocytes). The media only control culture produced 14 ± 8 PFCs per 10^6 lymphocytes. Non-haptenated lipopolysaccharide elicited insignificant numbers of anti-TNP PFCs at doses of 4 ng/ml or 0.4 ug/ml but stimulated a significant anti-TNP response at 40 ug/ml (235 ± 28 PFCs per 10^6 lymphocytes). The 40 ug/ml dose of TNP-LPS produced a response that was 76% (590 ± 136 PFCs per 10^6 lymphocytes) of the maximum response observed at 0.4 ug/ml. The induction of this response was still 2.5 fold greater than the response to non-haptenated LPS at 40 ug/ml. Presumably the observed anti-TNP response caused by LPS at 40 ug/ml is due to nonspecific polyclonal activation.

Demonstration of Polyclonal Activation by PHA, LPS and TNP-LPS

The T-independent antigen, TNP-LPS, was compared to the mitogens, phytohemagglutinin (PHA) and lipopolysaccharide (LPS), with respect to their ability to stimulate cellular proliferation, non-specific immunoglobulin production, and TNP-specific PFCs (Figure 13). Cellular proliferation was measured by tritiated thymidine uptake. Total immunoglobulin production was assessed by the use of an ELISA system specific for fish immunoglobulin and specific anti-TNP antibody responses were assessed employing the PFC assay. Dose responses of both LPS and PHA clearly stimulated these three functions in a dose dependent and parallel manner. At the PHA

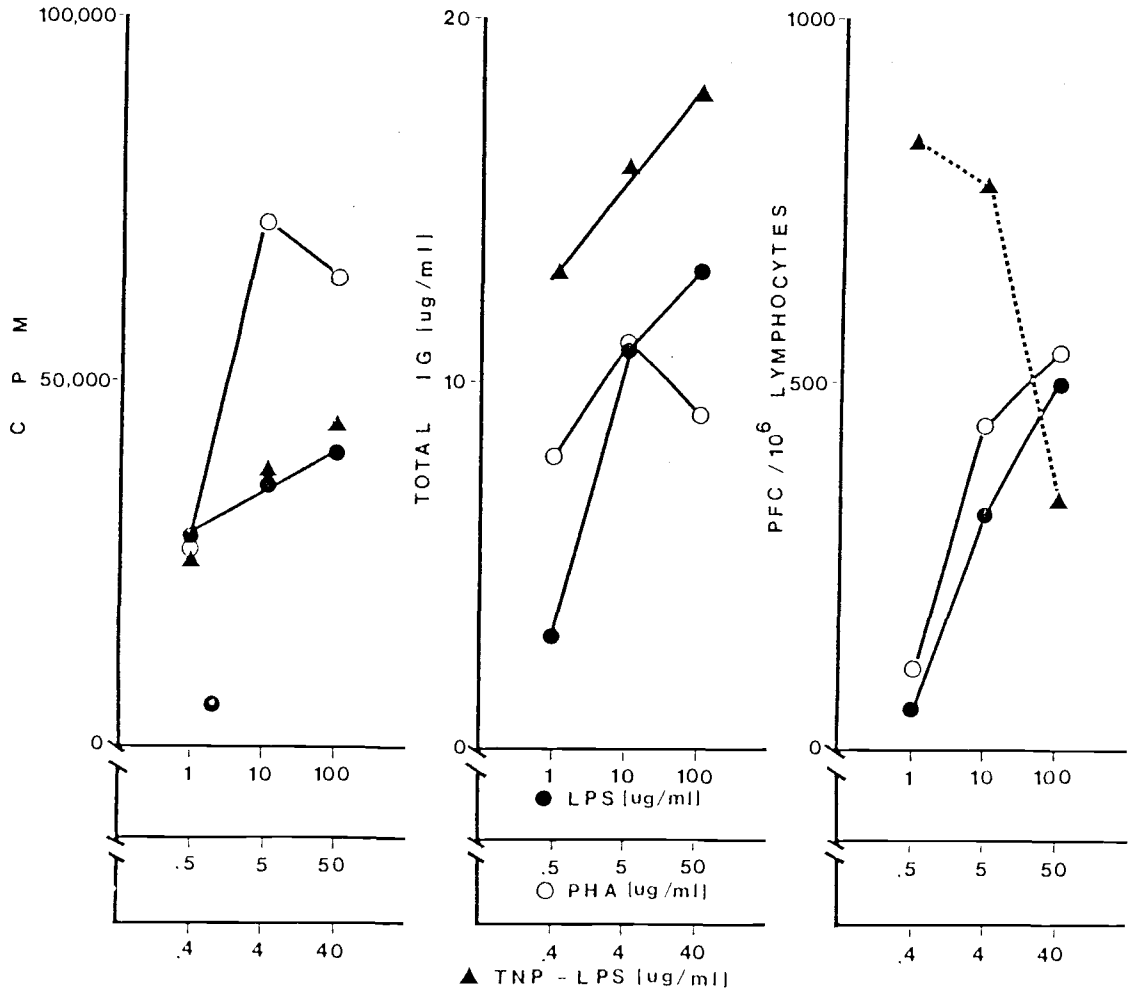


Figure 13. Polyclonal and antigen specific activation of splenic lymphocytes. Polyclonal activation of salmonid splenic lymphocytes by phytohemagglutinin (○), lipopolysaccharide (●) and trinitrophenyl lipopolysaccharide (▲). Polyclonal activation was measured by the amount of cellular proliferation and by total immunoglobulin produced. Cellular proliferation was measured by cellular uptake of ^{3}H-thymidine and counting the amount of radioactivity which was incorporated into the cells. Counts per minute (cpm) are reported after subtracting background cpm resulting from unstimulated control cultures. Measurement of total immunoglobulin levels were determined using a standard calibrated ELISA assay and reported as ug/ml of total immunoglobulin. Specific, anti-TNP responses were measured by PFC assay. Numbers of anti-TNP PFC are reported generated at a particular dose of antigen or mitogen.

concentration which caused optimal cellular proliferation, both specific and total immunoglobulin produced were also maximal. The same phenomenon held true for the LPS dose response. Conversely, at a TNP-LPS concentration of 0.4 ug/ml, TNP specific PFC production was greatest while cellular proliferation and total immunoglobulin produced were at a minimum. At high TNP-LPS levels (40 ug/ml) specific PFCs generated were lowest but cellular proliferation and total immunoglobulin levels were at a maximum.

Inhibition of B Cell Induction by Monovalent Hapten

The hapten specific nature of the induction of TNP-specific PFCs was investigated. A monovalent form of TNP, trinitrophenyl-lysine, (TNP-LYS) was introduced with TNP-LPS in spleen cell cultures at their initiation. Cultures treated with 40, 8, and 1.6 ug/ml TNP-LPS (Figure 14, panels A, B and C respectively) were each coincubated with graded amounts of TNP-LYS and assayed for their ability to produce TNP-specific PFCs. The highest concentration of TNP-LYS was capable of inhibiting B cell induction by all tested doses of TNP-LPS, while comparable concentrations of the non-haptenated lysine control caused no inhibition of B cell induction. Panels A, B and C illustrate that as the dose of TNP-LPS decreased, the concentration of TNP-LYS necessary to cause 50% inhibition of B cell induction (H_{50}) also decreased in a parallel manner. At 40, 8 and 1.6 ug/ml the H_{50} concentrations of TNP-LYS were 40, 18 and 9 (x 10^{-5} M) respectively.

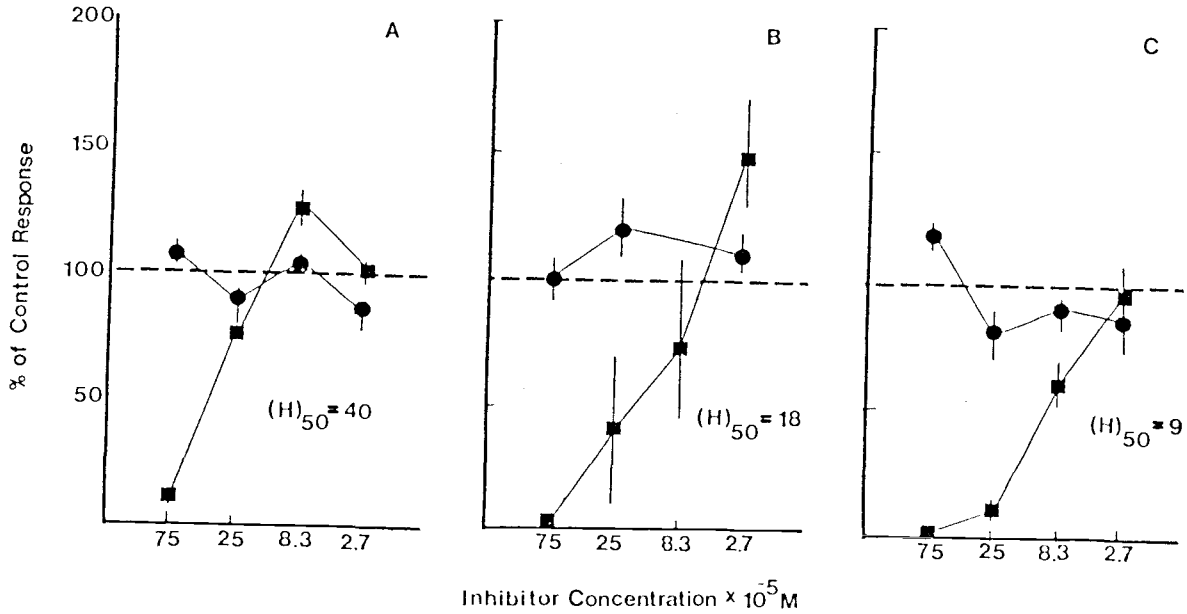


Figure 14. Inhibition of PFC induction by monovalent hapten. Salmonid splenic lymphocyte cultures were stimulated with TNP-LPS 40 ug/ml (panel A), 8 ug/ml (panel B), and 1.6 ug/ml (panel C). Each TNP-LPS stimulated culture was coincubated with a graded dose of TNP-lysine (■) nonhaptenated lysine control (●). The dashed line represents the anti-TNP PFC control response coincubated with tissue culture medium. Each point represents the mean of triplicate cultures and the vertical bars represent two standard errors about the mean of three cultures. All cultures were incubated 9 days. The hapten concentration necessary to inhibit 50% of PFC formation $(H)_{50}$ is reported for each stimulating TNP-LPS concentration.

Effects of gamma Irradiation on Lymphocyte Function

The effect of cobalt 60 gamma irradiation exposure on spleen cell responses to TNP-LPS and TNP-KLH was examined (Figure 15A and B). These experiments demonstrated an augmentation of the TNP-LPS response at radiation doses of approximately 400 rads. Doses greater than 600 rads reduced the PFC response to 75% or less of the control response. It was also observed that TNP-KLH responses were more radiosensitive than TNP-LPS responses. At low radiation doses (400 and 500 rads) the TNP-LPS response was 180% and 120% of the control, respectively (Figure 15A), whereas the TNP-KLH responses were 50% and 20% of the control. In panel B, showing a dose of 500 rads, the TNP-LPS response was 75% of the control and the TNP-KLH response was 25% of the control.

The Effects of Antigen Addition on the Anti-TNP Response In Vitro

T-independent and T-dependent antigens have been shown to stimulate different subpopulations of TNP-specific B cells in murine systems (Tittle, 1978). To determine if TNP-LPS and TNP-KLH stimulate different subpopulations of salmonid lymphocytes, optimal concentrations of these antigens were added to splenic cultures separately or in combination. If separate subpopulations of B cells responded to the two forms of antigen, then a PFC response equivalent to the sum of the responses to each antigen used separately would have resulted. However, if the same B cell subpopulation responded

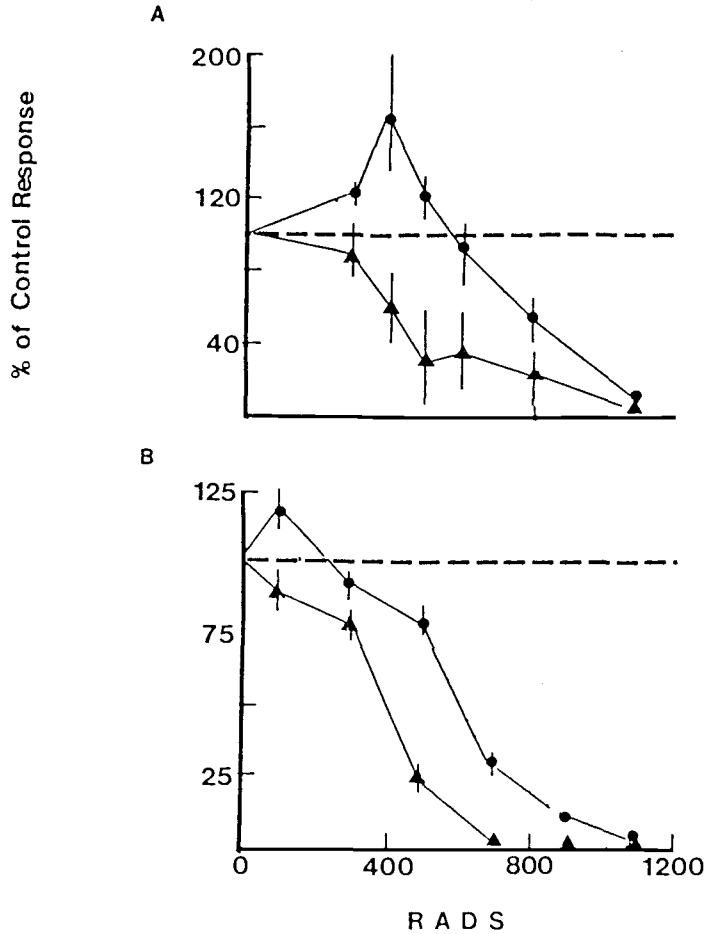


Figure 15. Effects of gamma irradiation on salmonid TNP-KLH and TNP-LPS generated PFCs. The PFC response to TNP-LPS (●) and TNP-KLH (▲) of splenic lymphocytes after exposure to various doses of gamma radiation are depicted. The dashed line represents the control response (non-irradiated lymphocytes) to TNP-LPS or TNP-KLH. Vertical bars represent two standard errors about the mean of three cultures. Lymphocyte cultures were incubated for nine days post-irradiation and culture initiation. Panels (A) and (B) represent the responses of coho lymphocytes.

to both forms of antigen, then addition of the two forms of antigen to the same culture would have resulted in a supraoptimal antigen dose. Thus, no change in the response or a lowered response would have occurred. Figure 16 shows plaque forming cell responses for the T-dependent antigen and the T-independent antigen. The responses for each antigen were approximately 375 $\pm$ 51 PFCs (A) and 186 $\pm$ 52 PFCs (B) per 10^6 lymphocytes for TNP-KLH and TNP-LPS respectively. These two different forms of antigen were added simultaneously to a cell culture and resulted in a response of only 83 $\pm$ 44 PFCs per 10^6 lymphocytes (Figure 16D), indicating no additive response. If the antigens could stimulate two separate subpopulations of B cells then the expected additive response would be nearly 550 PFCs per 10^6 lymphocytes as shown by the hatched bar (C) in figure 16.

Analysis of B Cell Functional Heterogeneity In Vitro

The heterogeneity of in vitro generated antibody was assessed in a similar manner as the determination of in vivo antibody heterogeneity, by antigen inhibition analysis. It was important to explore the intrinsic differences between splenic and anterior kidney PFCs in the more controlled, in vitro system. Figure 17 illustrates inhibition histograms of TNP-LPS stimulated splenic and anterior kidney cell cultures from an individual animal. No obvious in vitro differences were apparent between the two different cell cultures tested if 2,4,6-trinitrophenyl lysine (TNP-LYS) inhibition profiles were examined. Spleen and anterior kidney PFCs showed a heterogeneous range of antibodies encompassing both high and low

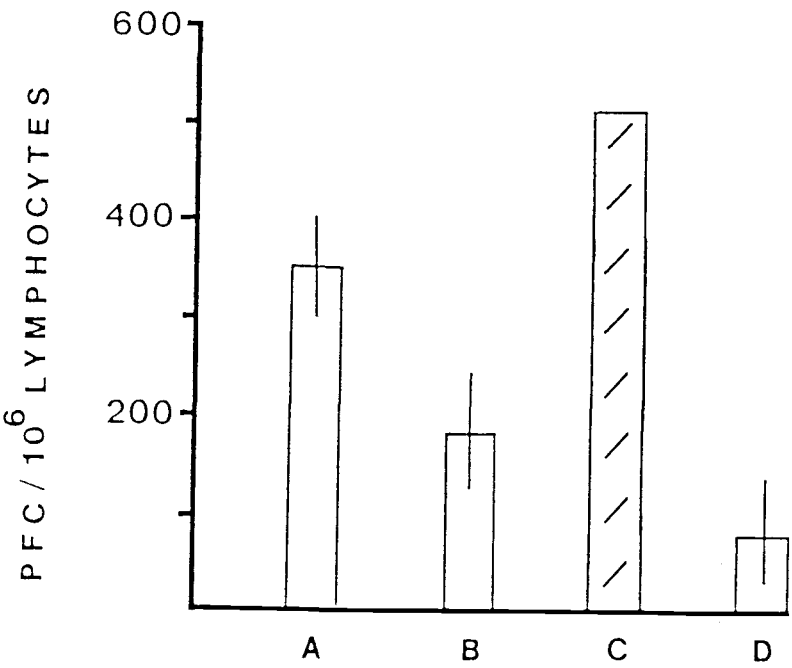


Figure 16. Effects of antigen addition on splenic lymphocyte cultures. The bars represent PFC per 10^6 lymphocytes generated in 9 day cultures to TNP-KLH (A) and TNP-LPS (B). The cross-hatched bar (C) depicts the expected PFC response if the response to TNP-KLH added in conjunction with TNP-LPS to a culture were additive. The observed PFC response to TNP-KLH and TNP-LPS added simultaneously at culture initiation is reported (D). The bars in A, B and D represent mean values of triplicate cultures and the vertical line depicts two standard errors about the mean.

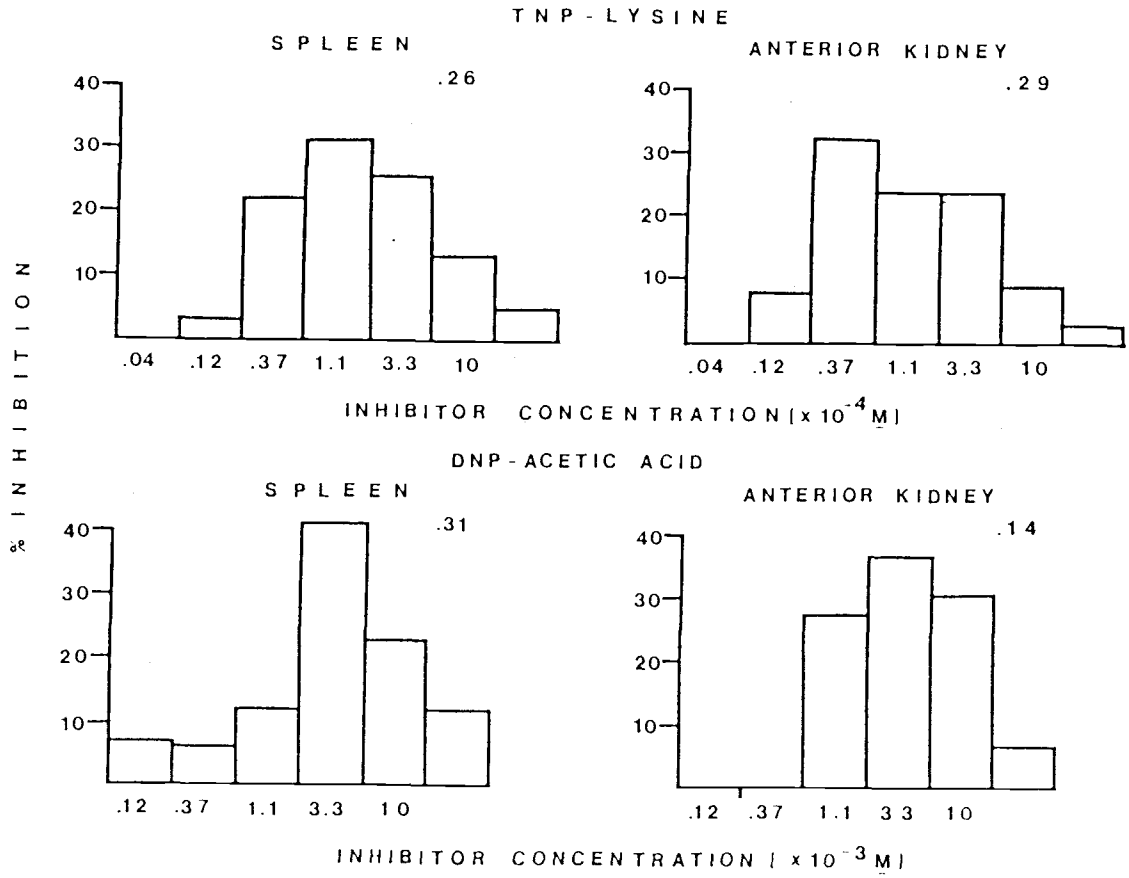


Figure 17. Inhibition profiles of <u>in vitro</u> generated splenic and anterior kidney PFCs. Salmonid splenic or anterior kidney PFCs, stimulated with TNP-LPS (0.5 ug/ml), were inhibited using DNP-acetic acid or TNP-lysine. Graded doses of TNP-LYS or DNP-acetic acid (abscissa) were employed to generate the inhibition profiles shown.

affinities. Heterogeneity, as measured by the variance from the mean of both histogram plots, is shown at the top right corner of each plot. However, inhibition profiles generated by 2,4-dinitrophenylacetic acid (structure in appendix II) did reveal differences between splenic and anterior kidney generated PFCs. The splenic inhibition profile illustrates the presence of both high and low affinity PFCs whereas the anterior kidney plot shows an absence of high affinity antibody. Furthermore, the heterogeneity index for the anterior kidney plot was twofold less than the splenic index. Inhibition profiles from the spleen and the anterior kidney from four animals generated from TNP-LYS inhibitions are plotted in figure 18 and those for 2,4-dinitrophenylacetic acid (DNP-acetic acid) are shown in figure 19. Data from these inhibitions are consistent with that presented in figure 17. Both splenic and anterior kidney plots demonstrate high and low affinity PFCs and equivalent heterogeneity indices with TNP-LYS (Table III). Inhibition profiles using the heterologous hapten, DNP-acetic acid, reveal a cluster of inhibition in the high antigen inhibitor concentrations and at least twofold greater heterogeneity with splenic lymphocytes than that found with lymphocytes from the anterior kidney (Table III).

B Cell Specificity Differences Determined by Relative Affinity Constant (Krel) Comparisons

The relative affinity constants (K_{REL}) calculated for splenic and anterior kidney PFCs again demonstrate differences between

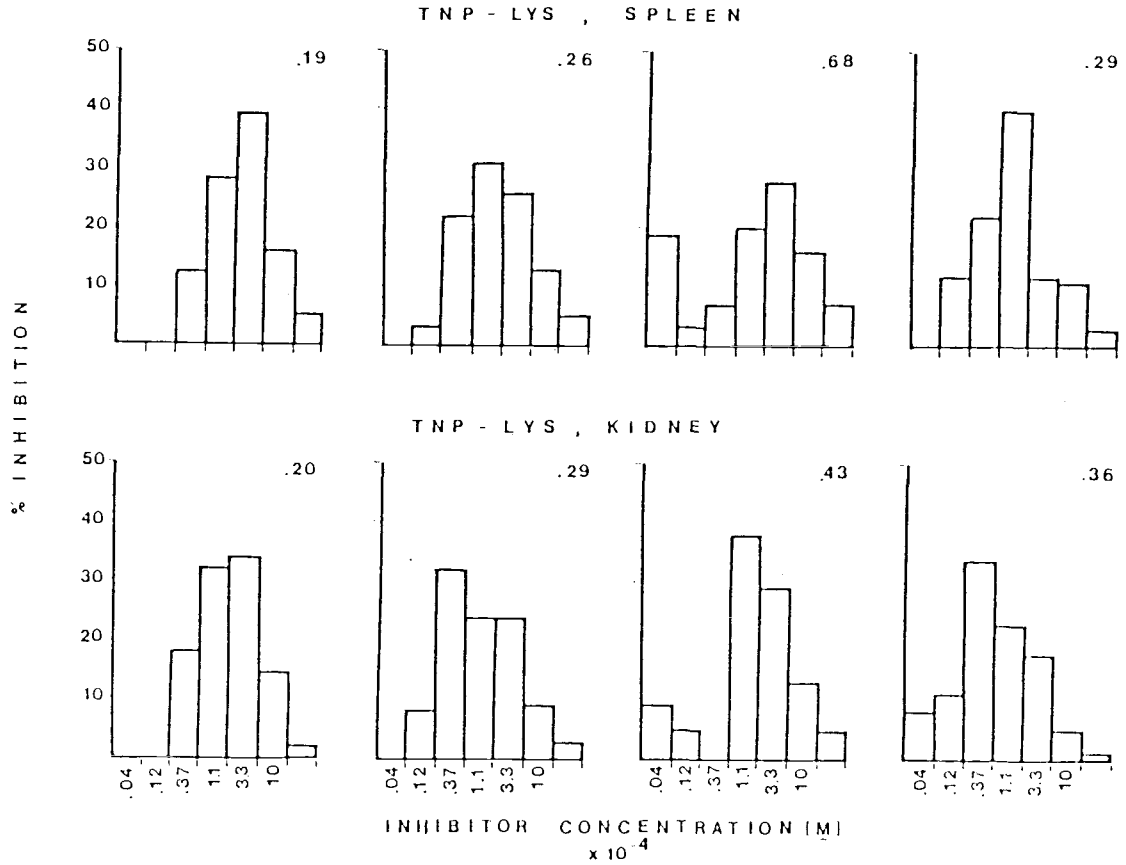


Figure 18. Composite of inhibition profiles employing TNP-LYS. Splenic and anterior kidney cell cultures were stimulated with TNP-LPS (0.5 ug/ml) and generated anti-TNP PFC on day 4 through day 11 post culture initiation. Resulting PFC were inhibited with graded doses of TNP-LYS (abscissa) and inhibition profiles were generated. The variance from the mean of log transformed histogram data is reported in the top right corner of each histogram. Plots of organs from the same animal lie above and below one another. For a more thorough explanation of inhibition profile generation or heterogeneity index calculation refer to Appendix I.

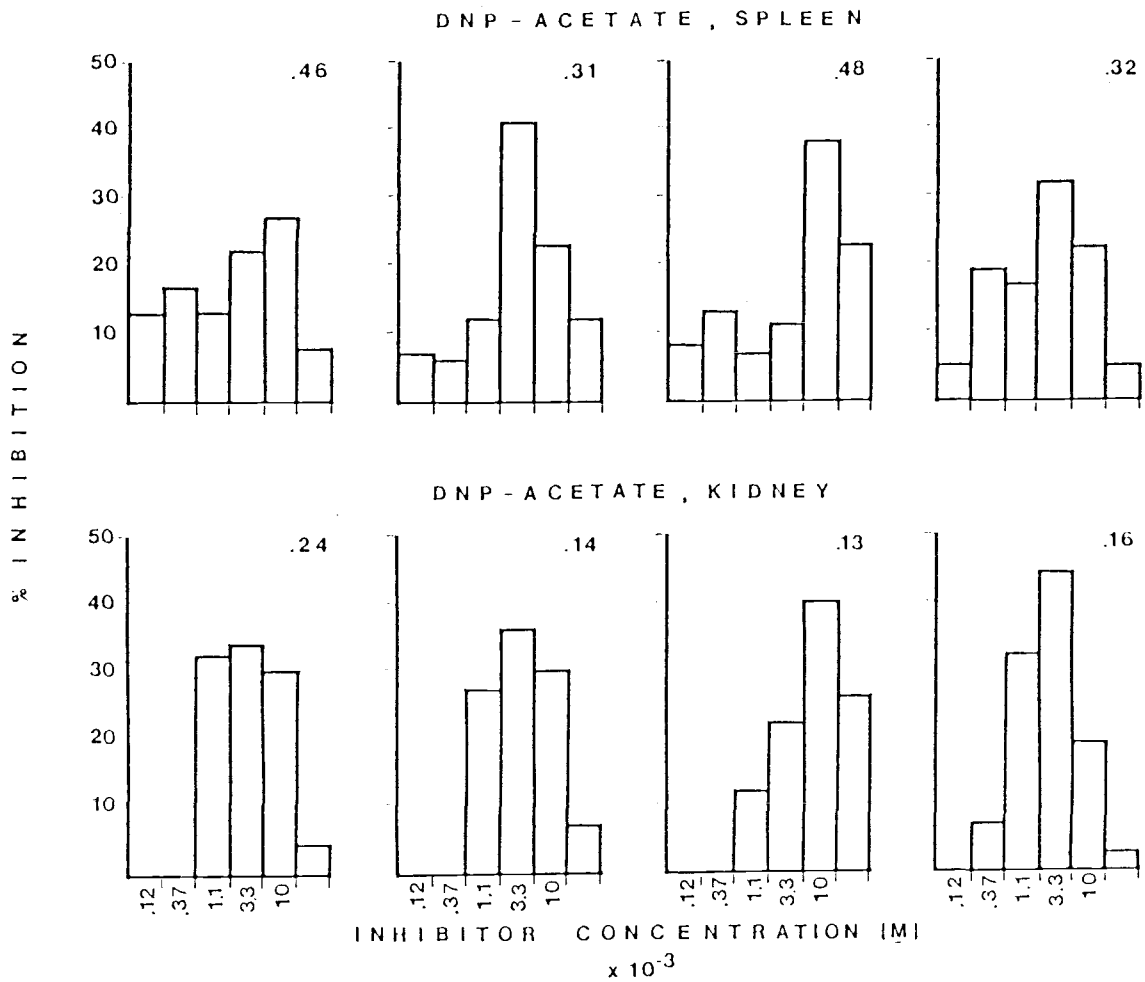


Figure 19. Composite of inhibition profiles employing DNP-acetic acid. Splenic and anterior kidney cell cultures were stimulated with TNP-LPS (0.5 ug/ml) and generated anti-TNP PFC on day 4 through day 11 post culture initiation. Resulting PFC were inhibited with graded doses of DNP-acetic acid (abscissa) and inhibition profiles were generated. The variance from the mean is reported in the upper right corner of each histogram. Plots of organs from the same individual lie above and below one another and are the corresponding individual organs, from left to right, of Figure 19 data. For a more thorough explanation of inhibition profile generation or heterogeneity index calculation, refer to Appendix I.

Table III. Heterogeneity indices from composite inhibition profiles of the anterior kidney and the spleen. Heterogeneity indices were calculated from inhibition profiles generated by inhibition both TNP-lysine and DNP-acetic acid.

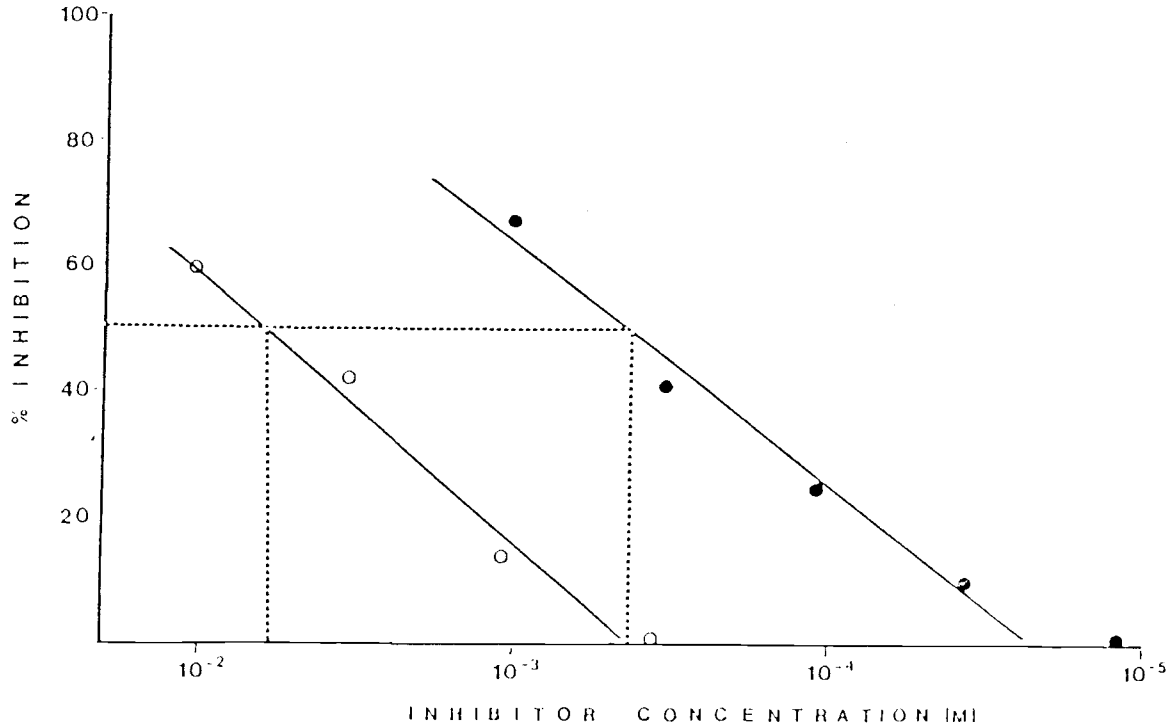
HETEROGENEITY INDICES

TNP-LYSINE		DNP-ACETIC ACID	
SPLEEN	KIDNEY	SPLEEN	KIDNEY
.46	.14	.72	.42
.90	.77	.56	.41
.65	.57	.26	.15
.17	.40	.31	.14
.26	.29	.46	.24
.19	.20	.32	.16
.47	.26	.48	.13

antibody populations (see appendix III and Figure 20). The relative affinity constant illustrates the average affinity a particular antibody population has for a heterologous hapten with respect to the homologous hapten. Table IV provides a list of K_{REL} values for 4-nitrophenylacetic and 2,4-dinitrophenylacetic acid inhibition with respect to trinitrophenyl-lysine (TNP-LYS). Splenic K_{REL} values were observed to be consistently greater than anterior kidney K_{REL} values. The data indicates that the average of all antibody produced in the spleen has from two to five-fold higher affinity for heterologous haptens with respect to TNP-LYS than the average antibody produced in the anterior kidney.

Nature of the Anti-TNP Antibody Binding Site

The use of various analogs of the trinitrophenyl hapten as inhibitors of an anti-TNP response can be used to predict which features of the hapten are essential for antibody binding. Figure 21 depicts the inhibition curves that resulted when either 2,4-dinitrophenyl-lysine (DNP-LYS) or 2,4,6-trinitrophenyl-lysine (TNP-LYS; structures drawn in appendix II) were employed to inhibit TNP-LPS generated PFCs. The coincident curves indicate that splenic generated antibody populations bind both TNP-LYS and DNP-LYS with the same affinity over inhibitor concentrations from 10^{-3} M to 4 x 10^{-6} M. Splenic generated antibody populations exhibited a lack of affinity for 2,4-dinitrophenylacetic acid (DNP-acetic acid; Figure 22). An approximate tenfold decrease in affinity of the antibody for this heterologous hapten, compared to DNP-lysine, was observed. An



$$K_{REL} = \frac{\text{Concentration of homologous hapten for 50\% inhibition}}{\text{Concentration of heterologous hapten for 50\% inhibition}}$$

$$\text{Splenic } K_{REL} = \frac{.61 \times 10^{-3} \text{ M}}{7.1 \times 10^{-3} \text{ M}} = .078$$

Figure 20. Example data for K_{REL} calculation. Inhibition curves generated by the heterologous hapten, 4-nitrophenylacetic acid and the homologous hapten, TNP-lysine, generated against splenic in vitro PFC. Splenic PFC inhibition curves are designated by the solid lines for TNP-Lys (●) and 4-NP acetic acid (O). The dashed line depicts 50% inhibition and the concentration of hapten at which this line intersects the inhibition curves is used in the calculation of the relative affinity constant. The 50% inhibition concentrations for 4NP-acetic acid and TNP-lysine are indicated by where the vertical, dashed lines cross the abscissa. Hapten concentrations causing 50% inhibition are 6.1×10^{-4} M and 7.8×10^{-3} M for TNP-Lys and DNP acetic acid respectively.

Table IV. Composite K_{REL} values generated with 4-nitrophenylacetic acid and 2,4-dinitrophenylacetic acid. K_{REL} values were calculated with respect to the homologous hapten 2,4,6-trinitrophenyl lysine.

INHIBITOR	SPLEEN	KIDNEY
4-NITROPHENYLACETIC ACID	.075	.015
	.014	.004
	.007	.003
2,4-DINITROPHENYLACETIC ACID	.058	.013
	.018	.006
	.016	.008
	.028	.019

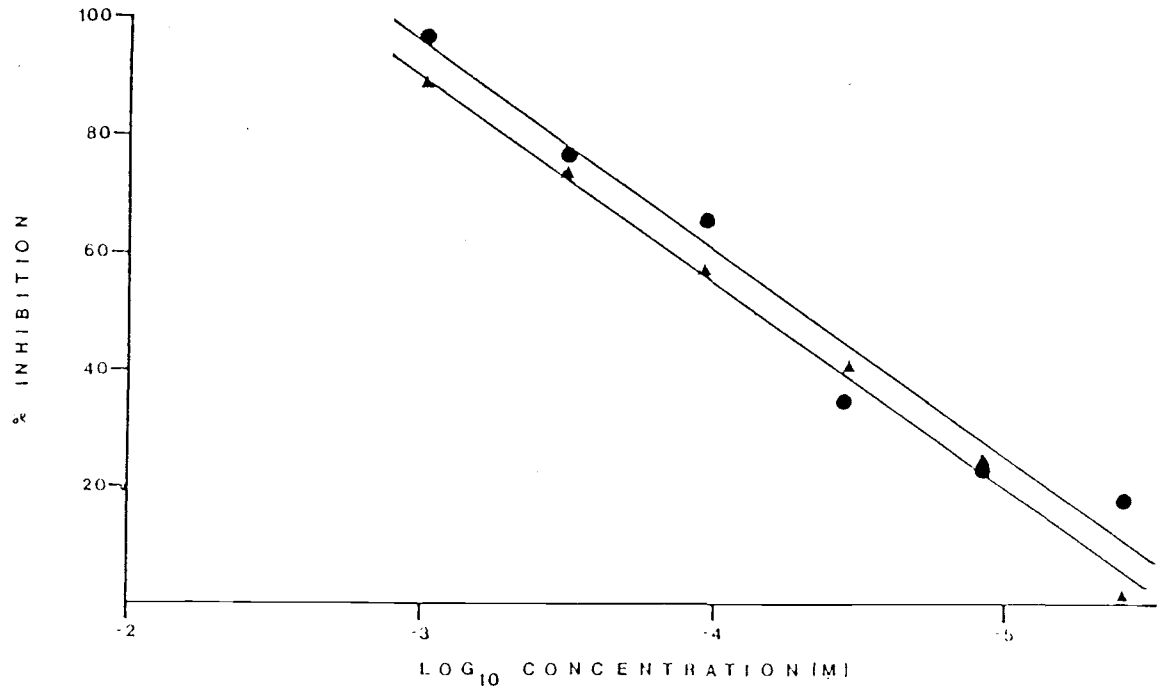


Figure 21. Inhibition curves generated by TNP-LYS and DNP-LYS. Splenic cultures stimulated by TNP-LPS (0.5 ug/ml) demonstrate equivalent inhibition curves employing TNP-Lysine and DNP-lysine. Inhibition curves were generated by inhibiting splenic PFC responses with serial 3 fold dilutions of TNP-Lys (●) or DNP-Lys (▲) from 10^{-3} M. Results are plotted as the percent inhibition of the control response resulting from the corresponding free hapten inhibitor concentration.

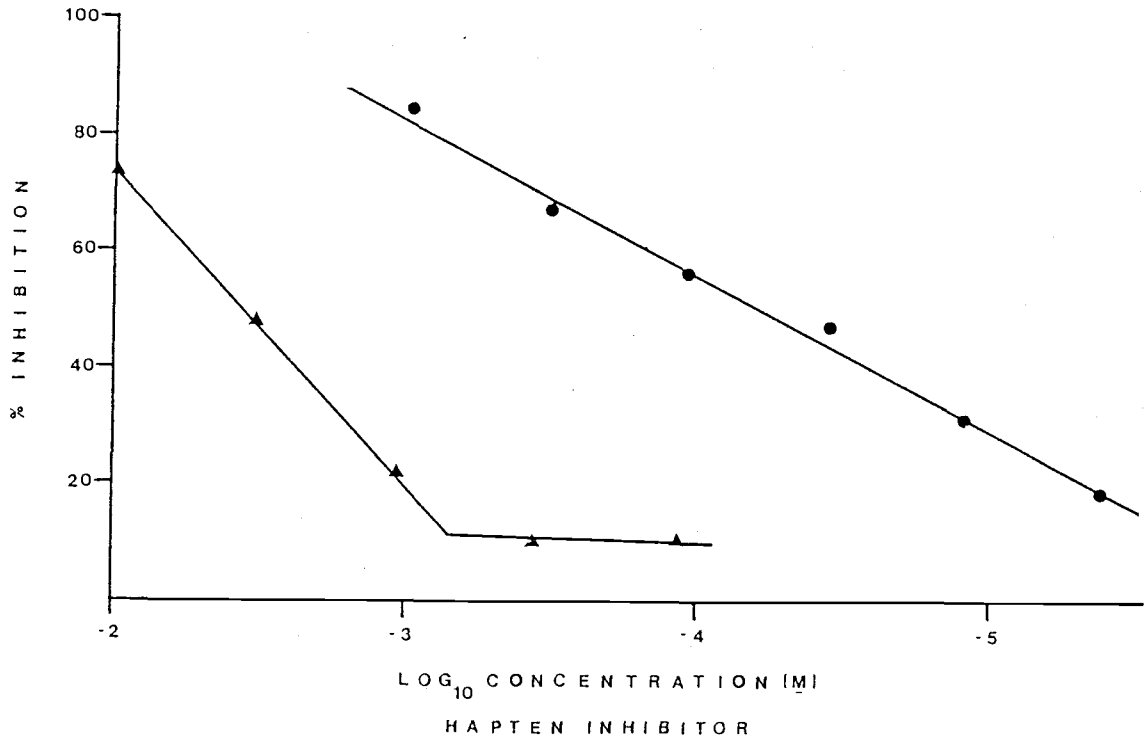


Figure 22. The effect of DNP-acetic acid as an inhibitor of anti-TNP PFCs. In vitro cultures of splenic lymphocytes produced PFC to TNP-LPS (0.5 ug/ml) which were characterized by PFC inhibition analysis, with DNP-acetic acid and compared to 2,4-dinotrophenyl-lysine. The inhibition curve generated by DNP-acetic acid (▲) illustrates at least of 10 fold loss of antibody affinity for the DNP molecule when the acetic acid substituent replaces lysine in DNP-lysine (●).

acetic acid (2.6×10^{-3} M) inhibition control demonstrated 0% inhibition. This indicated the hapten dependent nature of DNP-acetic acid inhibition. Use of 2,4-dinitrophenylbutyric acid (DNP-butyric acid), where the carboxyl group is positioned two methylene units more distal from the benzene ring of DNP than for DNP-acetic acid restored binding affinity to the same level as exhibited toward DNP-lysine (Figure 23). An inhibition curve for 2,4-dinitrophenyl-phenylalanine was also plotted in figure 23. The bulky phenyl group of phenylalanine (structure drawn in appendix II) exhibited no steric hinderance on the binding of antibody as its inhibition curve was observed to be identical to DNP-lysine.

Figure 24 contains two interesting inhibition curves generated by 2,4-dinitrophenol and para-nitrophenol (structures drawn in figure II). It is of particular interest that all previous hapten inhibitors generated curves with an approximately equivalent slope. The slopes for dinitrophenol and para-nitrophenol, however, were markedly greater. At hapten concentrations from 10^{-2} M to 2.5×10^{-3} M, inhibition by para-nitrophenol remained at almost 100% of the control. Inhibition by para-nitrophenol at lesser concentrations sharply decreased reaching 3% inhibition at only 6.7×10^{-3} M. Inhibition of the plaque forming cell response by dinitrophenol was also 100% at 10^{-2} M but its slope was intermediate to that of para-nitrophenol and DNP-lysine.

The inhibition curve generated by para-nitrophenol demonstrates that all PFCs were inhibited within a very small concentration range (2.5×10^{-3} M to 1.25×10^{-3} M). The possibility that these

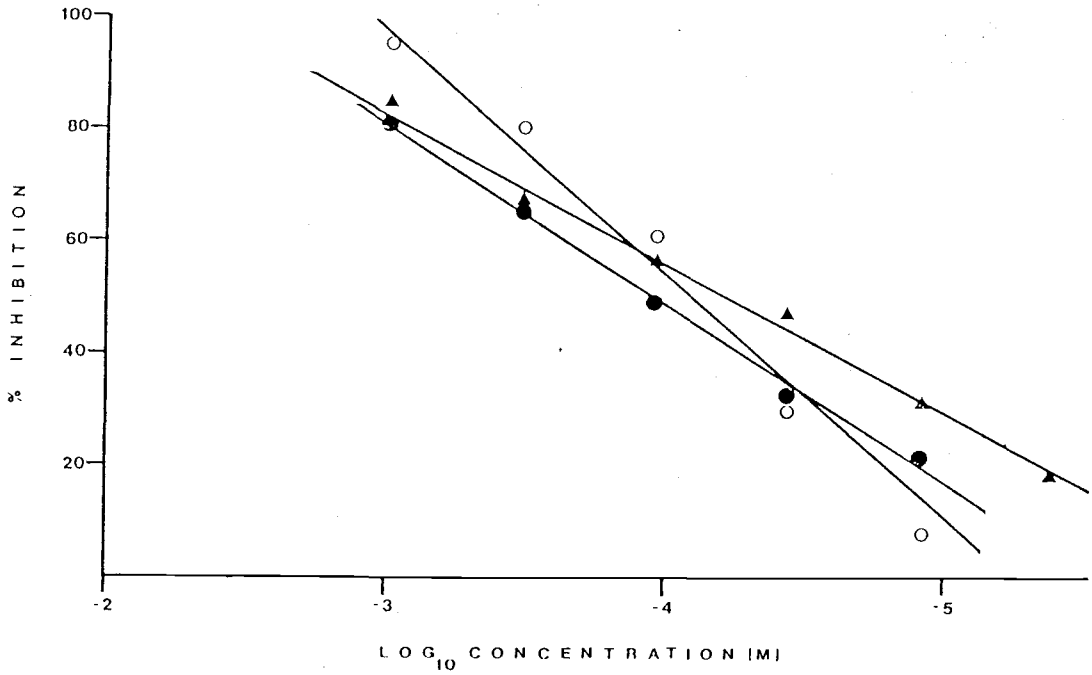


Figure 23. Inhibition curves generated by DNP-butyric acid and DNP-phenylalanine. The effect of butyric acid and phenylalanine as substituents attached to DNP, on the inhibition of anti-TNP PFCs responses are shown. 2,4-dinitrophenyl butyric acid (●) and 2,4-dinitrophenyl phenylalanine (○) were investigated for their ability to inhibit splenic PFC responses generated by TNP-LPS. Both inhibition curves are quite similar to the curve demonstrated by DNP-Lysine (▲). All three inhibitors were employed in a series of 3 fold dilutions from 10^{-3} M to 1.2×10^{-5} M, (or to 4.6×10^{-6} M for DNP-Lys).

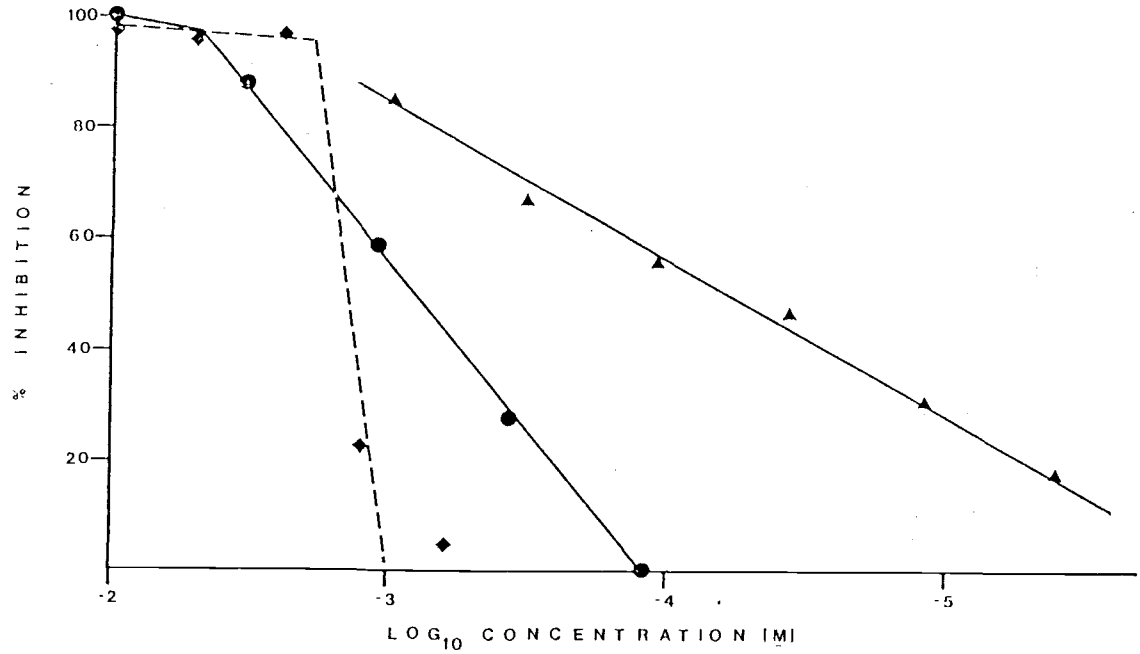


Figure 24. The effects of paranitrophenol and dinitrophenol as inhibitors of anti-TNP PFCs. Serial dilutions of each analog from 10^{-2} M (for paranitrophenol and dinitrophenol) and 10^{-3} M (for DNP-Lys) were used to generate inhibition curves for TNP specific PFC from cultures of coho splenocytes. A series of 2 fold dilutions of paranitrophenol (10^{-2} M) down to 6.8×10^{-4} M were employed to investigate this simple hapten analog (◆). Serial 3 fold dilutions of dinitrophenol (●), from 10^{-2} M to 1.2×10^{-4} M, were also used in this study. The curve for DNP-LYS (▲) employed 3 fold dilutions of this inhibitors from 10^{-3} M to 4×10^{-6}M

concentrations of paranitrophenol were lymphocytotoxic, thereby causing an apparent hapten specific inhibition of PFC formation, was tested. Para-nitrophenol (10^{-2} M) was preincubated with the lymphocytes for two hours, the duration of a normal plaque assay. Lymphocytes were washed to remove residual inhibitor and plated in a standard PFC assay in the absence of inhibitor. A response which was 97% of the control response indicated that cytotoxicity was not responsible for the inhibition curve generated by para-nitrophenol.

DISCUSSION

The study of salmonid B cell induction and antibody production necessitated the development of an assay system in which the particular cell producing a specific antibody molecule could be identified and characterized. A modified hemolytic plaque assay, such as that developed by Cunningham (1968) is such a system, therefore, it was adapted for the determination of salmonid antibody responses. Antibody responses were quantitatively monitored, after intraperitoneal immunization with an O-antigen extract from Vibrio anguillarum. The assay employed the plaque forming cell (PFC) assay with Vibrio O-antigen coated sheep red blood cells (VSRBC). An example of a typical hemolytic plaque is shown (Figure 2), which is characterized by a zone of lysis within the VSRBC lawn surrounding the PFC. This PFC (see arrow, Figure 2) is central in the zone of hemolysis and is secreting antibody specific for the Vibrio antigen.

Plaque forming cell responses generated in vivo and specific for Vibrio anguillarum were detectable by day seven post-immunization in both splenic and anterior kidney derived lymphocytes, but not until day 16 in the thymus of immunized animals (Figure 3). Maximum PFC levels were reached by day 16 post-immunization for the spleen and anterior kidney lymphocyte samples (Figure 3). The maximal thymic PFC response cannot be determined from this figure since the number of plaque forming cells (PFCs) per thymus was still increasing when the assay was terminated, 21 days post-immunization. PFC responses for all three lymphocyte sources from immunized animals were significantly greater (50 to 100-fold) than responses from non-

immunized control animals. Responses to uncoated sheep red blood cells (SRBC) from both immunized and control animals were also insignificant compared to the responses from immunized animals. Plaque forming cells were demonstrated to also occur in the posterior kidney, however kinetic studies were not done with cells from this organ.

The kinetics of the maximum PFC response from the spleen and anterior kidney (16 days post-immunization) appear delayed when compared to *in vivo* PFC responses generated in mammalian systems. Such kinetics are not so surprising considering that salmonids are poikilothermic animals. A number of studies have demonstrated the temperature dependence of the immune response kinetics of ectothermic vertebrates. Peak PFC responses in the carp (*Cyprinus caprio*) were found to be equivalent in number, but increasingly delayed as the water temperature was decreased from the optimal holding temperature for these animals (Rijkers, 1980). Groberg (1983) studied salmonid antibody titers to *Vibrio anguillarum* at 18°, 12° and 6°C and found that at each sequential drop in water temperature there was a corresponding increase in the time necessary to reach the peak agglutinating antibody titer. Since, in this study, the responding animals were held at 12°C, the apparent slow onset of maximal PFC responses is not extraordinary.

The demonstration of PFCs present in thymic tissues raises a question as to this organ's function. The occurrence of antibody producing B cells from thymocytes is perplexing in that the thymus is considered to be a T cell organ in mammals and this classification

has been extended to rainbow trout (Etlinger, 1976). However, PFCs have been demonstrated in the thymus of another fish species, Tilapia mossambica (Sailendri, 1975). The cellular composition of the piscine thymus, therefore appears to differ from that of mammals. DeLuca (1983) has demonstrated, with a specific anti-fish immunoglobulin monoclonal antibody, that immunoglobulin positive thymocytes comprise only 5% of the total thymocyte population. Such findings may point to the thymus being somewhat like a mammalian lymph node with regard to B and T cell composition. It is possible that at this stage of the evolution of the immune system, the thymus, as characterized in mammalian systems, may not have evolved into a primary lymphoid organ devoted to T cell differentiation. The structural organization of the salmonid thymus does not possess cortical and medullary regions, thus it appears more like a mammalian lymph node than a thymus (Yasutake, 1984). The structure and location of the rainbow trout thymus has been suggested to possess important immune function although not primarily as a T cell developmental organ (Tatner, 1982).

PFCs have been demonstrated among rabbit thymocytes, although this cell population was very small and did not alter the characterization of the thymus as a T cell developmental organ (Jentz, 1979). Thus, an alternate view is that, like in the rabbit, a small population of antibody-producing B cells may reside within the Salmonid thymus accounting for the observed PFC responses. Since the developmental stages of a putative salmonid T cell are not known, it is impossible to ascertain the effect that thymic microenvironments may have on salmonid thymocytes.

The kinetics of the thymic PFC response are delayed, even when compared to the salmonid splenic and anterior kidney responses. Furthermore, significant PFC responses are not detectable until day 16, which is the peak PFC response in these two main salmonid B cell organs. Although data was obtained demonstrating that thymic PFC responses were not due to inadvertant peripheral blood cell contamination, the possibility arises that during the onset of the PFC response, PFCs generated in the spleen and anterior kidney migrated and slowly accumulated in the thymus.

Alternatively, the method of intraperitoneal immunization might delay the arrival of antigen to the thymus compared to the spleen and anterior kidney. Nelson (1985) studied the distribution of intraperitoneal injected Vibrio bacterin at selected time intervals in the rainbow trout. Shortly after intraperitoneal immunization (5 min), all tissues of the rainbow trout contained demonstrable Vibrio bacterin, including the thymus. Thus, delayed PFC responses due to delayed antigen exposure to thymic tissues is unlikely.

The development of the PFC assay system was essential to the study of salmonid B cell induction and the antibody produced by these B cells. Although numerous PFC assays have been reported in various fish species (Chiller, 1969; Sailendri, 1975; Smith, 1967; Ingram, 1980; Anderson, 1979 and 1983), only one other report of a PFC response from a salmonid species in response to Vibrio anguillarum antigens has been published (Sakai, 1984). Therefore, it was necessary to determine that plaques produced by these PFCs were antibody (immunoglobulin) dependent and also that the antibody

produced was specific for the inducing antigen (Vibrio anguillarum O-antigen). Rabbit antiserum specific for coho immunoglobulin reduced the PFC response by 100% (Figure 4) presumably by binding secreted coho immunoglobulin and thus, interfering with the ability of this Vibrio specific molecule to bind to a VSRBC. This result was controlled by employing nonimmunized rabbit serum and demonstrating minimal inhibition of PFCs (Figure 4; 11% of the control response).

Another essential component necessary to cause plaque formation was the addition of salmonid serum as a complement source. Titration of various complement sources (spring chinook, fall chinook, coho, steelhead trout and rainbow trout) revealed an absolute dependence on salmonid complement for PFC formation (data not shown). Complement added to VSRBC without the addition of immune lymphocytes caused no PFC formation. Finally, when comparing dilutions of fresh serum samples from all salmonid species listed above (chinook, coho and trout), rainbow trout and steelhead trout serum in general yielded the best results. An ideal complement source was determined as that which possessed high titer activity and yielded the greatest number of PFCs, without causing nonspecific SRBC lysis.

Once the immunoglobulin dependency of the assay had been established, the specificity of this immunoglobulin was addressed. Addition of a soluble antigen extract from Vibrio anguillarum eliminated PFC formation (0% of the control response; Figure 5A). Vibrio antigen, free in solution, blocked Vibrio specific antibody from binding to the VSRBC in a competitive manner. An equivalent concentration of a closely related fish pathogen, Vibrio ordalii, caused partial inhibition of the PFC response (20% inhibition of the

control; Figure 5B). Partial inhibition is indicative of cross-reactivity between O-antigen components from these two Vibrio species.

Neither a soluble antigen extract from a gram positive fish pathogen, Renibacterium salmoninarum, (Figure 5C), nor an E. coli lipopolysaccharide (not shown) preparation exhibited inhibition of Vibrio specific PFCs. Thus PFC inhibition by a soluble antigen extract from any salmonid pathogen does not occur (no inhibition by R. salmoninarum). The inability of LPS to inhibit the PFC response illustrates that the anti-Vibrio PFC response is not simply directed at this component common to all gram negative bacterial cell walls.

It was important to demonstrate that inhibition of PFC formation was not simply occurring by effects of the Vibrio extract on the viability of lymphocytes. Demonstration that B cells were still able to produce hemolytic plaques after preincubation with this antigen indicates that a cytotoxic mechanism of PFC inhibition does not seem reasonable (Figure 5D).

Characterization of the anti-Vibrio PFC response was accomplished by free antigen inhibition analysis of splenic, anterior kidney and posterior kidney responses. Analyses showed inhibition of PFCs from the spleen and posterior kidney to be similar in that they possessed high and low affinity antibody producing cells (PFCs). The inhibition profiles of these two lymphocyte sources were also relatively heterogeneous, compared to the anterior kidney (Figure 7A, B and C). Average heterogeneity indices (Figure 8) for the spleen ($0.64 \pm .12$) and posterior kidney ($0.86 \pm .4$) were both significantly

greater than the anterior kidney index (0.20 ± .12).

Goidl (1974) investigated the ontogeny of B cell responses in fetal and neonatal mice employing this PFC inhibition analysis. In the fetal animal most inhibition profiles lacked heterogeneity and were skewed to the high free antigen end of the histogram plot (low affinity). However, early in neonatal life PFCs from secondary immune organs (spleen and lymph nodes) began to show high affinity antibody production and their inhibition profiles took on a more heterogeneous appearance. At this same particular point during ontogeny the fetal liver and bone marrow (primary immune organs) exhibited inhibition profiles which remained restricted, containing predominantly low affinity antibody. Later in ontogeny, the fetal liver and bone morrow were able to exhibit high affinity antibody and their inhibition profiles were more heterogenous (increased heterogeneity indices), the cause of which was suggested to be via recirculation of mature lymphocytes through the primary organs.

The inhibition profiles of the salmonid anterior kidney (Figure 7A) are similar to those of the bone morrow and fetal liver in the murine system (Goidl, 1974). The histogram data for the spleen and posterior kidney more closely resemble murine secondary organ inhibition profiles. The patterns of organ dependent B cell functional heterogeneity described here support histological data presented by Zapata (1979). Zapata has designated the anterior region of the fish kidney as being the primary hematopoietic tissue comparable in function to the mammalian bone morrow. Thus, B cells in a piscine primary immune organ appear histologically and functionally like their murine counterparts. Therefore, as in

mammalian immune systems, it is likely that immature lymphocyte populations in salmonids produce antibodies with less heterogeneity and which lack high affinity. Conversely, mature lymphocyte populations, as found in secondary immune organs like the spleen and posterior kidney, produce antibodies with greater heterogeneity, demonstrating both high and low affinities.

Observing functional differences between B cell populations in the anterior and posterior regions of the kidney was not surprising considering histological and functional characterizations of this organ (Zapata, 1979; Hendricks, 1983). The anterior region of the kidney is almost completely lymphoid in nature, possessing hematopoietic function. In the more posterior regions of the kidney, more renal structure and function is found. The lymphocyte population which does occur in the posterior kidney does not demonstrate the variety of different blood cell types nor do lymphocytes appear in the heterogeneous states of maturity that they do in the anterior kidney (Zapata, 1979; Hendricks, 1983).

Since these experiments were conducted <u>in vivo</u>, cellular trafficking could occur providing an alternative explanation for the differential inhibition profiles. Brahim (1970) envisions that immature lymphoctes may develop in the primary immune organs to particular stages of maturity and then are signalled to move, via the circulation, to the secondary immune organs for further development and differentiation. Similarly, lymphocytes in the anterior kidney may mature and enter the circulation to relocate in the secondary immune organs (spleen and posterior kidney). Further B cell

maturation might then take place in a similar fashion as with mammals. Therefore, this model may explain the observed lack of high affinity antibody producing PFCs in the anterior kidney. High affinity antibody production is generally considered to be a characteristic of more mature B cells. Thus, B cells destined to produce high affinity antibody and residing in the anterior kidney may be continually signalled to leave the anterior kidney prior to reaching this level of maturity. However, Goidl (1974) showed that the fetal liver and bone marrow inhibition profiles take on a more heterogeneous appearance later in neonatal life due to recirculation of mature lymphocytes back through the primary immune organs. Since the fish used in this study were immunologically mature (Agius, 1985) and restricted inhibition profiles were still observed from the anterior kidney, recirculation of mature B cells through the anterior kidney may not occur as suggested in the mammalian model (Brahim, 1970). Consequently, another explanation for the lack of high affinity PFCs in the anterior kidney may be necessary regardless of whether or not lymphocyte trafficking occurs.

Thymocytes have been demonstrated in numerous systems to exert dramatic effects on different types of B cell responses including T independent types 1 and 2 (Doenhoff, 1979; Mond, 1982; Mond, 1983; Zubler, 1982). Although in fish systems a strictly defined T cell has yet to be demonstrated, T cell like functions have been reported in fish (Ruben, 1977), and T cells can play a role in the generation of antibody responses (Miller, 1985). In the murine system, T cells have been demonstrated to be required for a heterogeneous antibody response to occur (Doenhoff, 1979). Tatner (1985) studied the

migration of lymphocytes from the thymus to the peripheral immune organs. This work showed a preferential migration of thymocytes to the spleen but to a much lesser extent the anterior kidney. Furthermore, responses to T cell mitogens have been reported to be lower in anterior kidney cell cultures compared to splenic cell cultures (Etlinger, 1976). Therefore, the observed heterogeneous inhibition profiles demonstrated in the spleen may be due to a cell-cell interaction which simply cannot take place in the anterior kidney due to an under-representation of crucial T cells. The study by Tatner (1985) however did not entail an investigation of thymocyte migration to the posterior kidney. Since functional differences in the B cell responses from the anterior and posterior regions of the kidney have been observed, it would be of interest to investigate the extent of thymocyte migration to the posterior kidney with respect to migration to the anterior kidney.

The use of a complex bacterial antigen, especially in vivo raises questions as to the distribution of this complex antigen after immunization. If different concentrations of antigen are distributed to the different immune organs, certain B cell subpopulations may reflect these dose differences by not responding. Therefore inhibition profile differences may simply reflect a difference in how antigen is distributed to the various fish immune organs. This is unlikely, however, considering the studies of Secombes (1980) and Nelson (1985) on the distribution and sequestering of antigen by rainbow trout immune organs. Both groups reported that the antigens under investigation could be detected in the spleen and anterior

kidney at the earliest time points assayed. Nelson (1985) found that Vibrio bacterin could be demonstrated in all fish tissues five minutes post-immunization and by day 14 this antigen had been cleared from all tissues but the spleen and throughout the kidney. Therefore on the basis of these studies, it seems unlikely that the Vibrio O-antigen extract, in the in vivo PFC reponse, was unevenly distributed to either the anterior kidney, spleen or posterior kidney. Furthermore, if the observed restricted inhibition profiles from the anterior kidney were caused by a lower amount of antigen sequestered in this region, then a selective stimulation of high affinity surface immunoglobulin bearing cells would be expected. Thus, a restricted response (homogeneous) would occur, but it would be skewed toward the low free antigen (high affinity antibody) end of the plot since lymphocytes bearing these high affinity receptors would best compete for a low antigen dose. Obviously, this is not the case because anterior kidney inhibition profiles are skewed toward low affinity (Figure 7A). If however, the distribution of antigen to the anterior kidney is in excess and lesser concentrations reach the spleen and posterior kidney, then we might observe a high dose tolerance effect that would inactivate high affinity clones in the anterior kidney and account for the observed inhibition profiles. The spleen and posterior kidney, by not receiving excess antigen doses, would respond to their full capacity and generate the heterogeneous plots which were observed.

The in vivo antibody response to Vibrio anguillarum O-antigen, and subsequent characterization of PFCs by antigen inhibition analysis indicated that functionally different B cell populations

exist in the different salmonid immune organs. However, examination of *in vivo* responses would necessarily leave essential questions unanswered. Why were different B cell populations residing in different regions of the same organ (i.e. the kidney)? How did splenic and posterior kidney lymphocytes generate heterogeneous responses which contained high affinity antibodies? *In vivo* analysis does not allow for answers to be obtained experimentally. It was therefore essential to develop an *in vitro* antibody production system for the generation of salmonid immune responses by the cells from these separate immune organs.

The *in vitro* antibody production system eliminated many biological and environmental parameters which affected *in vivo* antibody production. Immunization was performed in tissue culture wells, allowing for precise administration of an antigenic dose. Cellular trafficking as well as antigen trafficking was eliminated in this static situation. The *in vitro* antibody production system facilitated the manipulation of cells and antigens enabling more extensive studies into the nature of the signals required for salmonid B cell activation. Finally the *in vitro* antibody production system that was developed employed a chemically defined, small molecular antigenic determinant or hapten. The haptenic unit chosen was the trinitrophenyl molecule (TNP). Knowing the precise chemical structure of the antigen enables investigations into the fine specificity of the antibody which is generated. Affinity measurements of the antibody for antigen can be more accurately determined with precise amounts of pure haptenic inhibitors.

The *in vitro* antibody production system referred to above yielded PFC responses specific for trinitrophenylated sheep red blood cells (TSRBC). Figure 9 depicts the results of a dose response of trinitrophenylated-lipopolysaccharide (TNP-LPS) on splenic and anterior kidney derived lymhocyte cultures. Both splenic and anterior kidney lymphocyte cultures exhibited maximal PFC responses at TNP-LPS concentrations of 0.5 ug/ml. The presence of TNP-LPS was required to induce these responses as illustrated by the low response from control wells. Although the maximum anterior kidney lymphocyte response is fourfold greater than the maximum splenic lymphocyte response, the dose response curves are remarkably similar, indicating the lymphocytes from both organs respond to both suboptimal and supraoptimal doses of TNP-LPS in the same manner. Due to the limited lymphocyte numbers in the posterior kidney, B cells from this organ were not investigated in the *in vitro* system.

The ability of a T cell independent (TI) form of antigen, such as TNP-LPS, to stimulate a particular subpopulation of B cells is well documented (Coutinho, 1974; Mond, 1982). Employing a T cell dependent (TD) protein antigen, enables one to assess the presence of piscine T cells or whether salmonids react to different forms of antigen in a typically TI or TD manner.

Figure 10 illustrates maximal anti-TNP splenic PFC responses at a 100 ug/ml concentration of TNP-KLH while no stimulation of an anti-hapten response occurs for any dose of the non-haptenated KLH. T cell populations in mammals must be first primed to the antigen to elicit any PFC responses to protein antigens from B lymphocytes. In the case of TNP-KLH, the protein carrier, KLH, must first be exposed

to the helper T cell population, prior to B and T cell exposure to TNP-KLH. If this T cell priming step does not take place, then an anti-TNP response will not occur. The results shown in Figure 10 contradict this TD immune response dogma in that responses to TNP-KLH were demonstrated without a prior exposure of salmonid lymphocytes to KLH (carrier priming). In vitro responses to TNP-KLH, without previous carrier priming, have also been demonstrated in channel catfish (Miller, 1985). Furthermore, Miller has shown that depletion of a surface immunoglobulin negative lymphocyte population will not yield an anti-TNP response to TNP-KLH unless this cell type is added back to the culture. Since mammalian T lymphocytes do not possess surface immunoglobulin molecules, these data suggest that a T cell like lymphocyte is required for responses to a TD antigen. However, in two completely different fish systems responses to the same TD antigen (TNP-KLH) did not require previous carrier priming. This data indicates that fish T cell dependent B cell responses may be activated differently than mammalian TD responses.

The optimal kinetics for TNP-LPS stimulation at 0.5 ug/ml were reported to be day 6 of in vitro culture (Figure 11). However in a number of different kinetic experiments, the optimal incubation period ranged from day 6 post culture initiation through day 9 for both splenic and anterior kidney cell cultures (Table II). Such a broad range in time, for the generation of an optimal response, could reflect variation between individual animals since salmonids are an outbred population of organisms. Peak kinetics between the spleen and anterior kidney were relatively consistent within an individual,

further suggesting that variation likely originates between animals or different experiments, and not between immune organs.

TNP-LPS is a T-independent antigen capable of stimulating relatively large immune responses. Lipopolysaccharide, the carrier of TNP-LPS, is a well-documented, potent polyclonal B cell activator causing cellular proliferation and antibody production (Andersson, 1972; Chiller, 1973; Melchers, 1973). Coutinho (1974) has reported that activation of anti-TNP specific lymphocytes occurs by focusing of the polyclonal activator onto the lymphocyte surface by binding the TNP substituent of TNP-LPS.

Experiments were conducted to demonstrate that salmonid splenic lymphocyte responses to TNP-LPS were antigen specific and not caused by polyclonal activation. A dose response curve was generated (Figure 12) which demonstrated that TNP-LPS generated an optimal TNP-specific PFC response at a dose of TNP-LPS that was 100 fold less (0.4 ug/ml) than the dose of unhaptenated LPS (40 ug/ml) necessary to produce an anti-TNP specific PFC response. The TNP-LPS response at 40 ug/ml, although not maximal, still generated more TNP-specific PFC than LPS at 40 ug/ml (590 $\pm$ 136 PFC/10^6 lymphocytes compared to 235 $\pm$ 28 PFC/10^6 lymphocytes respectively). Data from figure 12 illustrate that TNP-LPS is far more specific than LPS because it activates peak PFC responses at concentrations at least 100-fold less than the unhaptenated molecule, similar to the results of Coutinho (1974).

Considering the dose response curves of TNP-LPS and LPS it appeared that TNP-LPS was stimulating anti-TNP receptor bearing lymphocytes in an antigen specific manner and LPS was stimulating

lymphocytes in a polyclonal manner. Other ways in which polyclonal activation was assessed was by measuring the amount of cellular proliferation in a mitogen assay and also by measuring total immunoglobulin production. Figure 13 illustrates parallel dose responses for cellular proliferation, total immunoglobulin production and TNP-specific PFCs generated for the mitogens, phytohemagglutinin (PHA) and LPS. However, the effects of the TNP-LPS dose response on these functions differ. At low doses of TNP-LPS cellular proliferation and total immunoglobulin levels remain low and just as with LPS, at high concentrations, the indicators of a non-specific immune response (proliferation and total immunoglobulin levels) rise dramatically. However, at low TNP-LPS concentrations the level of the specific immune response (anti-TNP PFC formation) is maximal. At high TNP-LPS concentrations the specific anti-TNP PFC response drops significantly, once again separating polyclonal activation responses from antigen specific ones.

Polyclonal activators such as PHA and LPS are postulated to act by binding specific mitogen receptors which then deliver intracellular stimulatory signals (Andersson, 1976; Moller, 1976). The induction of TNP-specific B cell responses by TNP-LPS, however, has been implicated to occur by the focussing of the polyclonal activator, LPS, onto the B cells by interaction of TNP with surface immunoglobulin receptors specific for the hapten (Moller, 1973; Moller, 1976).

Inhibition of B cell activation by monovalent antigen was employed to demonstrate that TNP-LPS activates B cells through the

TNP specific surface immunoglobulin receptors. Monovalent TNP-lysine (TNP-Lys) was added to individual cultures of splenic lymphocytes at a series of different concentrations to determine if binding of hapten could block binding and activation by TNP-LPS in a competititve manner. Three different concentrations of TNP-LPS were administered to the splenic cultures. The highest concentration of TNP-LYS (7.5×10^{-4} M) effectively eliminated the ability of TNP-LPS at 3 successive doses (40 ug/ml, 8 ug/ml and 1.6 ug/ml) to induce B cell activation to generate PFCs (Figure 14). The nonhaptenated lysine control at $7.5\ 10^{-4}$ M caused no inhibition of PFC generation, therefore, activation by TNP-LPS is demonstrated to be dependent on the availability of cell surface receptors specific for TNP. Panels A through C (Figure 14) illustrate that each successive drop in TNP-LPS concentration corresponds to a similar drop in the concentration of TNP-LYS necessary to cause 50% inhibition of the total control PFC response. This suggests that simple competitive inhibition by TNP-LYS for the TNP specific lymphocyte surface receptor has occurred thus blocking the ability of TNP-LPS to activate B cells.

Gamma irradiation has been employed in mammalian studies to selectively eliminate lymphocyte subpopulations (Anderson, 1975; Andersson, 1976). Therefore, gamma irradiation can be used as a tool to determine the contribution of certain lymphoid populations to various immune responses. Figure 15 illustrates such differential effects of radiation upon coho splenocyte cultures with respect to stimulation by either a TD antigen (TNP-KLH) or TI antigen (TNP-LPS). The TI response of coho splenic lymphocyte cultures appears enhanced by radiation doses of 400 rads or less. TD responses of the same

cell cultures however are inhibited by all radiation doses. These results are similar to those seen with irradiation of rat lymphocytes by low doses. This phenomenon was concluded to be caused by the elimination of a radiosensitive T suppressor cell (Tada, 1971). Although a strictly defined T cell has yet to be defined for any fish, let alone a T suppressor cell, low dose gamma irradiation demonstrates the elimination of some presumed down regulatory signal which apparently is present in control cultures.

Responses to both TD and TI forms of antigen have been demonstrated in in vitro salmonid splenocyte cultures. In an attempt to determine whether the same or different subpopulations of B cells were responding to TNP-LPS (TI) and TNP-KLH (TD), antigen addition experiments were performed on salmonid splenic lymphocytes (Figure 16). If responses were observed for antigens added to the same cell cultures which were the sum of the responses to each individual antigen (Figure 16C), then the existence of at least two separate responding subpopulations of B cells would be implied. However, responses below or equivalent to the sum of individual TNP-KLH and TNP-LPS responses would strongly suggest that the same B cell subpopulation is activated by both forms of the antigen. Thus, as in the dose response curve for TNP-LPS or TNP-KLH, once the optimal dose of an antigen has been reached, a more concentrated dose of antigen may actually cause a decrease in the PFC response.

Data in figure 16 indicate that the same population of salmonid B cells responds to TD and TI forms of antigen but these responses are affected differently by low dose irradiation (Figure 15). Thus

another cell type, other than a TNP specific B cell which is both radiosensitive and down-regulates TNP-LPS responses is likely to be present.

Antigen inhibition analyses of in vivo generated PFCs were used to functionally characterize B cell populations from the anterior kidney, spleen and posterior kidney. These analyses lent functional support to Zapata's histological characterization of the anterior kidney of the teleost (which include salmonids) as being a primary immune organ, similar to the mammalian bone morrow. The in vivo studies demonstrated differences between PFCs generated in splenic and posterior kidney tissues compared to anterior kidney PFCs. Development of an in vitro antibody production system eliminated certain inherent problems present in the in vivo experiments. Cellular trafficking after exposure to antigen could not occur in vitro. A chemically defined antigenic unit, the trinitrophenyl hapten, and precise doses of this antigen could be administered to each lymphocyte source in vitro. Due to the defined nature of the antigen, more accurate relative affinity determinations could be made as well as fine specificity analyses.

Splenic and anterior kidney in vitro lymphocyte cultures stimlulated with the optimal dose of TNP-LPS, were characterized by PFC inhibition analysis. Figure 17 illustrates inhibition profiles of splenic and anterior kidney lymphocytes generated by TNP-LYS and 2,4-dinitrophenylacetic acid (DNP-acetic acid). Splenic and anterior kidney inhibition profiles generated by TNP-LYS are apparently equivalent in that both demonstrate high and low affinity antibody. The heterogeneity indices of these plots are quite similar also

(Figure 17). The inhibition profiles generated from DNP-acetic acid however, reveal differences between splenic and anterior kidney PFCs. Splenic lymphocyte inhibition profiles generated by DNP-acetic acid maintain the ability to demonstrate high and low affinity antibody, which is reflected in the heterogeneity index (Figure 17). The anterior kidney inhibition profile generated by DNP-acetic acid exhibits a lack of high affinity antibody, with inhibition bars clustered at the high free antigen inhibitor end of the plot, indicating low affinity (Figure 17). The heterogeneity index reflects the difference between anterior kidney and splenic inhibition profiles in that at least a two-fold greater heterogeneity of antibody producing PFCs occur in the spleen in response to a heterologous hapten. Inhibition profiles from lymphocytes from four individuals confirm what has been indicated in figure 17. Splenic and anterior kidney inhibition profiles from TNP-LYS inhibition are similar in the four individual animals examined (Figure 18). Both lymphocyte sources demonstrate high and low affinity antibody with equivalent heterogeneity indices. Inhibition profiles of the same individuals shown in figure 18 were generated with DNP-acetic acid (Figure 19). DNP-acetic acid demonstrate PFC inhibition profile differences between all splenic and anterior kidney plots. High affinity antibody producing PFCs are present in the spleen but not the anterior kidney (Figure 19) and splenic heterogeneity indices from DNP-acetic acid inhibition are consistently greater (1.8 to 5 fold greater) than anterior kidney indices (Table 3).

The trinitrophenyl molecule has been implicated as being a very

cross-reactive antigenic epitope. It is an immunologically small molecule by virtue of its ability to fit completely inside a portion of the antigen binding site. Therefore, TNP most likely stimulates a very broad and diverse antibody response with a wide variety of antibody affinities represented. If this is true, then a diverse antibody response could originate, even from an immature set of B-cells presumed in the anterior kidney. Thus, PFC inhibition analyses may not be sensitive enough to detect subtle differences in antibody populations using a homologous form (TNP-LYS) of the hapten inhibitor. The anterior kidney may respond to TNP and produce a heterogeneous response which may be difficult to distinguish from that seen in the spleen. By roughly categorizing the total anti-TNP antibody spectrotype into only 7 inhibition groups (the number of graded inhibitor concentrations), the anterior kidney PFC inhibition profiles may appear very similar to splenic profiles, even though in actuality they are not.

Use of a heterologous hapten inhibitor, DNP-acetic acid, more stringently probes those antibodies which recognize TNP as well as DNP-acetic acid and thus can allow a more precise demonstration of heterogeneity within the population of antibodies generated. Hapten analog inhibition reflects the heterogeneity of the PFC population by determining if antibodies are being produced in the anti-TNP PFC population which can also recognize analogs of TNP. Figure 18 suggests that this may be true. Panels A-D are paired for organs from the same animals in complimentary panels of figure 19. In each animal (Figure 18, A-D) similar plots and heterogeneity values are obtained from paired splenic and anterior kidney PFC inhibitions,

however, for the same animal's cells (Figure 19 A-D), DNP-acetic acid produces greater heterogeneity indices and a greater ability to demonstrate higher relative affinity antibody. Therefore, anterior kidney lymphocytes are more restricted to TNP specific antibodies, whereas splenic lymphocytes produce a greater variety of specificities. This is reflected in the presence of high affinity antibodies to a similar hapten.

Mammalian systems have provided examples of more mature B cell populations producing antibody with a higher average affinity. This has been demonstrated in B cells from primary immune organs as compared to B cell populations from secondary organs and within the same B cell populations at different points during ontogenetic development (immature versus mature).

The relative average affinity constant (K_{REL}) was developed to describe the relative affinity that an antibody population, specific for a certain antigen, has for an analog of that antigen (Appendix III). This constant can be derived from a hapten inhibition analysis system to describe the relative average affinity that an antibody population has for a heterologous hapten with respect to its affinity for the homologous hapten, employed to generate the response. Inhibition curves were generated for two heterologous haptens, paranitrophenylacetic acid and 2,4-dinitrophenylacetic acid, and compared to the inhibition generated by the homologous hapten, TNP-LYS. Calculation of K_{REL} values were determined (refer to Appendix III) and shown in Table IV. The splenic PFC population generated <u>in vitro</u> by TNP-LPS shows greater relative average affinity (2 to 5

fold) over anterior kidney (K_{REL} values 2 to 5 times greater). Again, splenic lymphocyte populations demonstrate the ability to generate a more heterogeneous response than the anterior kidney. Relative average affinities of antibody generated from splenocyte cultures are greater than anterior kidney generated antibody affinities for two heterologous hapten molecules with respect to the homologous hapten. Therefore, the spleen likely contains a subpopulation of B cells which demonstrates characteristics of a mature population which is apparently difficult to detect in the anterior kidney B cell population, or is absent altogether (a lack of mature B cells).

The fine specificity of salmonid anti-TNP PFC populations was investigated for both splenic and anterior kidney lymphocytes. The data presented were generated exclusively from splenic in vitro cell cultures, however anterior kidney lymphocyte experiments were done in parallel with the spleen and show equivalent fine specificity. Fine specificity analyses allow for the characterization of the antigen binding site present in the antibody spectrum. This type of immunochemical investigation can lead to a rough model of the antigen binding site by assessing which epitopes of the antigen are required for optimal antibody recognition.

Figure 21 illustrates that 2,4,6-trinitrophenyl-lysine and 2,4-dinitrophenyl-lysine are equivalent inhibitors of the anti-TNP response (structures illustrated in appendix II). Binding affinity is not enhanced when the third nitrite group (added to the benzene ring at the 6 position) is present. Figure 21 leads to the suggestion that TNP generated antibody does not interact with TNP by

engulfing the total hapten molecule.

Examining the simplest analogs of TNP that are recognized by the antibody population demonstrates which features of the haptenic structure are absolutely essential to antibody binding. The unique inhibition curve generated by paranitrophenol (Figure 24) indicates uniform recognition (100% inhibition) by the total spectrum of antibody affinities from 10^{-2} M to a concentration of 2.5×10^{-3} M. The curve sharply drops at the next successive 2 fold dilution of inhibitor (1.2×10^{-3} M) to approximately 23% inhibition suggesting that almost all antibody affinities in this diverse antibody population recognize the features of paranitrophenol with equivalent low affinity. Employing 2,4-dinitrophenol demonstrates a more gradual decline in the inhibition curve compared to paranitrophenol (Figure 24). This indicates the importance of the second nitrite group present on the benzene ring (position 2) for greater antibody binding affinity. Coupling this result with figure 21, suggests two binding regions, associated with the nitrite groups at positions 2 and 4 on the benzene ring, are important for anti-TNP antibody recognition.

The inhibition curve of dinitrophenol compared to DNP-LYS illustrates a greater slope in the decline of its inhibition curve. Dinitrophenol inhibition is shifted somewhat to higher inhibitor concentrations necessary for equivalent inhibition (a reflection of lower affinity of the antibody for 2,4-dinitrophenol) compared to the DNP-LYS curve (Figure 21). The hydroxyl group attached to the benzene ring at position 1, examining 2,4-dinitrophenol, may be

responsible for the antibody population's inability to bind this molecule with the same affinity as 2,4-dinitrophenyl-lysine. The lack of a long hydrocarbon chain at position 1 on the benzene ring which is present in DNP-LYS, but absent from dinitrophenol might also be important in antibody recognition. Eisen (1964) has shown this for murine anti-DNP antibodies. Eisen found that 2,4-DNP-LYS was 38 times more effective an inhibitor of anti-DNP antibody than 2,4-dinitrobenzene. Substituent groups, attached to a TNP or DNP molecule, therefore, also affect antibody binding affinity for the hapten and suggest a third region of antigen antibody interaction in the anti-TNP antibody population.

The lack of antibody affinity demonstrated by the inhibition curve for 2,4-dinitrophenylacetic acid implicates a role for the substituent molecule in antibody recognition of the hapten (Figure 22). Compared to the inhibition curve of 2,4-dinitrophenyl lysine, antibody binds 2,4-dinitrophenylacetic acid with approximately 10 fold lower affinity (structures illustrated in appendix II). The structural reason for this loss of antibody recognition of DNP in the presence of an attached acetic acid substituent was investigated at the molecular level (Figure 23). Steric hinderance, caused by the carboxyl present in 2,4-dinitrophenylacetic acid, of the nitrite group at position 2 of the benzene ring of DNP might account for the inability of antibody to bind with high affinity. Thus, inhibition by 2,4-dinitrophenyl-phenylalanine attempted to mimic the presumed steric hinderance by positioning a bulky phenyl group proximal to the nitrite at position 2 of the benzene ring of DNP (structures illustrated in appendix II). Figure 23 illustrates the DNP-

phenylalanine inhibition curve, which is shown to closely resemble the curve of DNP-LYS. No steric hindrance by the phenylalanine substituent occurs, necessitating an alternate explanation for the lack of antibody affinity for DNP-acetic acid.

Thus far, electronegative substituents proximal to the nitrite group at the 2 position of the benzene ring of DNP have been implicated in low antibody binding affinity in two examples (the hydroxyl of dinitrophenol and carboxyl of DNP-acetic acid). The effects of another carboxyl containing substituent on anti-TNP antibody binding affinity, using 2,4-dinitrophenylbutyric acid, was investigated by PFC inhibition. Comparing the structures of 2,4-dinitrophenylacetic acid and 2,4-dinitrophenylbutyric acid (appendix II) illustrates that the butyrate carboxyl group is removed two methylene groups distal to the benzene ring of DNP, compared to the carboxyl positioning in 2,4-dinitrophenylacetic acid. Examining the resultant inhibition curve for 2,4-dinitrophenylbutyric acid (Figure 23), a curve essentially identical to DNP-LYS is shown. This indicates that removing the carboxyl group 2 methylene groups distal to the DNP substituent (switching from 2,4-dinitrophenylacetic acid to 2,4-dinitrophenylbutyric acid), antibody recognition of DNP is restored to the level observed for DNP-LYS. This also indicates that the proximity of the carboxyl group to the DNP substituent, as in DNP-acetic acid, has deleterious effects on an antibody's ability to bind to DNP.

Considering murine antibodies, using equilibrium dialysis, Eisen (1964) has shown that antibodies specific for the 2,4-dinitrophenyl

molecule can have dramatically lower binding affinity if the 2 nitrite groups are placed together on the benzene ring as in 3,4-dinitrophenyl or 2,3-dinitrophenyl. In the salmonid *in vitro* antibody response to TNP, and subsequent inhibition by DNP-acetic acid, the proximity of the carboxyl group to the 2 position of the nitrite on the DNP ring has been discussed previously. Considering Eisen's work (1964) and the similar three-dimensional structures of a carboxyl and a nitrite group, a loss of affinity of antibody for 2,4-DNP-acetic acid may be expected. The two proximal electron dense clouds may affect antibody binding just as 2,3-dinitrophenyl substituents caused lower binding affinity by antibody than 2,4-DNP substituents.

Summarizing the fine specificity data indicates a number of requirements the antigen must reach to be recognized by TNP-specific antibody. Two nitrite groups are necessary for a high level of antibody recognition, however a third nitrite group cannot augment this level of antibody affinity. For optimal binding affinity, the substituent molecule attached to DNP plays an important role in diminishing antibody affinity if this substituent contains a carboxyl group proximal to the benzene ring of DNP. Finally, a substituent hydroxyl group attached to the DNP ring may have the effect of diminishing antibody affinity and may imply that any electronegative group proximal to the benzene ring at position 1 may cause lowered anti-TNP antibody affinity.

SUMMARY AND CONCLUSIONS

Salmonids are excellent animal models for the study of comparative and developmental immunology. Fish arise at a pivotal point in evolution with respect to the appearence of the immune system. Fish possess the first true immune system (McKinney, 1976). B cell responses were generated *in vivo* and *in vitro* and detected with the PFC assay system. Both *in vivo* and *in vitro* produced PFCs from the anterior kidney were demonstrated to be functionally immature, however splenic responses were shown to be functionally mature. These functional characterizations corroborate histological data describing salmonid B cell populations (Zapata, 1979; Yasutake, 1983).

Fine specificity analyses described the *in vitro* generated, anti-TNP antibody. These characterizations indicate an anti-TNP binding site which possess comparable affinity for DNP-lysine and TNP-lysine. The simplest analog of TNP possessing the ability to reveal heterogeneity in the antibody population was dinitrophenol. Electronegative substituents proximal to the hapten molecule could exert substantial hindrance of antibody to bind the haptenic determinant.

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APPENDICES

APPENDIX I

Analysis of heterogeneity by graded antigen inhibition

Sequential inhibition of plaque forming B cells can be plotted in a histogram form of the data. Each individual histogram bar represents the amount of inhibition of the PFCs that a particular antigen concentration can produce. The histogram plot generates what is referred to as an inhibition profile of a particular antibody population. Histogram bars resulting from the lowest antigen concentrations represent PFCs producing the highest affinity antibody, this is due to the fact that high affinity antibody can best compete for lower concentrations of free antigen inhibitor. Conversely, histogram bars occuring at high free antigen concentrations represent the inhibition of low and high affinity antibody producing PFCs. Additionally, in the analysis of each lymphocyte population each histogram plot becomes a representation of the relative heterogeneity, since the variation in inhibition is due to the heterogeneous distribution of antibody affinities within a population of PFCs. In short, the inhibition profile is a measure of the ability to produce high and low affinity antibody by the PFCs, and of the heterogeneity of a particular PFC response (Davie, 1972; Claffin 1973; Goidl, 1974).

Andersson (1970) developed and employed the PFC inhibition analysis to analyze PFC populations from different animals. Quantitative results were obtained using the PFC inhibition analysis where the individual B cells responsible for producing high and low

affinity antibody could be enumerated. Since individual B cells produce monoclonal antibody, the PFC assay is a measure of the polyclonality of a response.

A mathematical approach to describe heterogeneity has been used to define what may be empirically apparent in the inhibition profiles. Goidl (1974) used the Shannon heterogeneity index (formula shown in figure 6) as a measure of the heterogeneity in murine B cell populations. The Shannon heterogeneity index, when calculated for inhibition profiles from salmonid lymphocyte populations, was an inappropriate statistical tool to measure differences in antibody heterogeneity. The evaluation of heterogeneity by the measurement of variance (S^2) is preferred to the Shannon heterogeneity index (Goidl, 1974) due to the ordered distribution of the categories measured in this study. The Shannon heterogeneity index is a measure of dispersion or diversity of unordered categories (Zar, 1984), and thus may not always accurately reflect the degree of heterogeneity revealed by PFC inhibition studies.

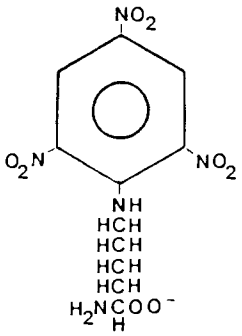
An example of shortcomings of the Shannon method is provided in Figure 6. Figures 6A and B show that when plots are extremely different; one showing maximal heterogeneity (Figure 6A) and one lacking heterogeneity (Figure 6B), the Shannon heterogeneity index is able to demonstrate heterogeneity differences between the plots. A higher index indicates greater heterogeneity. However, in Figures 6C and 6D, data was depicted with histogram bars of similar magnitude ordered about a central mean in one plot, but in a second plot histogram bars were scattered randomly. The Shannon heterogeneity

index demonstrated the identical heterogeneity indices for these obviously different plots (H = 0.64). However, when the variance is calculated for these two plots, intuitive heterogeneity differences between plot 6C and D are only discernable by this alternative statistical description for the heterogeneity index. The variance is 0.78 for figure 6C and 0.27 for figure 6D.

APPENDIX II

Hapten Inhibitors

TNP - LYSINE



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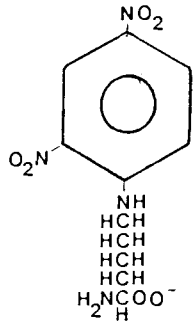

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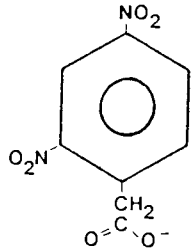
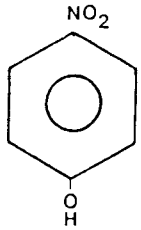
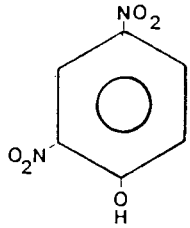


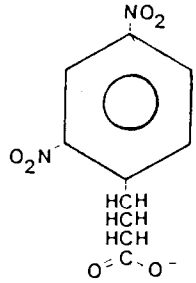
PARANITROPHENOL



DINITROPHENOL



DNP-BUTYRIC ACID



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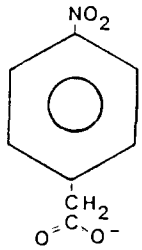
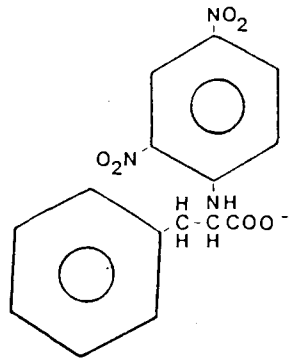


DINITROPHENYLALANINE



APPENDIX III

Relative Affinity Constant Calculation

The relative average affinity constant, or K_{REL} value is a measure of the relative average affinity an antibody population has for a heterologous hapten molecule with respect to the homologous hapten. Using serum antibody and equilibrium dialysis employing homologous and heterologous haptens, Pressman determined K_{REL} values using analogs of a particular hapten and repeated these studies for a number of different haptenic systems. This form of fine specificity analysis allows one to devise structural models of the crucial molecular features of the representative antibody binding site for a particular hapten.

The PFC inhibition analysis system lends itself particularly well to the determination of relative average affinity constants. From an inhibition curve of PFCs, the hapten concentration at which 50% of the PFCs in a population are inhibited is defined as the average affinity of that PFC population for that particular hapten. Therefore from inhibition curves of PFCs, average affinities can be calculated. A ratio between two average affinities of related hapten analogs can be made and this ratio is the average affinity an antibody population has for a particular hapten relative to the second hapten. The formula of K_{REL} calculation from PFC inhibition curves is shown in figure 20. A sample K_{REL} calculation is provided (Figure 20) using data from splenic PFC inhibition curves for TNP-LYS and 4-nitrophenylacetic acid. The concentration of homologous hapten

(TNP-LYS) necessary for 50% inhibition is estimated to be 6.1×10^{-4} M. The concentration of 4-nitrophenylacetic acid necessary for 50% inhibition of PFCs is 7.8×10^{-3} M. Thus the average affinity of antibody produced in this population of PFCs for 4-nitrophenylacetic acid with respect to TNP-LYS is 0.078. Alternatively the affinity of these antibodies for 4-nitrophenylacetic acid is .078 of that seen for TNP-LYS.

APPENDIX IV

Buffers and Bacterial Growth Medium

Cacodylate Buffer

Cacodylate buffer (0.28M) was prepared as described by Rittenberg and Amkraut (1966). Cacodylic acid (38.2 g/l) was dissolved in distilled water (1 l) at room temperature with constant stirring. Nine pellets of anhydrous sodium hydroxide (approximately 0.9 g) were dissolved with stirring and the pH was adjusted to 7.0 by dropwise titration of HCl (2N). Cacodylate buffer was filter sterilized (0.45 um) and stored at 4^{o}c.

Modified Barbital Buffer (MBB)

MBB (5X) was prepared by dissolving 1 vial (0.05 moles Sodium Barbital, 0.01 moles Barbital) Barbital Buffer (Sigma, St. Louis, MO.) in one liter of distilled water at room temperature with constant stirring. The buffer solution was then supplemented with anhydrous calcium chloride (0.083 g/l), magnesium chloride (0.508 g/l) and sodium chloride (42.5 g/l). The 5 X stock was filter sterilized (0.45 um) and stored at 4^{o}c. The MBB stock solution was diluted to 1X with cold (4^{o}c) saline (0.85%) and the pH adjusted to 7.4. Fresh, (1X) buffer solutions were prepared weekly.

Phosphate Buffered Saline

Phosphate buffered saline (PBS) was prepared by dissolving monobasic potassium phosphate, KH_2PO_4 (1.0 g/l) and dibasic sodium phosphate·7 hydrate, $Na_2HPO_4 \cdot 7H_2O$ (17.8 g/l) in one liter of distilled water. To this solution sodium chloride NaCl (8.5 g/l) were added. The pH of the resulting solution was reduced to 7.4 with hydrochloric acid, HCl (1 N). A 0.7 M solution resulted.

Kidney Disease Medium II

Kidney disease medium II (KDM II) was prepared by dissolving peptone (10 g/l), cysteine (1 g/l) and yeast extract (0.5 g/l) in distilled water. The pH of the resulting solution was raised to 6.5. This solution was autoclaved to sterility in a stoppered flask and cooled before bacterial inoculation.