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Stephen L. Kaattari

This study was undertaken to develop a solid phase, enzyme based immunoassay to characterize affinity maturation and antibody affinity heterogeneity in rainbow trout (*Oncorhynchus mykiss*). The few existing reports examining affinity maturation in rainbow trout failed to show changes in antibody affinity during an immune response. A competitive ELISA was established in which the binding of serum or (leukocyte) culture supernatant antibodies to serially decreasing, plate-bound, multi-haptenated antigen is inhibited by graded doses of a free hapten inhibitor. The assay yields an estimate of the distribution of affinities within polyclonal mixtures of antibodies, as well as estimates of the apparent affinity constants (aK) for each antibody subpopulation. In addition, a method for the calculation of an average affinity (K) from affinity subpopulation data is described. The assay was used to characterize increases in average affinity during the primary and secondary immune response to both a T-dependent form of the trinitrophenol hapten (TNP-KLH) and a T-independent form (TNP-LPS) in trout. Significant increases in affinity were documented as early as three weeks post immunization in these animals. The sensitivity of the assay allowed detection of small differences in average antibody affinity produced

against a T-dependent antigen in populations of rainbow trout embryonically exposed to 1 ppm aflatoxin B_1 versus controls. The ELISA was also adapted for use in measuring leukocyte culture antibody affinities. Armed with this tool, it became possible to demonstrate antigen specific stimulation of high affinity B cell clones *in vitro*, suggesting that the mechanism of affinity maturation *in vivo* in rainbow trout may be similar to that previously described in mammals. Finally, murine IgM and IgG anti-TNP antibodies were characterized during the first three weeks of an immune response to TNP-KLH.

Development of a Novel Affinity ELISA and Its Application to the Analysis of Affinity Maturation in Trout

by

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David A. Shapiro, Author

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DEDICATION

This manuscript is dedicated to my family, whose friendship and support has been unconditional.

Development of a Novel Affinity ELISA and Its Application to the Analysis of Affinity Maturation in Trout

INTRODUCTION

The development of mammalian antibody affinity is a highly complex and regulated phenomenon. Most of our knowledge of the regulation of affinity generation in the mammalian immune response has come from studies performed within the last twenty years. During an antibody response, the acquisition of affinity is a dynamic process wherein the antigen may selectively induce proliferation of high affinity B cells leading to affinity maturation, an increase in the average affinity of antibodies over time (Paul et al., 1967). Alternatively, cellular induction by antigen can lead to somatic mutation (Kochs and Rajewsky, 1989), which in turn leads to increases in antibody affinity.

One measure of antibody affinity is termed "intrinsic affinity. This is a measure of the binding constant of an isolated antigen combining site with a univalent antigen. Alternatively, antibody avidity takes into account the stabilizing and enhancing effects of multivalency on antigen binding. It is not uncommon for an IgG molecule to bind a multivalent antigen a thousand-fold more tightly when both binding sites engaged than if only one binding site were occupied (Feibig, 1977; Hornick and Karush, 1972). Similarly, pentameric mammalian IgM, with 10 functional binding sites, may bind an antigen with much greater avidity, even though each binding site may bind

with less strength than an analogous IgG binding site. The binding constants and affinities of these interactions are difficult to define, as the binding of one site stabilizes the complex, affecting the binding of all other sites.

Antibody affinity has been shown to correlate well with disease prognosis, making its measure a useful tool for diagnosing active infections (Polanec, 1994; Suenaga, 1993). Antibody affinity has also been shown in some cases to correlate with survival of pathogenic insult (Mulcahy et al., 1992).Classical affinity measurement techniques, such as equilibrium dialysis (Eisen and Karush, 1949) and fluorescence quenching (Eisen and Siskind, 1964) are inadequate for the study of heterogeneous mixtures of antibodies (such as serum) because they both yield only a single, statistical estimate of antibody affinity termed the average affinity constant (K_o), obscuring the contributions of different antibody subpopulations to the average. Also, the requirement for relatively large quantities of purified antibodies is incompatible with repeated sampling of blood from small animals.

LITERATURE REVIEW

The Generation of Antibody Binding Site Diversity

Four elements and/or features of the mammalian immune system are responsible for the generation of antibody binding site diversity, namely 1) the large number of variable region (V) genes in the germline, 2) the random fusion of V region-encoding gene elements, 3) the random assortment of H and L chains, and 4) somatic mutation of sites within mature, recombined variable region genes.

In all mammals studied to date, distinct immunoglobulin heavy and light chain loci have been identified. The heavy chain genes are arranged in clusters of V (variable), D (diversity) and J (joining) segments. Both V-D and D-J rearrangements provide junctional diversity leading to increased variability in the antibody repertoire. Initially, a D-J recombination occurs. This product subsequently combines with a V_H gene segment (Alt et al., 1984) Significant variability is provided both by imprecise joining with the diversity segment and any of a large assortment (hundreds) of V_H gene segments. Once assembled, VDJ segments combine with a C region gene (Dreyer and Bennett, 1965). The constant region genes are invariant, and are arranged in a defined linear series 3′ to V, D, and J clusters (Shimizu et al., 1982). The constant region defines an antibody's isotype, specifying its function (e.g., complement fixation, transport to mucus, recognition by F_c receptors, etc.).

Light chain genes are arranged in clusters of V and J segments. Rearrangement of a V region with a J segment, and subsequent combination with a constant region gene forms a functional light chain gene. Though light chain loci do not possess diversity segments (Schilling et al., 1980) diversity is again provided by junctional diversity as described for heavy chain gene rearrangement.

Somatic hypermutation of the rearranged V genes provides nearly a limitless source of variability (Griffiths et al., 1984). Further antibody diversity is generated by the random assortment of any H chain with any L chain (Tonegawa, 1983).

Primary immunization of an animal with a protein antigen (immunogen) stimulates B-cells to secrete antibodies, initially of the IgM (μ) class. These antibodies are heterogeneous with respect to affinity (Eisen and Siskind, 1964). Further, subsequent immunizations (boosts) with the same antigen typically result in the production of serum antibodies of increased affinity (and specificity) for the inducing antigen (Eisen and Siskind, 1964; Siskind et al., 1968). These antibodies are primarily of the IgG (γ) class. The switch to producing IgG from IgM antibodies is called class, or isotype switching, while the increase in affinity is known as affinity maturation. Both of these phenomena are considered to be quintessential characteristics of the mammalian "memory" response. Affinity maturation allows an animal

to "fine tune" it's response to yield antibodies which bind with maximum efficiency.

Initially, affinity maturation occurs when B-cells selectively bind antigen based on the affinity of their cell surface receptors. Antigen-bound T-cells then elaborate soluble factors which induce clonal expansion of antigen stimulated B-cells into antibody secreting plasma cells. Further, this interaction also induces the maturation of some of the B-cells to long-lived, higher affinity memory cells. Upon secondary stimulation, these memory cells rapidly proliferate, effectively raising the average affinity (MacLennan and Gray, 1986).

Continued stimulation by antigen, accompanied by T-cell elaborated cytokines causes a switch in the isotype of antigen-specific antibodies. Using the same set of V, D, and J genes, DNA encoding one constant region (e.g., μ) is spliced out and another C gene (e.g., γ) is spliced in, thus effecting an IgM to IgG switch. In mice, this happens within the first month of antigen stimulation. As IgM is a pentamer, while IgG is a monomer, it would seem that a net loss in avidity would be suffered by the switch to IgG. This does not appear to be the case. Induction of somatic mutation in γ^+ (surface IgG bearing) B-cells can produce receptors and antibodies of higher affinity. These high affinity receptors provide for preferential antigen binding and, thus, stimulation. Therefore, the overall affinity of the serum IgG antibodies increases. The magnitude of this affinity increase in mice has been observed

to be greater than four orders of magnitude (Hornick and Karush, 1972). Such an increase in binding site affinity can make up for the loss in avidity due to the shift from a pentameric to monomeric form. Using the techniques described herein, the phenomenon of affinity maturation has now been observed in fish, though the magnitude of the shift is significantly lower than that seen in mammals. As there is only one expressed isotype in rainbow trout, isotype switching has not been observed (reviewed in Warr, 1995). A few teleosti have been reported to express more than one isotype (Håvarstein, et al., 1988; Lobb and Clem, 1983), but in these animals, no isotype switching has yet been observed.

Factors Influencing Affinity Maturation

It is now widely accepted that antigen driven selection of B-cell clones is responsible for affinity maturation, at least in mammals (MacLennan and Gray, 1986). As antigen levels decrease, cells with high affinity receptors compete more effectively than cells with low affinity receptors for available antigen. These cells are selectively stimulated to proliferate. Thus, even in the absence of somatic mutation, selection of high affinity clones raises the average affinity of the antibody population as the concentration of antigen diminishes over time.

Somatic mutation provides an even wider repertoire from which to select high affinity clones. The induction of somatic mutation minimally

requires: 1) B-cell stimulation by antigen, 2) the infrastructure of germinal centers (Jacob et al., 1991), and 3) the presence of helper T cells in the surrounding lymphoid sheath (Gershon and Paul, 1971).

Germinal centers are areas of extensive B-cell proliferation within the follicles of secondary lymphoid organs. Follicular dendritic cells are antigen presenting cells, located near germinal centers. These cells have the capacity to take up antigen and present it on their surface in an undegraded form for years (Tew et al., 1990). Germinal centers were first suspected of being microenvironments in which mutation was taking place because secondary, or memory B-cells were known to reside there (Coico et al., 1983).

Somatic mutation is a tightly controlled process that can begins three days after immunization (Levy et al., 1989) and continues for approximately the first two weeks after immunization. High mutation rates cease near the end of the primary response (Shlomchik et al., 1987; Claflin et al., 1987). Further mutations have been reported in memory B-cells during secondary immunizations (Berek and Milstein, 1988), though it is unclear how these cells re-initiate the process.

Antibody gene mutation rates under antigen stimulation have been shown to reach levels of 10^{-3} per base pair per generation (McKean et al., 1984) in a viral antigen model system, though lower mutation rates were reported for an Abelson virus-transformed pre-B-cell line (approx. 10^{-5} per base pair per generation; Wabl et al., 1985). Computer model calculations of mutant

distributions of proliferating B-cell clones (Allen et al., 1987) suggest that mutation rates in excess of 1.5×10^{-3} per base pair per generation prevent the expansion of functional cells altogether, while rates significantly lower than 1.5×10^{-5} per base pair per generation would require strong selection to allow them to become the dominant fraction; unselectable mutations would have little chance to appear in these mutant populations.

Time and dose dependency play important roles in affinity maturation, though the extent to which these factors affect production of IgG and IgM antibodies has been controversial. Early affinity maturation studies clearly showed maturation of IgG affinity after immunization (Andersson, 1970; Eisen and Siskind, 1964), but reports citing affinity maturation of IgM antibodies were conflicting, both at the cellular level (Claflin and Merchant, 1972; Huchet and Feldmann, 1973) and by serum antibodies (Kimball, 1972; Sarvas and Mäkelä, 1970). One study, using equilibrium dialysis (Neri et al., 1978), reported that IgM affinity increased with time after immunization, with maturation rates increasing with increasing antigen dose.

The absolute need for T-cell help in the generation of higher affinity has been brought into question due to the work with certain T-independent antigens, such as NP-ficoll (Maizels, 1988) and dextran B512 (Fernandez and Möller, 1991). These unique antigens were shown to promote the secretion of IgG antibodies late in the response with affinities much higher than IgM affinities measured early in the response. Though the participation of T-cell

help in the induction of specific antibodies by T-dependent antigens is unquestionable, these studies indicate that T-cell help is not an absolute requirement for isotype switching events or somatic hypermutation. Fernandez and Möller suggested that the induction of high affinity memory B-cells to these antigens could be simply a consequence of the process of antigen stimulation, irrespective of T-cell help. T-cell cytokines may only be necessary to stimulate the proliferation of memory B-cell clones by classical T-dependent antigens (Fernandez and Möller, 1991).

Immunological competence is known to vary with age and affect the development of high affinity clones. Studies indicate that the ability to form high affinity antibodies increases with age from low capacity in the neonate to a maximum in adulthood, decreasing in old age, probably due to significant changes in populations of helper and suppressor T-cells (Doria et al., 1978; Marshall-Clarke and Playfair, 1975). Older mice develop a deficiency in high affinity antibody-producing cells and overall level of IgG. It has been noted that spleen cells from aged mice could also suppress high affinity cells from young mice (Goidl et al., 1976). It has also been demonstrated that suppressor cells (T_s) selectively suppress high affinity B-cells (Takemori and Tada, 1974). The mechanism(s) for the selective suppression of high affinity B-cell clones has not yet been elucidated, but may be due to interactions of antigen-specific T_s receptors with greater amounts of antigen bound by high affinity B-cells, or

to a physiological state of heightened susceptibility due to the degree of antigenic stimulation.

Persistent soluble antigens, such as those seen during chronic malarial parasite infections, have been shown to inhibit affinity maturation and memory cell generation in germinal centers (but not in extrafollicular pathways; Pulendran et al., 1995). Pulendran suggests that inhibition of both germinal center formation and localized affinity maturation due to an unlimited supply of soluble antigen may be due to increased susceptibility to apoptosis within germinal centers (for, as yet, undefined reasons). This was a mechanism of self tolerance proposed by Linton et al. (1991) intended to explain how emergent anti-self mutant clones may escape early tolerance generation. A similar finding was reported for trypanosome infections, in which large amounts of coat proteins are shed into the blood of hosts (Cross, 1990).

Rainbow Trout Immunoglobulins and the Genes Encoding Them

Rainbow trout heavy chain immunoglobulin gene arrangements are similar to their mammalian counterparts. Clusters of V segments are followed by D and J clusters, with one or more C genes. The heavy chain variable gene locus of rainbow trout is believed to possess at least nine V_H gene families, likely encoding more than 100 V_H genes (Roman and Charlemagne, 1994; Matsunaga et al., 1990). No D_H or J_H sequences have been

cloned or analyzed, but their multi-copy presence is currently accepted (Roman and Charlemagne, 1994; Bengtén et al., 1994). Only one constant region segment has been identified (Hansen et al., 1994; Lee et al., 1993; Anderson et al., 1993; Hordvik et al., 1992). Thus, to date, only one isotype has been defined for rainbow trout. This isotype has been called IgM, based on superficial structural and physicochemical similarities to multimeric mammalian IgM. Light chain gene arrangements are less clear, but have been reported to exist in a multicluster V, J, C arrangement. At least two V_L families have been reported in rainbow trout (Daggfeldt et al., 1993). Regulation of the expression of these loci to avoid simultaneous expression of multiple genes has yet to be defined, but has been shown to involve 5' upstream regulatory promoters which share some sequence similarities to their mammalian counterparts (reviewed in Staudt and Lenardo, 1991). Several enhancers have also been identified (Lee et al., 1993; Ghaffari and Lobb, 1992).

Rainbow trout produce both a membrane receptor form of antibody (Hansen et al., 1994) and a secreted antibody superficially resembling mammalian IgM (Wilson et al., 1992). Four bivalent monomers, each of approximately 190 kdal, are linked to form a functional tetrameric antibody product (O'Leary, 1981). The monomer has been shown to consist of two identical light chains (approx. 26 kD) and two identical heavy chains (approx. 72 kD), linked via disulfide bridges.

Early data suggested that tetrameric IgM was the only immunoglobulin present in some fish species (Acton et al., 1971). However, Lobb and Clem (1988) defined at least three antigenically distinct isotypes (and probably a fourth unidentified isotype) in catfish using monoclonal antibodies. Only one isotype, however, has thus far been identified in rainbow trout (Wilson et al., 1992).

Various reports using classical affinity measurements place the binding constants for salmonid antibodies at around 1-2 x 10^5 M^{-1} (O'Leary, 1981; Voss et al., 1978). While these values are not uncommon for antibodies of the mammalian IgM class, they are significantly lower than the affinity constants associated with IgG antibodies ($K_{eq} \leq 10^{11}$). To date, affinity shifts during the course of an immune response have not been reported in rainbow trout (O'Leary, 1981; Arkoosh 1989).

Antibody Affinity at the Molecular Level

Antibody affinity is a measure of the binding capacity of an antigen binding site. This measure reflects the goodness of fit between the antibody binding site and the homologous epitope. The strength of this interaction is dependent on the sum of the weak (non-covalent) interactive forces between amino acid residues in the antibody binding site and the epitope on the antigen molecule. These forces include electrostatic interactions, hydrogen bonding, and van der Waals interactions. Additionally, abrogation of

hydrogen and ionic bond competition by exclusion of water molecules when there is a good fit serves to strengthen these tight binding interactions. Table I summarizes the attractive forces between antigen and antibody combining site described below.

Hydrogen bonding results from the interaction of good H-bond donors and acceptors in close proximity. Though standard free energies of formation of hydrogen bond are around -2 to -5 kcal mole^{-1}, the sum total of the gain in stability brought about by the formation of hydrogen bonds within the binding structure and the breaking of hydrogen bonds with the solvent (water), is usually on the order of -1 kcal/mole per bond.

Hydrophobic interactions result from the interaction of non-polar groups in an aqueous environment. These moieties (such as the non-polar amino acids leucine, isoleucine, valine, and phenylalanine) find it energetically more favorable to interact with similar residues. Acquisition of a lower energy state and a gain in entropy is achieved by the exclusion of water, resulting in a net attractive force.

Electrostatic (ionic or coulombic) interactions either attract (opposite charges) or repel (like charges) charged groups in close proximity. These interactions are highly dependent on pH, as amino acids take on different charged (or uncharged) characteristics based on the pH of their surroundings. It is thought that these are not dominant forces in the stabilization of antibody-antigen complexes (Karush, 1962).

Table I. Major forces of attraction between antigen and antibody in the antibody combining site.

	Strength (kcal)*	Relationship between force and distance (d)
Electrostatic	5 - 10	$1/d^2$
Hydrogen	2 - 5	$1/d^2$
Hydrophobic	1 - 5	$1/d^7$
Van der Waals	0.5	$1/d^7$

* Compare with covalent bonds: strength of interaction = 40 - 140 kcal

From Devey, M.E. (1986).

Van der Waals interactions may be thought of as weak interactions between the oscillating dipoles produced by polar electron clouds. Every atom has a van der Waals radius defining its sphere of interaction. The sum total of these spheres defines the van der Waals "outline" of the molecule. These forces, are thought to play a major role in determining antibody affinity (Pressman, 1973).

Strong electron cloud interactions (steric repulsion), though not responsible for the type of attractive force defined above, are included here for completeness. Steric factors are extremely sensitive (proportional to the twelfth power of distance) and form the basis for antibody discrimination, or specificity. The sum of these weak attractive forces acting within a combining site yield optimal affinity only if the electron clouds between antibody and antigen are complementary (or at least "compatible").

Each of the interactions mentioned above can contribute, at most, a fraction of a kcal per mole (a few kJoules per mole) to the stability of the complex, but the sum total of the interactions may have enormous effects on the measured affinity of the interaction.

The contributions of many of these forces to antibody-antigen binding have been studied in a few model systems including, but not limited to, the p-azobenzoate group (Nisonoff, 1959) and the 2,4-dinitrophenyl group (Eisen, 1966; Eisen and Siskind, 1964; Eisen, 1962; Carsten and Eisen, 1955). The 2,4-

dinitrophenyl group is structurally very similar to the 2,4,6-trinitrophenyl group used in all of the experiments in this thesis.

In a series of fluorescence quenching investigations, Eisen determined the effects of different molecular substitutions on relative binding affinities of antibodies produced to 2,4-dinitrophenylated protein. The existence of a strict requirement for both nitro groups for good combining with antibody was evidenced by a decreased relative affinity (K_{rel}) upon binding of nitroanilines containing only one nitro group. Lysine ε-amino group participation in protein conjugation was suggested by large K_{rel} values for ε-DNP-L-lysine conjugate binding. Finally, a slightly reduced K_{rel} for binding of the D-amino acid conjugate suggested specificity for the L form.

In another experiment, the importance of non-polar or van der Waals interactions in determining binding force was revealed by the fact that antibodies with relatively high binding constants bound ε-DNP-L-lysine with 10-fold greater affinity than 2,4-dinitroaniline (Eisen and Siskind, 1964). Low binding constant antibodies bound both haptens with only a two-fold difference in relative affinity. Experiments such as these in which binding site specificity was determined by antigen substitution artfully described the forces involved with antibody-antigen binding at the molecular level.

The Relationship Between Free Energy and Equilibrium Constants

There is a free energy of combination associated with the binding of a antigen with a combining site. This free energy is related to the tightness of bonding of any two molecules (at equilibrium) by the thermodynamic equation:

$$\Delta G^{o} = -RT \ln K_{eq} \quad \text{(Equation 1)}$$

where R is the gas constant, T is the (absolute) temperature and ln K_{eq} is the natural logarithm of the equilibrium constant. Thus, it can be seen that large equilibrium constants correlate with large negative free energy values. To understand why large negative free energies are energetically favorable, one need only consider the thermodynamic equation:

$$\Delta G = \Delta H - T\Delta S \quad \text{(Equation 2)}$$

As the free energy of a reaction decreases, some combination of a negative change in enthalpy (ΔH, a measure of the change in heat content of a system) or positive change in entropy (ΔS, the state of randomness or disorder of a system) takes place, both thermodynamically favorable conditions. Similarly, any conformational change or bond formation affects ΔH and ΔS to yield a net negative (favorable) or net positive (unfavorable) free energy change which, according to Equation 1 may be translated into a change in binding affinity. Based on the logarithmic relationship between K_{eq} and ΔG (Equation 1), and knowing R and T, one can estimate that at 25°C, a tenfold increase in affinity

corresponds to a free energy change of -1.36 kcal/mole. Thus, a very high affinity, 10^{10}, for example, corresponds to a free energy change of -13.6 kcal/mole. This is slightly more than the free energy change associated with three hydrogen bonds (this refers to three theoretical bond energies, not the net energy yield from the formation of an average hydrogen bond within an antibody combining site, -1 kcal/mole). The sum of all the relatively small free energy changes from each of the acting forces results in the considerable difference between a high affinity and a low affinity antibody. Thus, a minute change in the structure of an epitope or the antibody binding site can lead to a change in the binding energies supplied by the above mentioned forces which, in turn, can have dramatic effects on binding affinity.

Empirical Determination of Affinity Constants

It is possible to measure the entropy, enthalpy, and free energy changes associated with substrate-ligand binding reactions, and thus, calculate binding constants (Mukkur, 1980). However, more typically affinity constant determinations have involved measurement of bound and free substrate and ligand concentrations directly, calculating binding constants using the Law of Mass Action (Equation 5). A creative manipulation of this form was first used by Scatchard to determine binding constants (Equation 9). Affinity constants (also known as intrinsic association constants, K_a or K_{aff}) between

antibody and hapten are quantified by measuring the concentrations of reactants and products in the reaction:

$$Ab + H \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AbH \qquad \text{(Equation 3)}$$

$$\text{where, } \frac{k_1}{k_{-1}} = K_{eq} \qquad \text{(Equation 4)}$$

$$\text{Thus, } K_{eq} = \frac{[AbH]}{[Ab][H]} \qquad \text{(Equation 5)}$$

If reactants and products are expressed in moles•liter^{-1}, *Keq* will be expressed in liters•mol^{-1}. The stronger the bond between antibody [Ab] and hapten [H], the more antibody-hapten product [AbH] will be present at equilibrium, and the greater the value of *Keq*.

Experimentally, there are a variety of ways to determine *Keq*. Generally, a series of reactions employing a constant antibody concentration while varying (labeled) antigen concentration are permitted to reach equilibrium. Bound and free antigen are measured either by determining the concentrations of bound and free antigen in separate compartments (equilibrium dialysis) or by separating bound antigen from free antigen by precipitation of antibody (Stupp et al., 1969; Goidl et al., 1968). Alternatively, the antigen concentration can be held constant while varying the concentration of antibody; this is particularly useful if antibody concentration is unknown.

Equilibrium Dialysis

In the past, equilibrium dialysis was extensively utilized in measuring equilibrium constants (Eisen and Karush, 1949; Pinckard, 1978). Its primary disadvantages, besides being the most labor intensive procedure are that it requires relatively large quantities of purified antibody and a source of membrane permeable, radiolabeled antigen. In addition to these shortcomings, the procedure is incapable of resolving the affinity distributions within a heterogeneous population of antibodies, as is found within sera. An important feature of equilibrium dialysis, however is that measurements of bound and free antigen can be made without perturbing the equilibrium.

In equilibrium dialysis, radiolabeled antigen is allowed to equilibrate between two chambers, only one of which contains antibody, separated by a semipermeable membrane impermeable to antibody. After a suitable incubation, the concentration of free radiolabeled antigen, [H], is measured. The concentration of bound hapten (equal to the concentration of bound antibody) may be determined by:

$$[AbH] = [H]_t - [H]$$

Once bound and free ligand concentrations have been determined, affinity constants may be calculated by either of the two methods described below (Karush, 1962).

The law of mass action (Equation 5) may be rearranged as follows:

$[AbH] = K_{eq}[Ab][H]$ (Equation 6)

where $[Ab]=[Ab]_t-[AbH]$. Thus,

$[AbH] = K_{eq}([Ab]_t-[AbH])[H]$ (Equation 7)

This may be rearranged to:

$$\frac{[AbH]}{[H]} = K_{eq}([Ab]_t - [AbH])$$ (Equation 8)

or

$$\frac{B}{F} = K_{eq}([Ab]_t - B)$$ (Equation 9)

where B = bound hapten and F = free hapten.

The resulting equation is the form used by Scatchard (1949) to analyze the binding of proteins to small molecules.

The important assumption in deriving this equation is that B, the concentration of bound hapten, is equal to [AbH] the concentration of bound antibody sites. This is true when the ligand behaves as a monovalent antigen, such as a hapten, since every bound hapten will occupy one and only one antigen binding site. This is a poor assumption when the ligand is multivalent (with multiple antibody binding sites). In fact, it has been determined that antibody may bind multivalent antigen with an overall affinity that is a few orders of magnitude greater than that observed with

monovalent antigen. Thus, intrinsic antibody affinity measurements are usually made with, and relate to binding of monovalent hapten.

Since K_{eq} is a constant, and $[Ab]_t$ is known, the equation may be rewritten in the form of a line with slope $-K_{eq}$:

$$\frac{B}{F} = -K_{eq}B + K_{eq}[Ab]_t \quad \text{(Equation 10)}$$

Two sample Scatchard plots are shown in Figure 1. The first (1A) is a hypothetical monoclonal antibody binding curve, and the second (1B) is a hypothetical serum binding curve. The straight line in 1A indicates that the monoclonal antibody has one type of binding site (i.e., one affinity). The curve in 1B indicates the heterogeneity of binding site affinities within the pool of serum antibodies. It is convenient to think of sera as composed of many monoclonal products of varying affinities. Thus, the steeper part of the curve corresponds to antibodies of higher affinity and the shallower part of the curve corresponds to antibodies of lower affinity. Graphical and mathematical (computer) models have been developed to analyze these relatively complex curves in an attempt to resolve the various contributions of the constituent antibody subpopulations (Rodbard et al., 1980; Thakur et al., 1980; Munson et al., 1980).

When measurements of bound and free antigen are made while varying the concentration of antibody in the reaction mixture (or while using variable dilutions of antibody of unknown concentration), one must first

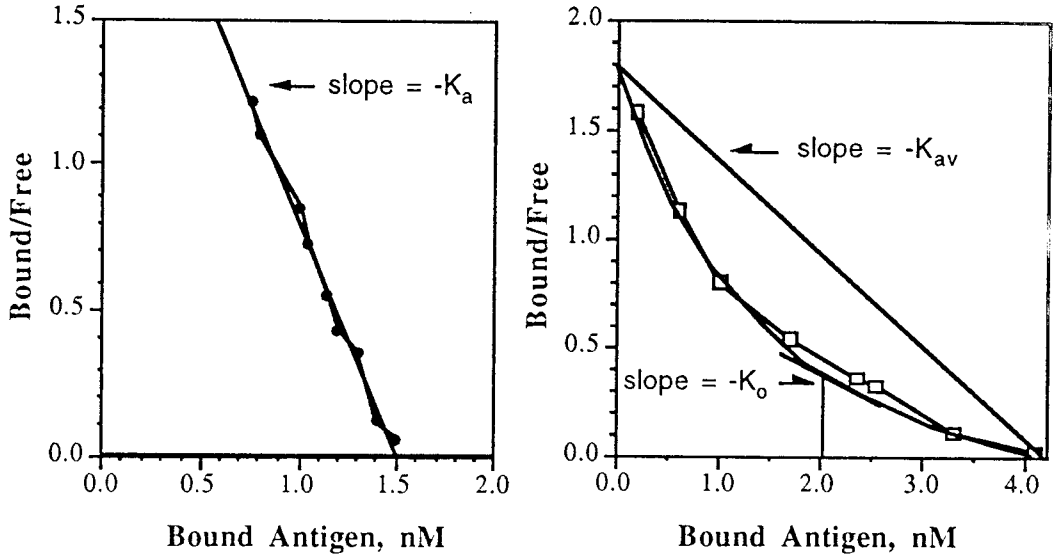


Figure 1. Scatchard analysis of the binding of antigen to monoclonal and polyclonal antibodies. The binding curves for the binding of monoclonal antibody (1A) or serum antibodies (1B) to radiolabeled antigen are shown. The monoclonal antibody (-•-) produces a linear binding curve whose slope is equal to $-K_a$ and whose intercept on the abscissa yields the concentration of antigen binding sites. Serum antibodies (-□-) produce a curved Scatchard plot. Two popular methods of determining average affinity for serum antibody populations include the determination of K_o, the median affinity and K_{av} which corresponds to a weighted average of the affinities, weighted by the concentrations of the antibodies with each affinity. K_o is the slope of the tangent to the curve at a point where bound antigen equals one half the concentration of antigen binding sites (x-intercept). K_{av} is the slope of the chord between the intercepts (adapted from Berzofsky, et al., 1980).

normalize the Scatchard equation for antibody concentration. If no assumptions are made about antibody valency (n), Equation 9 is rewritten as:

$$\frac{B}{F} = K_{eq}([S]_t - B) \quad \text{(Equation 11)}$$

where $[S]_t$ is the total number of binding sites, equal to twice the antibody concentration for monomeric IgG, and 10 times the antibody concentration for pentameric mammalian IgM. To normalize for antibody concentration, equation 11 is divided by total antibody concentration $[Ab]_t$, and the following is obtained:

$$\frac{B}{F} \times \frac{1}{[Ab]_t} = K_{eq}([S]_t - B) \times \frac{1}{[Ab]_t} \quad \text{(Equation 12)}$$

Rearranging,

$$\frac{r}{F} = K_{eq}(n - r) \quad \text{(Equation 13)}$$

where, $r = \frac{B}{[Ab]_t}$ and $n = \text{binding sites / Ab} = \frac{[S]_t}{[Ab]_t}$

A plot of $\frac{r}{F}$ vs r yields a straight line, again with slope $-K_{eq}$, but an x-intercept of n. As with the previous form of analysis, Scatchard plots produced by a mixture of more than one antibody type (serum, for example) yield curves due to the multiplicity of affinities. Average affinities and heterogeneity

indices are usually calculated for these complex mixtures, and the reader is referred to Berzofsky (1978) and to computer analyses performed by Munson and Rodbard (1980).

If purified antibody is used, the precise knowledge of [Ab] can yield a value for 'n', the valence of the antibody. Although this value is commonly known for the different forms of mammalian Ig (IgG, n=2, IgM n=10, etc.), the use of this analysis for n determination has value in comparative immunology where the number of binding sites may not be as firmly established for every immunoglobulin.

Fluorescence Quenching

Antibodies, like most proteins, fluoresce at 330-350 nm due to absorption of ultraviolet light, excitation of tryptophane residues, and emission of light energy when returning to a ground state. When an antibody forms a complex with certain kinds of antigens, including various haptens such as DNP (Velick et al., 1960; Karush, 1959) and p-azophenylarsonate (Epstein et al, 1956), this excitation energy is transferred to the antigen. The excitation energy is then released in some form other than the characteristic 330-350 nm fluorescence. In essence, the antigen quenches the fluorescence. This phenomenon was first exploited to measure the concentration of bound antibody in reaction mixtures under a wide range of conditions by Velick et al. (1960). It was subsequently adapted for the purpose

of determining antibody affinity by Eisen and Siskind (1964). In a brilliant series of experiments using this technique they examined the effects of graded doses of the antigen DNP-bovine γ globulin on serum antibody affinities of rabbits. These studies laid the groundwork for antigen-selection and affinity maturation studies in the future. This treatise also validated the use of fluorescence quenching, a highly empirical tool, by comparing representative K_{eq}s with those obtained by equilibrium dialysis. Experimentally, aliquots of purified antibody are mixed with increasing amounts of hapten. The quenching of fluorescence is then measured as a function of added hapten. Bound and free hapten are then determined based on the degree of quenching (Q) relative to the quenching when all binding sites are occupied (Q_{max}). Bound hapten (in moles) is taken as $Q/Q_{max} \bullet 2 \bullet Ab$, where Ab is the total amount of antibody (moles) and 2 is the number of binding sites per antibody. The concentration of free hapten is taken to be the difference between added and bound hapten. An average association constant (K_o) and heterogeneity index (a) describing the dispersion of association constants about the average constant were first calculated by Sips (1948) using the

equation:

$$\frac{r}{n} = \frac{(K_o c)^a}{1+(K_o c)^a} \quad \text{(Equation 14)}$$

where r is moles of hapten bound per mole antibody at free hapten concentration c, and n is the maximum number of moles hapten that can be

bound per mole antibody (antibody valency); a is the heterogeneity index constant described above. Karush (1962) recast equation 14 as:

$$\log\frac{r}{n-r} = a\log c + a\log K_o \quad \text{(Equation 15)}$$

A plot of $\log\frac{r}{n-r}$ vs $\log c$ (Figure 2) yields the average association constant from the intercept and the heterogeneity index from the slope of the line.

Advantages of this procedure include rapid assay performance (sometimes as little as 10 or 15 minutes) and the requirement for relatively small quantities of purified antibodies. Because the assay may be completed quickly, the reaction may be run under extreme conditions of pH and temperature which would not be possible in longer assays. Serious drawbacks to using this assay include the requirement for highly purified antibody and the fact that not all antigens (i.e., carbohydrates) quench fluorescence.

Inhibition of the Plaque Forming Cell Response

The Jerne hemolytic plaque assay has been widely used to analyze antibody production at the cellular level (Jerne et al., 1963). In this assay, the number of specific antibody-forming cells (plaque forming cells, or PFC) is enumerated by observing the ability of B-cells to lyse surrounding antigen-coated erythrocytes via secretion of opsonizing antibody (leading to plaque formation upon complement mediated cell lysis). Inhibition of plaque

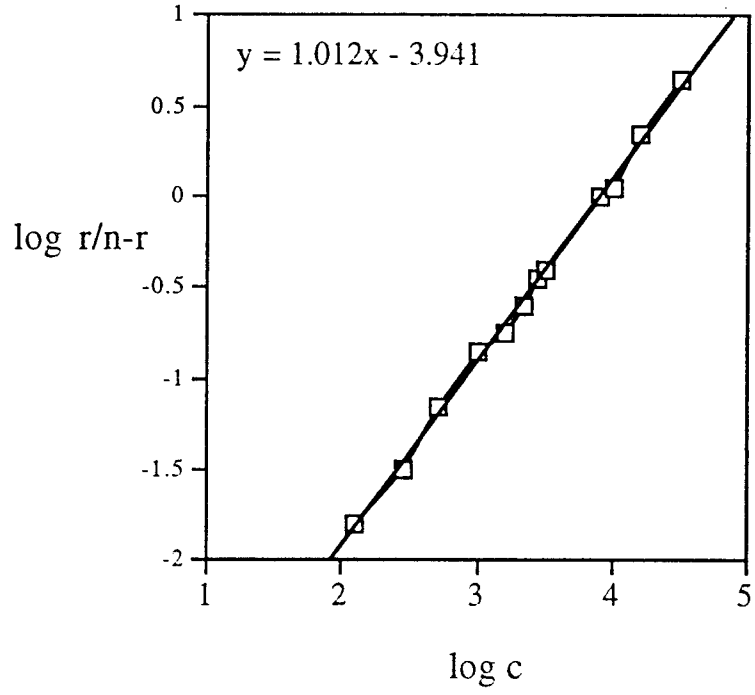


Figure 2. Sips plot of a purified anti-TNP antibody preparation. Hypothetical data plotted according to Equation 15. The heterogeneity index is obtained from the slope of the line and the average affinity constant, K_o, from the intercept (Karush, 1962). Figure adapted from Eisen and Siskind, 1964.

formation (Andersson, 1970) is based on the observation that the formation of hemolytic plaques can be inhibited by free antigen. Plaque formation around high affinity clones is inhibited by low concentrations of hapten. As high affinity antibodies are more competitive in binding when hapten is limiting (low concentration), only high affinity antibodies are inhibited by low concentrations of hapten. At high concentrations there is sufficient hapten to bind, or inhibit, both high and low affinity antibodies.

Using this technique, Claflin and Merchant (1972) determined the change in relative avidity of anti-TNP IgM antibodies over time after immunization. They observed a decrease in the amount of hapten required to inhibit 50% of plaque formation, indicating an increase in the percentage of high affinity B-cells. A decrease in the slope of the inhibition curves also indicated to them that a subset of high affinity precursor cells was being selectively stimulated, leading to a more homogeneous, high affinity antibody pool. Changes in the inhibition curves ceased 5-7 days after immunization.

Goidl and coworkers (1976) used this technique to reveal the restricted heterogeneity of the antibody affinity response to the T-dependent antigen, dinitrophenylated bovine gamma-globulin (DNP-BGG) in older mice after adoptive lymphocyte transfer. Graded concentrations of a soluble form of the dinitrophenyl hapten were used to inhibit the plaque forming response of splenic lymphocytes from young and old mice. The percent of total

(uninhibited) PFC was plotted for each inhibitor concentration, and a relative avidity was taken to be the inverse log of the concentration of hapten causing inhibition of 50% of plaque formation (a technique validated by DeLisi and Goldstein, 1974). Sample plots from the Goidl experiment are shown in Figure 3. Each plot illustrates the distribution of splenic indirect PFCs with respect to avidity. They concluded that older mice experience both a loss of high avidity PFC and a decrease in the heterogeneity of the response. In mixed cell transfer experiments, cells from older (24 month) mice were either transplanted alone or co-transplanted with young mouse (2 month) cells into histocompatible, lethally irradiated recipients. After immunization, it was observed that the inhibition profiles from irradiated and reconstituted mice more closely matched those of older mice than younger mice. Thus, evidence was presented for impaired helper and/or augmented suppressor cell activity in older mice which was transplantable into younger mice.

Precipitation Techniques

The Farr Technique

Farr (1958) pioneered another technique for measuring antibody avidity which was extended by others (Stupp et al., 1969; Goidl et al., 1968). The technique is based on the observation that some radiolabeled antigens (particularly haptens) are soluble in 50% saturated ammonium sulfate, whereas antibody and radiolabeled antibody-bound antigen will be

Figure 3. Anti-DNP-BGG affinity distribution in C57L/J mice by age. Three groups of C57L/J mice, 2, 12, and 25 months of age were immunized with DNP-BGG. Two weeks after immunization, mice were sacrificed, and the distribution of PFCs with regard to avidity was determined by hapten inhibition of plaque formation. Each histogram illustrates the distribution of splenic indirect plaque forminc cells (PFC) with respect to avidity. The abscissa represents the log of the free hapten concentration used in the inhibitory assay. The ordinate represents the percent of the total population of PFCs present in each subpopulation. PFCs from spleens of 2-month-old mice were most heterogeneous with regard to avidity and had the highest average avidity. In this study, Shannon heterogeneity indices were determined, as well as total splenocyte and PFC counts (not shown) (Goidl et al., 1976).

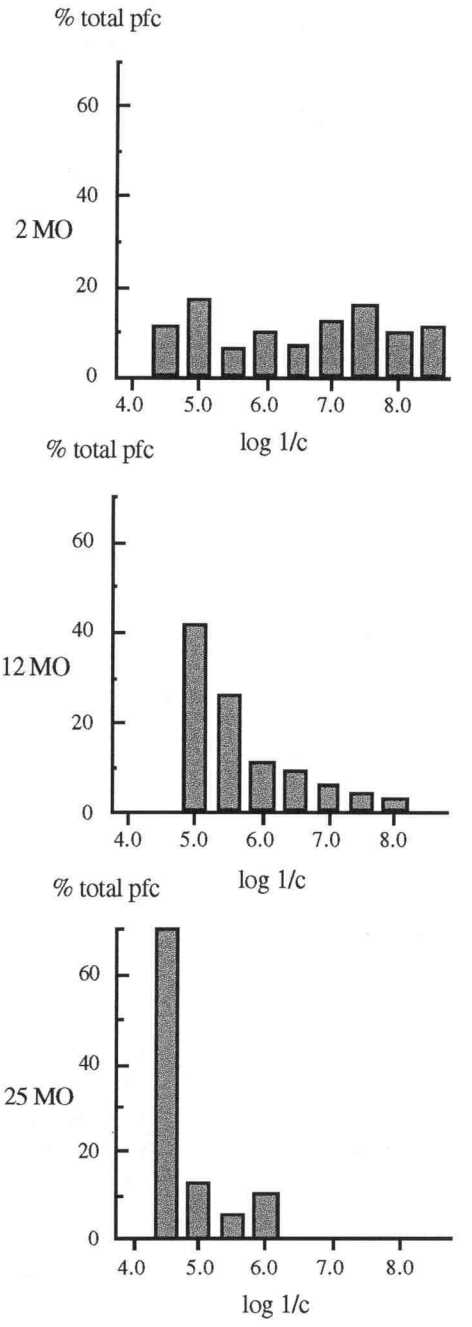

% total pfc
60
40
2 MO
20
0
4.0
5.0
6.0
7.0
8.0
log 1/c
% total pfc
60
40
12 MO
20
0
4.0
5.0
6.0
7.0
8.0
log 1/c
% total pfc
60
40
25 MO
20
0
4.0
5.0
6.0
7.0
8.0
log 1/c

Figure 3

precipitated under the same conditions. In practice, antiserum or purified antibody, usually at a 1:5 dilution in normal serum is mixed with varying concentrations of radiolabeled antigen and incubated to reach equilibrium. As antibody concentrations in the sample are minute, the addition of normal serum aids in the formation of large precipitates containing radiolabeled antigen-bound antibody. Ammonium sulfate is added, and supernatant radioactivity is measured after precipitate is removed. Control normal serum and radioactive hapten are also processed so that both total radioactivity and supernatant (free) radioactivity may be determined. The difference is equivalent to antibody-bound radioactivity (Stupp et al., 1969). Equilibrium constants are then determined as shown above (equilibrium dialysis) or by any of the classical techniques outlined in Karush (1962).

Hapten Inhibition of Precipitation

Precipitation by antibody-antigen complex formation is based on the fact that (relatively) large, multideterminant antigens become cross-linked to form aggregates when bound by multivalent antibodies. These aggregates, being insoluble, precipitate out of solution. Univalent haptenic molecules, however, can only occupy single binding sites and thus are incapable of cross-linking. An example of this would be the reaction of anti-TNP antibodies with haptenic TNP-lysine versus multimeric TNP-BSA. When homologous hapten is added to a mixture of antibody plus multi-haptenated antigen, the

amount of precipitate obtained is less than that obtained in the absence of hapten. The decrease in the amount of precipitate is due to the competition of hapten with antigen for antibody binding sites. As with inhibition of plaque formation, avidity is related to the inverse log of the hapten concentration required to inhibit 50% of precipitation. The specificity of binding to the homologous hapten may be determined by attempting the inhibition of the antibody-antigen binding reaction with heterologous haptens. The concentrations of test and reference haptens required to give 50% inhibition are used to determine the relative binding constants by the equation:

$$K_{rel} = \frac{\text{(total concentration of ref hapten added to give 50\% inhibition)}}{\text{(total concentration of test hapten added to give 50\% inhibition)}} \quad \text{(Equation 16).}$$

Thus, by assaying a panel of heterologous haptens with respect to their inhibition of antibody-antigen binding, one can determine what structures are important for antibody binding.

Solid Phase Assays

In the last ten years, the use of solid phase ELISA techniques to study antibody affinity have come into prominence, primarily due to the following advantages of this technology: 1) non-reliance on radiolabeled reagents, 2) increased sensitivity (minute amounts of antibody are required), and 3) the ability to partition and quantitate antibodies of different affinities.

Both inhibition of antibody-antigen binding by hapten inhibition or by resistance to chaotropic elution of bound antibody (the "eluting principle") have been widely used to study relative antibody affinity (Polanec et al., 1994; Sennhauser et al., 1989; Macdonald et al., 1988; Hall and Heckel, 1988; Jones et al., 1987; Pullen et al., 1986).

Pullen et al. (1986), used the chaotropic agent thiocyanate to determine the increase of immune serum average affinity constants over time. In this assay, ELISA plates were saturated with antigen, then incubated with serum. Bound antibodies were exposed to increasing concentrations of thiocyanate ion, and resistance to thiocyanate elution was used to determine relative serum affinities. Using this assay, they were able to discriminate between the efficacies of vaccine treatments by comparing changes in post-vaccination antibody affinities over time.

This technique was also used to distinguish primary rubella infections from secondary infections in a clinical setting (Polanec et al., 1994) by comparing the relative antibody affinities. In this experiment, the efficacies of various chaotropic agents, including urea, diethylamine, and guanidine as well as high pH buffers (pH 11) were examined for their antibody eluting properties. All but urea adequately dissociated antibody from plate bound antigen.

Two ELISA-based competition methods for determining affinity constants in homogenous solutions have been described (Friguet et al., 1985;

Rath et al., 1988). Both methodologies are based on methods originally proposed by Nieto et al. (1984). In the first (Friguet et al., 1985), monoclonal antibody is mixed with antigen in a test tube and incubated to reach equilibrium. The amount of free antibody is then determined in solution by comparison of the ELISA titer with a preliminary calibration curve, and equilibrium constants are determined by the law of mass action as described earlier. This assay is designed such that only a small fraction (at most 5-10%) of the unsaturated mAb at equilibrium is captured by the coating antigen, thus ensuring that equilibrium in solution is not significantly affected by the ELISA.

In the method used by Rath (1988), plates were uniformly coated with DNP-BSA. Monoclonal antibody preparations of different affinities were titered, and the dilution of antibody yielding 50% of maximum signal was determined. Serial dilutions of haptenic inhibitor (DNP-LYS) were then used to inhibit binding of the antibody to DNP-BSA (again, using one coating concentration). Molar inhibition concentrations required for 50% inhibition of antibody binding ($I_{0.5}$) were compared with antibody affinity measurements (K) made by equilibrium dialysis. There was an inverse relationship between $I_{0.5}$ and K. The relative rank order of monoclonal antibodies was maintained when comparing the two values, but no direct correlation between $I_{0.5}$ and K was established.

Examining immunological memory in rainbow trout, Arkoosh (1989) detected fine specificity shifts using K_{rel} (described above) in antibody production typical of affinity maturation. Using a modification of the above technique to study shifts in antibody affinity, Arkoosh demonstrated a lack of affinity maturation over time. Based on these results, it was suggested that either the presence of a limited clonotype did not provide the selective competitive pressure necessary to drive affinity maturation, or that active suppression was inhibiting the proliferation of high affinity clones. In retrospect, however, the form of assay used may not have provided the sensitivity required to examine the small shifts in affinity which are reported here.

MATERIALS AND METHODS

Animals

Shasta strain rainbow trout (*Oncorhynchus mykiss*) were supplied by the Freshwater/Marine Biomedical Center, Oregon State University. The trout were maintained at the Oregon State University Salmon Disease Laboratory, the Western Fish Toxicology Station of the Corvallis Environmental Research Laboratory, or at the Marine Culture Laboratory, Virginia Institute of Marine Science, School of Marine Science, College of William and Mary, Gloucester Point, Virginia.

Oregon-reared trout were maintained at approximately 12°C in a constant, flow-through supply of pathogen-free well water. The fish were fed a daily ration of Oregon Moist Pellet (OMP) which was kindly provided by Dr. Jerry Hendricks.

Virginia-reared trout were maintained at approximately 12°C in 370 liter tanks within a recirculating system supplied with biological and chemically-balanced, u.v.-treated recirculating tap water. Freshwater exchange was approximately 2% per day with 75% of the volume recirculated through the filter system every hour. The fish were fed a daily ration of dried pellet feed (Asb2, Ziegler Brothers, Gardner, PA) from belt feeders.

BALB/c mice were maintained at Oregon State University by the Laboratory Animal Resource Center, and in Virginia by technical staff in the laboratory of Dr. Stephen L. Kaattari. Mice were fed *ad libitum* with dried food pellets (RMH 3000 rat chow, Agway, Syracuse, NY) and water.

Antigen Preparation

Trinitrophenylated-keyhole limpet hemocyanin (TNP-KLH) was prepared as described by Rittenberg and Amkraut (1966). In brief, 105 mg KLH dissolved in 4.0 ml 10N NaOH was brought to 28.0 ml in borate buffered saline (BBS) (see Appendix 1) and dialyzed 2 h vs 1 L BBS. The pH was adjusted to 8.0 with 1N HCl. Trinitrophenylation of the KLH was accomplished by the addition of 262 µl picrylsulfonic acid (trinitrobenzenesulfonate) (5% w/v) (TNBS, Sigma Chemicals, St. Louis, MO) dropwise while stirring, and rotating the mixture overnight at 4°C. Haptenated protein was dialyzed extensively against three changes of 4 L phosphate buffered saline (PBS) (Appendix 1). KLH concentration and haptenation ratios were determined spectrophotometrically using the following equations:

$$A280 = [KLH]\varepsilon_{280}^{KLH(mg/ml)} + [TNP]\varepsilon_{280}^{TNP(M)}$$
$$A340 = [KLH]\varepsilon_{340}^{KLH(mg/ml)} + [TNP]\varepsilon_{340}^{TNP(M)}$$

where,

$$\varepsilon_{280}^{KLH(mg/ml)} = 1.21$$
$$\varepsilon_{340}^{KLH(mg/ml)} = 0.072$$
$$\varepsilon_{280}^{TNP(M)} = 1.25x10^{4}$$
$$\varepsilon_{340}^{TNP(M)} = (0.337)\varepsilon_{280}^{TNP(M)}$$

Using the above formulae and spectrophotometric readings for TNP-KLH at 280 and 340 nm, it was determined that the TNP-KLH conjugate was 3.56 mg/ml, approx. 75 TNP per 400,000 d monomer (373 TNP per KLH). The conjugate was sterile filtered through a 0.22 μm porosity filter and stored in a sterile stoppered serum bottle.

Trinitrophenylated bovine serum albumin (TNP-BSA) was prepared essentially as TNP-KLH except BSA was dissolved directly in BBS (20 mg/ml) and the following extinction coefficients were used to calculate the haptenation ratio: $\varepsilon_{280}^{0.1\%BSA} = 0.51$ and $\varepsilon_{340}^{0.1\%BSA} \cong 0$. Protein concentration and haptenation ratio were determined as for TNP-KLH. Protein concentration was 17.9 mg/ml, with a haptenation ratio of 8.1 TNP:BSA.

Trinitrophenylated lipopolysaccharide (TNP-LPS) was prepared as described by Jacobs and Morrison (1975). Briefly, 50 mg of *E. coli* lipopolysaccharide, serotype 0111:B4 (Sigma) was dissolved in 2.5 ml of 0.28

M cacodylate buffer (see Appendix 1) and adjusted to a pH of 11.5. A solution of thirty mg (600 µl of a 5% solution) of picrylsulfonic acid (TNBS) in 2.5 ml of 0.28 M cacodylate buffer was added dropwise to a 15 ml conical centrifuge tube and allowed to mix, covered from light, on a rotator mixer 2 hours at room temperature. After coupling, the mixture was exhaustively dialyzed vs. 3 changes of 4.0 L PBS with a final change against 1.0 L RPMI-1640 (Sigma). The solution was then pasteurized for 30 minutes at 70°C and stored at 4°C in a sterile stoppered serum vial.

Immunizations

For affinity maturation studies, seventeen normal rainbow trout were immunized intraperitoneally (i.p.) with 100 µg of TNP-KLH or 100 µg TNP-LPS emulsified in an equal volume of Freund's complete adjuvant (FCA; Difco Laboratories, Detroit, MI). The final volume injected into each fish was 0.05 ml. Fish were given an identical secondary i.p. injection of TNP-KLH twenty weeks post primary injection in Freund's incomplete adjuvant. Control fish received PBS in Freund's adjuvant. Approximately 400 µl of blood was drawn at weeks 0, 9, 22, and 31, representing nine weeks post primary, two weeks post secondary, and 11 weeks post secondary immunization. Three groups of twenty fish not designated for use in affinity maturation studies were immunized under the same protocol for stock anti-TNP antibody purification and repeatability studies. All trout were tagged for

identification at the time of immunization with either individually numbered tags or group specific color tags (Floy Tags, Seattle, WA).

In studies of the kinetics of initial affinity maturation, groups of nine normal trout received 100 µg of TNP-KLH i.p., emulsified in an equal volume of Freund's complete adjuvant, or Freund's adjuvant emulsified with PBS (control). All immunizations were administered in a volume of 50 µl. Blood was drawn at day 0 and 3 weeks post immunization. A minimum volume of 1.0 ml serum was collected from each animal at both time points. Based on prior experience in our laboratory, this collection volume was sufficient to disallow any further short-term affinity measurements using the same animals (large volume repeated bleeds caused a form of anemia within 9 to 22 weeks).

Plasma, Sera, and Leukocyte Harvesting

Trout were anaesthetized in approximately 5 L dilute benzocaine solution (Appendix 1). Serum samples were collected for antibody measurements, while plasma was harvested when peripheral blood leukocytes were also required for *in vitro* cultures.

Blood was collected from the caudal vein using a 1.0 ml syringe fitted with an appropriately sized needle, entering from the lateral surface of the caudal peduncle immediately below the lateral line. Serum samples were dispensed into 1.5 ml Eppendorf tubes on ice, allowed to clot for at least one

hour, centrifuged 10 minutes at 1600g in a microfuge (Beckman Instruments, Palo Alto, CA), and serum stored at -70°C until used. When peripheral blood leukocytes (PBL) were required, blood was collected from 150 to 300 g fish via the caudal vein using heparinized Vacutainer™ tubes (Becton Dickinson, Franklin Lakes, NJ) fitted with appropriately sized needles.

Peripheral blood leukocytes were separated from red blood cells (RBC) as described below and in Arkoosh and Kaattari (1991); plasma were separated and stored at -20°C. Rainbow trout anterior kidneys (HK) were aseptically removed, single cell suspensions prepared by grating the organ over a sterile wire mesh, and processed according to the protocol for PBL isolation.

Culture of Trout Leukocytes

Media components were purchased from Sigma Chemicals, St. Louis, MO unless otherwise noted. Tissue culture medium (TCM) was prepared essentially according to Mishell and Dutton (1967) with the following modifications. Four hundred ml RPMI-1640 (Irvine Scientific, Irvine, CA) was supplemented with 102 mL fetal bovine serum (FBS, Hyclone Laboratories, Logan, Utah), 464 µl gentamicin sulfate stock (50 mg/ml, Irvine Scientific), 4.64 ml nucleoside stock (1.0 mg/ml uridine, adenosine, cytosine, Sigma), 232 µl 2-mercaptoethanol stock (50 µM, Biorad), 464 µl guanine stock (10.0 mg/ml in 1N NaOH, Sigma). A nutritional supplement (Tittle and Rittenberg, 1978) was fed to cells every other day during leukocyte culture

incubations. This supplement was composed of 3.0 ml of stock medium (RPMI-1640, 66 ml; gentamicin sulfate, 50 mg/ml, 90 µl; RPMI-1640 amino acids, Sigma #R7131, 13.62 ml; dextrose stock, 200 mg/ml, 4.62 ml; L-glutamine, 100 mM, 4.62 ml) to which 1.5 ml fetal bovine serum, 200 µl nucleoside stock (1.0 mg/ml), 20 µl guanine stock (10 mg/ml in 1N NaOH) was added. All stock reagents were diluted in deionized water and sterile filtered through 0.22 µm unless otherwise indicated. Stock supplements were stored in 3.0 ml aliquots at -20°C until used. All other reagents were stored at 4°C for up to 2 weeks.

Fish were either anaesthetized in a 1:1000 dilution of benzocaine stock for peripheral blood collection or anaesthetized and sacrificed by cerebral concussion for anterior kidney harvests. After bleeding the fish using a heparinized Vacutainer™ tube, anterior kidneys were harvested and stored in cold RPMI-1640 on ice until used. All subsequent procedures were performed in a laminar flow hood under sterile conditions.

Anterior kidneys were ground over a fine wire mesh using a pair of forceps and the barrel of a 3.0 ml plastic syringe. Cells were pelleted at 500g for 10 minutes at 4°C. Subsequent steps were identical for peripheral blood or anterior kidney leukocytes. Cells were washed once by the addition of 15 ml RPMI-1640 and recentrifugation. Pellets were resuspended in RPMI-1640 at a total volume of 4 times the original blood volume (PBL) or 4.0 ml RPMI-1640 (HK) and carefully layered over an equal volume of Histopaque-1077 (Sigma).

Red blood cells were separated from white blood cells by centrifugation at 500g for 45 minutes. Cells at the RPMI/Histopaque interface were harvested, washed 3x with RPMI-1640, and resuspended in a total volume of tissue culture medium equal to 25% of the original blood volume (PBL) or 1.0 ml (HK leukocytes) Cellular viability was assessed using trypan blue dye exclusion. Cells were diluted to $2x10^7$ ml^{-1} in TCM and distributed 50 µl per well to 96 well, low evaporation culture plates (Costar Corporation, Cambridge, MA). Fifty µl of the appropriate antigen (LPS, TNP-LPS) or a negative antigen control in TCM was added to replicate wells in such a way as to use all available cells from a given harvest. Cells were cultured at 17°C in a blood gas environment of 80% N_2, 10% O_2, 10% CO_2 in gas box incubators (C.B.S. Scientific, Del Mar, CA). Twenty-four hours after the addition of antigen, plates were centrifuged, antigenic (or mitogenic) stimulation was removed by aspiration of the media using a multichannel pipettor, and refed with TCM. Cells were incubated for 9 days when assaying for plaque forming cells (concomitant with ELISA titration), or 12 days when determining antibody titer by ELISA only. Cultures were fed on alternate days with 0.01 ml nutritional supplement.

Measurement of the anti-TNP Antibody Forming Cell (AFC) Response

Trinitrophenylated sheep red blood cells (TNP-SRBC) were prepared according to the method of Rittenberg and Pratt (1969). Briefly, 10 ml SRBC

(in Alsevers solution) were washed 3x at 4°C with cold modified barbital buffer (1x MBB, Appendix I). Washes were performed by centrifugation at 500g for 10 min. Picrylsulfonic acid (TNBS) was prepared by mixing 200 µl of a 5% solution (50 mg/ml) with 3.5 ml cacodylate buffer in a foil wrapped 15 ml conical centrifuge tube. Washed cells were added to the tube and rotated 15 minutes at room temperature to allow coupling of TNP to SRBC. TNP-SRBC were then centrifuged at 1400g for 5 minutes at 4°C, the supernatant was removed and the pellet was mixed with 6 ml glycylglycine solution (10 mg glycylglycine in 15.75 ml 1xMBB). The mixture was centrifuged again, and washed with 1xMBB until supernatant was clear. TNP-SRBC were diluted to a final cell concentration of 20% (v/v) in 1xMBB, stored at 4°C, and washed immediately prior to use.

Stimulated cultures were assayed for generation of antibody-forming cells after nine days of incubation, previously determined to be the peak day for the antibody-forming cell (AFC) response (Irwin, 1987). Culture plates were centrifuged at 500g for 10 minutes at 4°C. Supernatants were removed and saved for analysis. A modification of the Cunningham plaque-forming cell assay (Cunningham and Szenberg, 1968) was performed. Wells were dosed with 50 µl cold RPMI-1640, 10 µl diluted steelhead serum in 1xMBB (previously determined to provide maximal complement activity with a minimum of autolysis at 1:8) and 10 µl 20% (v/v) TNP-SRBC. Following the addition of TNP-SRBC, the contents of each well were placed into

Cunningham slide chambers. Cunningham slide chambers consist of two slides, separated into two compartments by three pieces of double sided tape (3M, St. Paul, MN). The chambers were sealed with liquid paraffin (melted and kept liquid on a hotplate) and incubated 1 to 3 hours at 17°C until maximal plaques could be distinguished with a minimum of autolysis. Plaques were counted with the aid of a dissecting microscope.

Titration of Anti-TNP Activity and Total Immunoglobulin in Serum and Plasma by ELISA

Anti-TNP activity was determined according to the protocol of Arkoosh and Kaattari (1990). Briefly, 96 well microtiter plate wells were coated with 50 µl TNP-BSA (10 µg/ml) in a carbonate/bicarbonate coating buffer, pH 9.6. Plates were incubated overnight at 4°C, washed 3x with Tween 20 supplemented tris buffered saline (TTBS), and unbound sites blocked with 240 µl TTBS with 1% fetal bovine serum (FBS) (blocking buffer) for 1h at room temperature. Fifty µl aliquots of three-fold culture supernatant dilutions or five-fold serum dilutions, along with dilutions of a standard, purified trout anti-TNP antibody in blocking buffer were added to each well of the antigen coated plate. The plates were then incubated 90 minutes at room temperature. After removing unbound antibody by washing 3x with TTBS, 50 µl of a 1:1000 dilution of biotinylated mouse anti-trout monoclonal antibody (1.14) in blocking buffer was added to each well and again incubated 90 minutes at room temperature. Plates were washed 3x with TTBS. Fifty µl streptavidin

conjugated horseradish peroxidase, diluted appropriately in blocking buffer, was then added to each well and incubated 60 minutes at room temperature. Plates were washed extensively with TTBS and developed using 0.04% 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and hydrogen peroxide (7µl/10 ml reaction volume) in 0.2% citrate buffer, pH 4.0, 100 µl per well. Absorbance at 405 nm was determined using a Titertek® Multiskan® MCC/340 plate reader when the maximum O.D. reached approximately 1.2 to ensure that the reaction rate was still proceeding linearly. Titration curves were plotted and titers taken to be the reciprocal of the dilution at which the sample produced one half maximal optical density. All samples were then standardized by comparison to a purified trout anti-TNP antibody with a reference value of 1000 units/µl (see Arkoosh and Kaattari, 1989 for a detailed explanation of the data analysis).

Immunoglobulin determination were performed in much the same way, except 0.2 µg/well mouse anti-trout monoclonal (1.14), used to capture trout immunoglobulin upon incubation with serum or supernatant, was coated onto microtiter plate wells in coating buffer, 50 µl per well. The rest of the assay was performed as above, using the same standard, purified anti-TNP of known concentration (0.25 mg/ml). Titration curves were plotted, and titer was determined to be the reciprocal of the dilution at which the sample produced one half maximal optical density. Concentrations were determined by direct comparison with the standard.

Monoclonal Antibody Production

Monoclonal antibodies were developed essentially as described in Harlow and Lane (1988). Four mice were injected intraperitoneally (i.p.) with either 50 µg affinity purified trout anti-TNP antibody emulsified with FCA or 50 µg immunopurified normal trout immunoglobulin, purified by binding to Sepharose-bound 1.14 monoclonal, and eluted under low pH. Primary immunizations were made by emulsification of antigen in Freund's complete adjuvant, while all subsequent immunizations were administered in incomplete adjuvant. Blood was drawn every two weeks from the tail vein, and mice were either titered by ELISA for reactivity to solid phase trout immunoglobulin or polyclonal anti-H or L chain antibody was assessed for each serum by western blotting.

Upon detection of a positive titer, mice were challenged with 5 µg purified antigen in sterile phosphate buffered saline (PBS) i.v., and spleens were harvested three days later for fusion. Mice were euthanized by cervical dislocation, and spleens were aseptically removed into petri plates containing 10 ml RPMI-1640. Single cell suspensions were generated by grating the spleen over a fine wire mesh using the forceps and the plunger from a 3.0 ml plastic syringe. Red blood cells were lysed by incubation for 10 minutes at 37°C with 5.0 ml of a solution of tris-ammonium chloride (17 mM trizma, 155 mM ammonium chloride, pH 7.2). Following red blood cell lysis, cells were

washed three times with RPMI-1640, and live cells were enumerated by trypan blue dye exclusion. SP2/0 myeloma cells (American Type Culture Collection) were prepared by washing twice with RPMI and determining live cell counts using trypan blue. Spleen cells were chemically fused with SP2/0 myeloma cells in a 1:4 ratio using a low molecular weight polyethylene glycol (PEG 1450; Sigma). Hybrid cells were selected using hypoxanthine-azaserine (Foung et al., 1982; Sigma) according to the manufacturer's instructions. Cells were grown in culture until confluence, and screened by ELISA or western blot procedures (see below).

As immunization with intact rainbow trout immunoglobulin failed to produce monoclonal antibodies with light chain specificity, an alternative protocol was attempted, based on the solubility of nitrocellulose in DMSO (Knudsen, 1984). After electrophoresis and electroblotting onto nitrocellulose, trout light chain was excised with a razor blade. The nitrocellulose strip was dissolved in DMSO (approx. 1 µg light chain dissolved in 100 µl DMSO), mixed with an equal volume of FCA, and injected into naive mice. Blood was drawn every two weeks from the tail vein, and polyclonal anti-H or L chain antibody was assessed for each serum by western blotting a 1:1000 dilution of serum onto strips cut from an electrophoretic separation of reduced rainbow trout immunoglobulin.

Screening of Monoclonal Antibodies

ELISA

Ninety-six well microtiter plates were coated with immunopurified normal trout immunoglobulin, 0.1 μg/well in carbonate/bicarbonate coating buffer for two hours, then blocked 1 hour with 1% FBS in TTBS. Five microliters of supernatant were diluted into wells containing 50 μl of 1% FBS/TTBS (blocking buffer), and allowed to incubate for two hours. After washing with TTBS, 50 μl peroxidase labeled goat anti-mouse antibody in 1% FBS/TTBS was added to each well and incubated 1 hour at RT. Plates were extensively washed with TTBS, and positive clones were detected by the addition of 100 μl/well ABTS substrate solution (described above). The color change reaction was read spectrophotometrically at 405 nm in the TiterTek™ Multiscan as previously described.

Polyacrylamide Gel Electrophoresis and Immunoblotting

SDS-PAGE

Affinity purified trout anti-TNP antibody was electrophoresed under reducing conditions in BioRad mini Protein-II™ electrophoresis chambers (Biorad, Richmond, CA) according to the manufacturer's instructions to separate heavy and light chains. Protein concentrations were determined by

the Peterson modification of the Lowry total protein assay (Peterson, 1982; Lowry, 1956). Four µg antibody samples were mixed with an equal volume of reducing sample buffer (Appendix I), boiled 4 minutes in a boiling water bath, and applied to 12% polyacrylamide gels with 3% polyacrylamide stackers (in a discontinuous Laemli buffer system; Laemmli, 1970). Samples were electrophoresed approximately 200 Vh in a tris-glycine running buffer until the sample dye front reached the bottom of the gel. Following electrophoresis, proteins were transferred to Immobilon-P (polyvinyl difluoride - PVDF - membranes) using the Biorad wet cassette transfer system. Transfers were performed at 100V for 1 hour. Membranes were transferred to troughs containing TTBS and either stained with a colloidal gold total protein stain (Biorad), or incubated in TTBS overnight for immunoblotting. For screening hybridoma supernatants, strips were cut from the membrane, and incubated with approximately one ml of supernatant for 1 hour at RT. After rinsing the blots with TTBS, horseradish peroxidase-labeled goat anti-mouse Ig was added to each strip (1 ml in 1%FBS/TTBS, 1:10,000 according to manufacturer's instructions) and incubated 1 hour at RT. After extensive washing, blots were visualized photographically using enhanced chemiluminescence reagents and photographic film (Amersham International plc, Buckinghamshire, England).

2D-IEF

Two-dimensional polyacrylamide gel electrophoresis was used to visualize the spectrotype of heavy and light chains in trout and mouse serum or that of purified antibody preparations. Approximately 1 µg purified antibody or 4 µg serum protein was mixed 1:1 with first dimension reducing buffer and incubated 15 minutes at room temperature. Tube gels were cast according to the recipe in Appendix I and allowed to polymerize for one hour. No prefocusing of ampholytes was required. Samples were added to reservoirs at the anodic end, and electrophoresed 30 minutes at 250 volts, then 3.5 hours at 500 volts. Visible IEF standards were run in separate tubes to confirm accurate focusing of proteins within the matrix.

Upon completion of focusing, gels were extruded from the tubes and overlaid with 200 µl reducing SDS sample buffer. Tube gels were carefully layered onto 12% SDS PAGE gels containing preparative stacking gels. A portion of the original sample was added to the reference well of each gel for comparison. Electrophoresis, staining, and western blotting was performed as described above.

Immunopurification of Antibodies (Trout and Murine)

TNP-BSA or the 1.14 mouse monoclonal antibody were conjugated to cyanogen bromide activated sepharose 4B beads according to the manufacturer's instructions. Briefly, beads were washed alternately in 1 mM

HCl or coupling buffer (see Appendix I) 4 times (all centrifugations @ 600g for 5 min). The antigen ligand was then added, 0.5 ml TNP-BSA (41.5 mg/ml) to 3.5 ml of the hydrated beads, or 0.5 ml 1.14 antibody (6.0 mg/ml) to 1.75 ml beads. Each conjugate preparation was brought to 12.0 ml in coupling buffer, and rocked end over end, overnight at 4°C. The supernatant was removed, and unbound sites were blocked with a 1:4 mixture of 1M ethanolamine, 0.2M glycine, pH 8.0 (10.0 ml total volume), rocking end over end for four hours. Beads were washed alternately four times with coupling buffer or 0.1M sodium acetate, pH 4.0, and stored in PBS with 0.02% NaN_3 (preservative) at 4°C.

Anti-TNP antibodies were purified from serum after particulate matter was pelleted by centrifugation 15 minutes at 2600g. The serum was then combined with an appropriate volume of packed TNP-BSA beads (approximately 2 ml serum per ml packed beads) and rocked end over end, overnight at 4°C. After incubation with immune serum, beads were packed into an empty PD-10 columns (Pharmacia, Uppsala, Sweden) and washed with PBS until no absorbance at 280 nm was detectable in the eluate. The column was plugged and incubated 4 hours with 7.0 ml of a 10^{-3} M solution of TNP-LYS (#104793, ICN Biomedicals, Irvine, CA) in TTBS to allow competitive elution of antibodies. Six volumes of TNP-LYS were exchanged through the column, incubating 4 hours between exchanges. Elution fractions were combined and dialyzed extensively against four changes of 3 L

PBS over 48 hours @ 4°C. Protein concentration was determined by the Lowry assay, and the anti-TNP titer was determined by ELISA.

Murine anti-TNP antibodies were purified from the IgM and IgG isolations of one mouse (#10, see Gel Fractionation, below) using the same TNP-BSA beads and elution scheme as above. The volume, however, were scaled down to reflect the 75 µl serum volume from which the immunoglobulins were prepared. Thus, 300 µl TNP-BSA beads were used.

Trout immunoglobulin was immunopurified from thirteen ml normal trout serum after removal of particulate matter by centrifugation for 15 minutes at 2600g. This volume was then combined with 4.0 ml 1.14 (mouse anti-trout monoclonal antibody) conjugated sepharose beads and rocked overnight at 4°C. After incubation with serum, beads were inserted into an empty PD-10 column and unbound material was eluted by passing PBS through the column until no absorbance at 280 nm was detected. Bound immunoglobulins were eluted by passing three 8.0 ml aliquots of 100 mM Glycine, pH 2.5 and collecting the eluent directly into 2.0 ml aliquots of 1M Tris, pH 8.5. Elution fractions were combined and dialyzed four times against 3 L PBS over 48 hours @ 4°C.

Gel Fractionation of Murine IgG and IgM

A 17 cm bed of Sephacryl S300 (Pharmacia) was poured in a Biorad 20 cm x 1.2 cm Econo™ column. Beads were equilibrated with approximately 20

column volumes of PBS. The column was calibrated for separation of IgM from IgG using a 75 µl samples of purified blue dextran, 2 mg/ml (m.w. 2,000,000), thyroglobulin, 8 mg/ml (669 kd), ß-amylase, 4 mg/ml (200 kd), and cytochrome C, 2 mg/ml (12.4 kd). Each sample was diluted in PBS, and 0.6 ml fractions were collected under gravity flow.

Eleven mice were immunized intraperitoneally with 50 µg/mouse TNP-KLH (1.0 mg/ml final concentration) in an emulsification of Freund's complete adjuvant with PBS. One mouse received Freund's complete adjuvant/PBS without immunogen (negative control). All animals were bled from the tail vein prior to immunization, and at 1 and 3 weeks post immunization. Serum was stored at -20°C until used. Forty to seventy-five microliters from each bleed was passed over the Sephacryl S300 column. One ml column fractions were collected in PBS.

Detection of murine IgM and IgG

All fractions were assayed for the presence of IgM and IgG by ELISA titration. Briefly, 96 well microtiter plates were coated with goat anti-mouse $F(ab')_2$ (capture antibody, Jackson ImmunoResearch, West Grove, PA), 2.0 µg/ml, 50 µl per well in carbonate/bicarbonate coating buffer, 2 hours at room temperature. Unbound protein binding sites were blocked with 240 µl/well 1% FBS/TTBS 1 hour at room temperature. The plates were washed 3x with TTBS. Serial dilutions of column fractions or standard purified mouse IgM

or IgG (Jackson ImmunoResearch), 50 µl per well in 1% FBS/TTBS were added. Dilutions were 1:50, 1:250, 1:1250, 1:6250, 1:31250, 1:156250, 1:781250, and a buffer blank. After incubation at room temperature for 90 minutes, plates were again washed with TTBS to remove unbound material. The secondary reagents, horseradish peroxidase labeled goat anti-mouse IgM or IgG, were added, 50 µl per well in 1% FBS/TTBS, incubated 60 minutes, and washed extensively (6x) with TTBS. Absorbance at 405 nm was determined after development with 100 µl per well ABTS substrate. Peak fractions containing either IgG or IgM were pooled. Peak fraction anti-TNP titers for use in affinity analyses were performed essentially as described for detection of IgM or IgG, except plates were coated with 0.5 µg/well TNP-BSA in carbonate bicarbonate coating buffer. Affinity distribution analyses for each sample (next section) were determined.

Determination of Plate Antigen Saturation

TNP-BSA (antigen) saturation of protein binding sites on the ELISA plate was determined using the protocol of Muñoz et al. (1986). Briefly, peroxidase (from horseradish) was allowed to bind to 96 well microtiter plates after having been "blocked" with serial dilutions of TNP-BSA. A plot of A405 vs. TNP-BSA coating concentration yielded the theoretical saturation concentration, shown in Figure 9 to be approximately 6 µg/ml.

Determination of Antibody Concentration for Affinity Analysis

The assay is performed in two stages. In the first stage, the anti-TNP titer in a serum is calculated so that equal concentrations of anti-TNP binding activity may be applied to the affinity distribution assay in the second stage. Each well of the ELISA plate in the affinity analysis receives one unit of antibody activity in 50 µl of diluent (i.e., ELISA blocking buffer; Appendix I). Calculation of the dilution of the serum required to produce this level of activity was performed prior to affinity analysis, following the procedure outlined in Chapter 3 of FITC Volume I (Arkoosh & Kaattari, 1989). Fifty microliters of TNP-BSA in carbonate/bicarbonate coating buffer (10 µg/ml) were distributed to each well of a 96 well plate and incubated for 2 hours at room temperature. Unbound sites were blocked with 240 µl/well 1% FBS/TTBS 1 hour at room temperature. The plates were washed 3x with TTBS. Serial dilutions of trout antiserum and an anti-TNP standard were added to duplicate columns of the ELISA in 1% FBS/TTBS (50 µl per well) at the following dilutions: 1:50, 1:250, 1:1250, 1:6250, 1:31250, 1:156250, 1:781250, and a buffer blank. After incubation for 90 minutes at room temperature, plates were again washed 3x with TTBS to remove unbound material. Biotinylated mouse anti-trout monoclonal (1.14) was diluted appropriately in 1% FBS/TTBS. Fifty µl was added to each well of the assay and incubated 60 minutes at room temperature. The plates were washed three times with TTBS. Fifty µl of an appropriate dilution of horseradish peroxidase

conjugated streptavidin in 1% FBS/TTBS was added to each well and incubated 60 minutes at room temperature. The plates were washed 6x with TTBS. Absorbance at 405 nm was determined after development with 100 µl per well ABTS substrate (Appendix I). The titer was determined with respect to the standard anti-TNP preparation.

A sample calculation for determining the amount of antibody required for an affinity assay is provided. If a 1/1000 dilution of an antiserum contains one unit of activity (as defined in Arkoosh & Kaattari, 1990), then 0.05 µl of the undiluted serum contains 1 unit (1/1000 x 50 µl). As one ELISA plate contains 96 wells, the volume of serum required for the one assay is 4.8 µl (0.05 µl x 100). These calculation were made for each sample in advance of the execution of the affinity ELISA. Aliquots of test sera were stored at -20°C until used.

The Affinity ELISA

Serial 1/5 dilutions of TNP-BSA were prepared in coating buffer. A 'Worked Example' in which a rainbow trout antiserum was analyzed, is shown in Appendix 2. The plates were coated with 1, 0.2, 0.04, 0.008, 0.0016, 0.00032, 0.00064 µg TNP-BSA/ml and a no antigen control. Row A (Figure 4) received 50 µl/well of diluent (no antigen). Subsequent rows received 50 µl of each antigen dilution from highest dilution to lowest dilution. The plates were then incubated 2 hours at room temperature. After incubation, the

contents of the plates were disposed into a sink, and unbound sites were blocked with 240 µl per well of 1% FBS/TTBS. The plates were then covered with parafilm and set in a humid chamber for 1 hour at room temperature.

A solution of 10^{-3} M TNP-lysine (hapten inhibitor) was prepared in diluent (1% FBS/TTBS). The solution required incubation at elevated temperatures (50 - 70°C) with occasional swirling to complete the solubilization of the TNP-lysine powder.

Four serial 1/5 dilutions of the TNP-lysine solution were made in diluent buffer. The most informative range of hapten concentrations was derived empirically, depending upon the affinity of the serum tested. Modifications in dilution concentrations were made after an initial experiment revealed the most sensitive range for inhibition.

Following the blocking step, duplicate columns of each plate received 50 µl of 1/5 serial dilutions of hapten inhibitor (Figure 4). The final hapten inhibitor concentrations are reduced by a factor of two with the addition of an equal volume of diluted serum.

Fifty µl of diluted serum (containing one unit of antibody activity as determined by ELISA) was then deposited into each well. The plates were covered with parafilm and incubated at room temperature for 90 minutes. Plates were then washed 3x with TTBS.

One hundred µl of biotinylated anti-trout Ig (1.14), appropriately diluted, was then administered to each well, the plates were covered with parafilm,

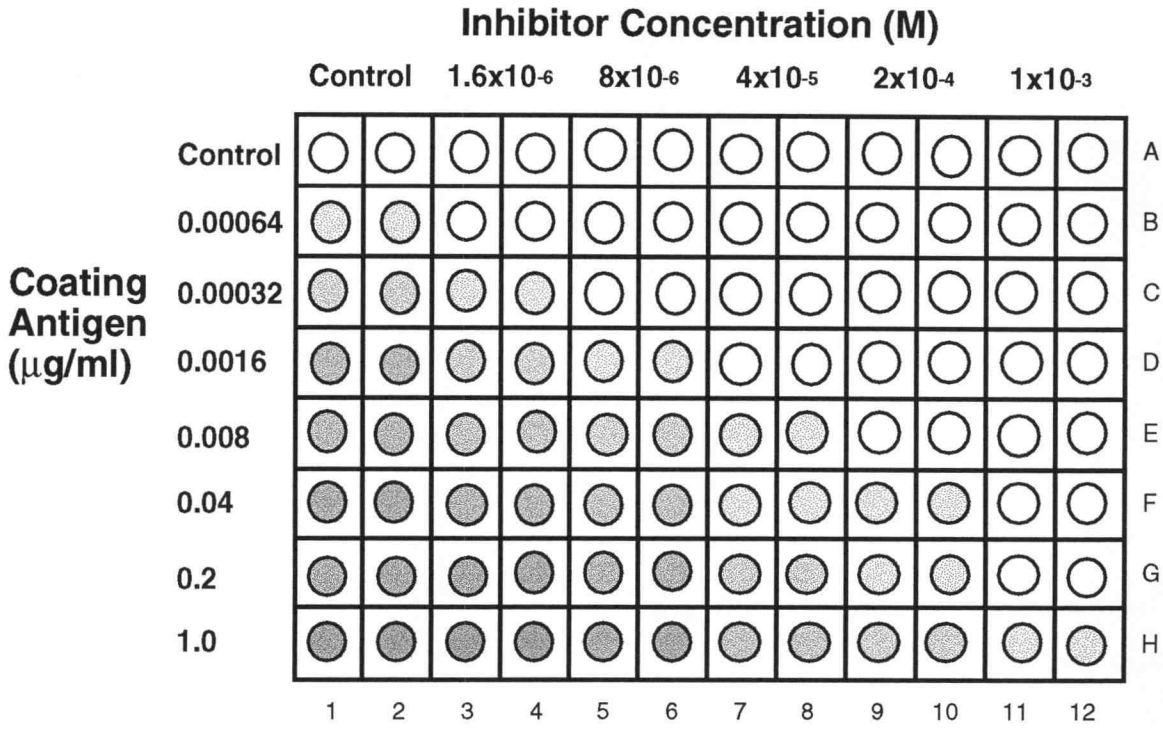


Figure 4. Sample microtiter plate layout. In the affinity assay, serial dilutions of TNP-BSA are bound within successive rows of the plate. Serial dilutions of TNP-LYS inhibitor are added to duplicate columns of the plate, starting with a high concentration of 10^{-3} M. Diluent alone (control) is added to the duplicate control columns. Fifty μl of serum at a predetermined dilution (see text) is then added to each well of the plate and allowed to bind TNP-BSA, subject to inhibition by TNP-LYS. The concentration of inhibitor responsible for inhibiting 50% of antibody binding in each row of the plate is proportional to the affinity of the subpopulation of antibodies bound in that row. The percent of the total population in each row is proportional to the decrease in absorbance from one row to the next in the control columns.

placed in the humid chamber, and incubated at room temperature for 1 hour. Plates were again washed.

One hundred µl of an appropriate dilution of streptavidin conjugated horseradish peroxidase (SA-HRPO) was added to each well, and the plates were incubated 1 hour at room temperature. After extensive washing, 100 µl of the ABTS substrate solution was added to each well and the plates were read at 405 nm as soon as a significant O.D. was readable (> 1.2 in wells H1 and H2). Average values were calculated from each replicate. It was most critical to use only O.D. values for analysis which were gained while the rate of reaction was still linear. Within this context, the highest O.D. values were obtained, allowing for the greatest discrimination between the various hapten inhibitor concentrations.

Determination of Antibody Subpopulation Sizes

The relative proportions of each antibody subpopulation were determined by performing a series of subtractions of the O.D. values derived from Columns 1 & 2 in the following manner (where 1^2 represents the average of wells 1 and 2):

$$\%\text{ of the antibodies with the highest affinity} = \frac{(\text{Row B } 1\text{^}2)}{(\text{Row H } 1\text{^}2)}$$

$$\%\text{ with the 2nd highest affinity} = \frac{(\text{Row C } 1\text{^}2) - (\text{Row B } 1\text{^}2)}{(\text{Row H } 1\text{^}2)}$$

$$\%\text{ with the 3rd highest affinity} = \frac{(\text{Row D } 1\text{^}2) - (\text{Row C } 1\text{^}2)}{(\text{Row H } 1\text{^}2)}$$

$$\%\text{ with the 4th highest affinity} = \frac{(\text{Row E } 1\text{^}2) - (\text{Row D } 1\text{^}2)}{(\text{Row H } 1\text{^}2)}$$

$$\%\text{ with the 5th highest affinity} = \frac{(\text{Row F } 1\text{^}2) - (\text{Row E } 1\text{^}2)}{(\text{Row H } 1\text{^}2)}$$

$$\%\text{ with the 6th highest affinity} = \frac{(\text{Row G } 1\text{^}2) - (\text{Row F } 1\text{^}2)}{(\text{Row H } 1\text{-}2)}$$

$$\%\text{ with the lowest affinity} = \frac{(\text{Row H } 1\text{^}2) - (\text{Row G } 1\text{^}2)}{(\text{Row H } 1\text{^}2)}$$

Determination of Antibody Subpopulation Affinities

The affinity of each antibody subpopulation was determined by plotting the inhibition data for each row of the plate (Figure 5). This permitted an estimation of the concentration of free hapten which resulted in 50% of the maximum O.D. obtainable for that subpopulation ($[H_{50}]$). The inverse log of this concentration is considered equivalent to the apparent affinity of the antibodies bound to that specific concentration of coating antigen (termed aK; Nieto et al., 1984). Figure 5 shows a typical set of inhibition curves resulting

from the analysis of one serum. Estimates of $[H]_{50}$ can also be performed mathematically using a logit-log transformation of the data (Banowetz, 1987; Arkoosh, 1989). An estimate of the average affinity from the data was also calculated as follows:

$$\text{Weighted Average} = \sum_{i=1}^{n}([aK_i]x[P_i])$$

Where, n = number of subpopulations

aK_i = apparent affinity of the i^{th} subpopulation

P_i = proportion of antibodies within the *i*th subpopulation.

Assay Repeatability

Individual sera were analyzed by up to six different investigators on different days using common (shared) stock reagents (TNP-BSA, TNP-LYS, biotinylated 1.14, SA-HRPO, BSA/TTBS). In the first experiment, an individual serum was given to a panel of investigators, each of whom determined the affinity profile and average affinity for the serum. The means and variances were calculated for each replicate experiment. In the second experiment, affinity profiles and average affinities for a panel of sera were determined in duplicate or triplicate. The means and variances were calculated for each replicate experiment.

Analysis of Antibody From Trout Lymphocyte Culture

Antibody titers from lymphocyte culture supernatants were sufficiently low that modification of the protocol was required. Enzyme conjugate reagent concentrations were increased four-fold. The highest concentration of plate coating antigen (TNP-BSA) was increased to accommodate binding of lower affinity antibodies (50 µg/ml). Substrate development reagent concentrations were increased ten-fold, and development times were extended (as necessary) in order to detect lower concentrations of antibodies in culture.

Analysis of Murine Serum Antibody Affinities

After chromatographic separation of IgM and IgG and the subsequent determination of their respective anti-TNP activities, affinity profiles were determined using a modification of the affinity ELISA used for trout. Optimization of plate antigen coating concentration dictated lowering the maximum plate antigen coating to 1.0 µg/ml, and using serial 1:5 dilutions. To accommodate antibodies of higher affinity, inhibitor concentrations were reduced (five-fold serial dilutions of TNP-LYS were made, after an initial 1:5 dilution of the stock, 10^{-3} M, inhibitor was prepared). Mouse antibodies were detected with a horseradish peroxidase labeled goat anti-mouse secondary reagent. The color change reaction with ABTS was read spectrophotometrically as described above.

Aflatoxin Experiments

Rainbow trout embryos were exposed to aflatoxin B_1 as described by Hendricks et al. (1981). In brief, groups of 100 embryos were exposed to an aqueous mixture of aflatoxin B_1 at 1.0, 0.5, 0.1, or 0.01 ppm (mg/L) for 60 minutes. The embryos were rinsed in fresh water several times and their development was allowed to proceed as normal. Control rainbow trout were treated as above except they were not exposed to aflatoxin B_1. Fish were immunized and bled when they reached the experimental size of approximately 100g on the same schedule as normal fish. Blood was collected at day 0 and three weeks post immunization.

RESULTS

Development of the ELISA-Based Affinity Assay

Introduction

The successful development of an ELISA based affinity assay required satisfaction of a number of prerequisites. First, the anti-trout immunoglobulin reagent was shown to recognize all antibodies that could be induced (no isotype specific reactivity). Second, the limits of resolution and the repeatability of such measures were established before comparing results from different assays. Finally, means were developed to resolve affinity subpopulations in sera expressing either very low or very high affinities.

Recognition of All H Chains by 1.14

The first prerequisite for the assay (i.e., universal recognition of trout antibodies) was established by determining whether every isoelectropherotype visualized by a protein stain was also reactive with the immunoreagent. The mouse anti-trout Ig (with specificity for heavy chain) monoclonal antibody (1.14), a generous gift from Dr. G. Warr (Medical University of South Carolina, Charleston, SC), has been shown to bind a large fraction of total rainbow trout immunoglobulin based on precipitation of radioiodinated Ig (DeLuca et al., 1983). In order to determine the utility of

this monoclonal antibody in the detection of all trout Ig, reduced trout immunoglobulin chains were electrophoresed two dimensionally on the basis of isoelectric point and molecular weight, and detected with the 1.14 monoclonal antibody. Figure 6A, a stain of all proteins from the electrophoretic separation of reduced antibody chains from one trout (#150) reveals 11 heavy chains and 6 light chains used by the trout from which the anti-TNP antibodies were purified. The immunoblot of the same electrophoretic run detected all heavy chains present in the sample (6B). The cocktail of anti-TNP antibodies (#395) produced a sequence of heavy and light chains similar in isoelectric focusing pattern to the purified antibody from the individual fish, revealing 9 heavy chains and 5 light chains present in the sample (Fig. 7A). Again, the 1.14 monoclonal bound all detectable isoelectropherotypes.

Development of Alternate Anti-Trout Monoclonal Antibodies

Although recognition of all intact antibodies by 1.14 was concluded to be possible, it was deemed advantageous to attempt the production of an anti-light chain reagent. If trout antibodies could be distinguished on the basis of possessing different L chains, then a possible association of affinity with light chain isotype might be possible.

Production of a specific anti-rainbow trout light chain monoclonal antibody proved elusive, probably because of the immunodominance of

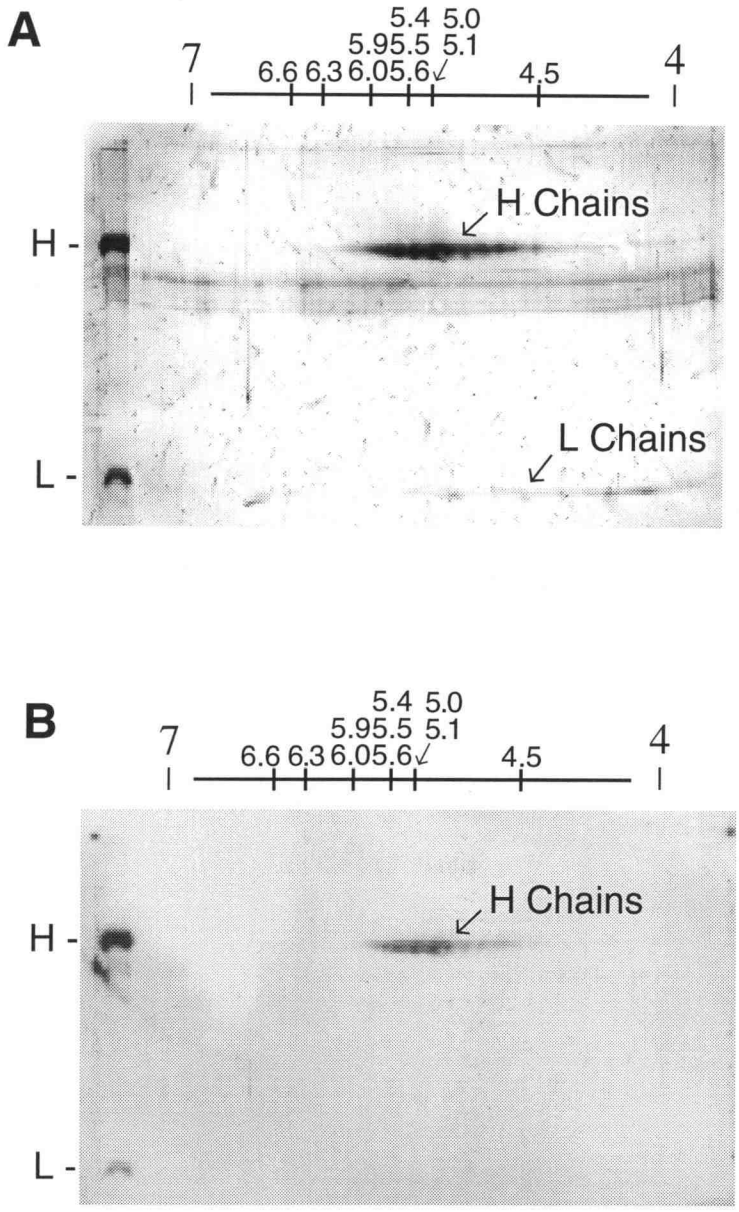


Figure 6. Two-dimensional electrophoretic separation of anti-TNP antibodies from a single rainbow trout (#150). Antibodies were electrophoresed in a pH 4-7 gradient and blotted onto PVDF membranes as described in the text. The control lane on the left of Figure A shows the relative molecular weights of heavy and light chain. All proteins (heavy and light chain protein spots) are visualized by the colloidal gold stain in (A). Western blotting of the same transfer with the 1.14 monoclonal (B) shows the H chain specificity of the monoclonal. All 9 identifiable heavy chains and 6 light chains (A) were bound by the monoclonal antibody in (B).

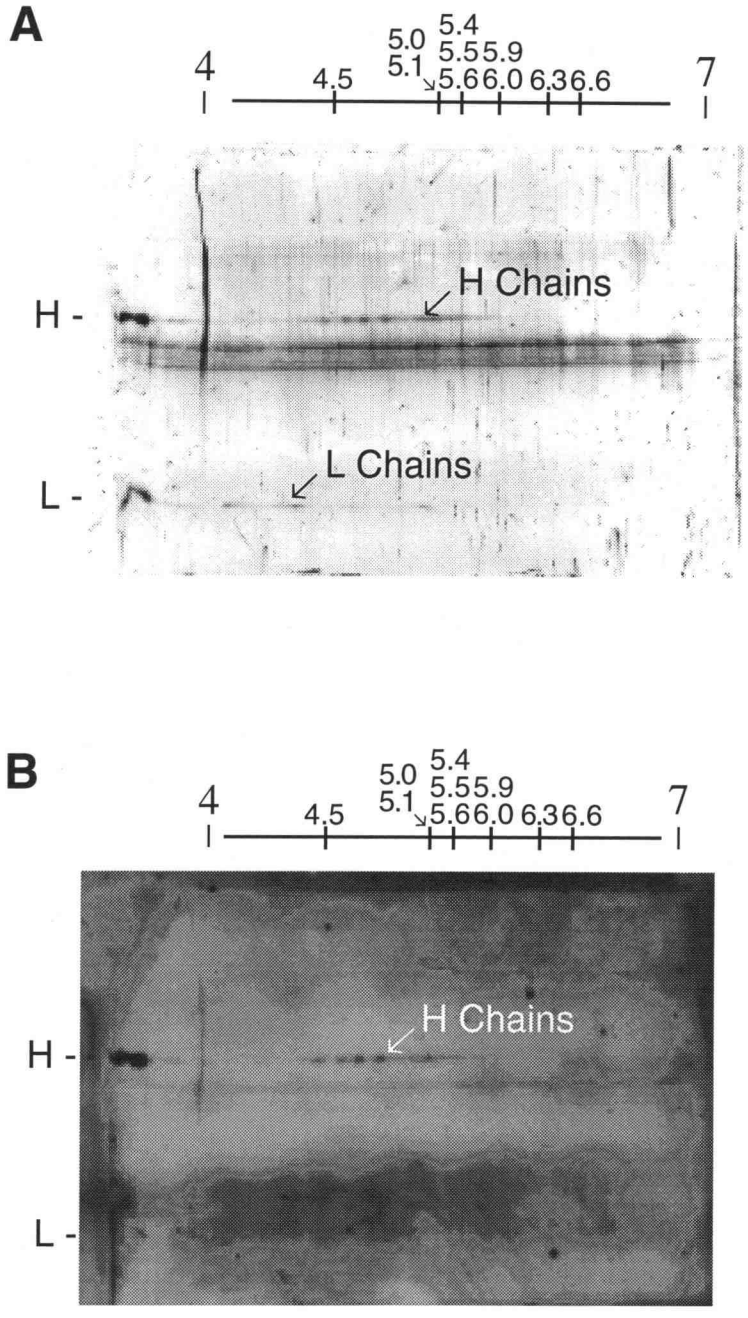


Figure 7. Two-dimensional electrophoretic separation of anti-TNP antibodies from a mixture of trout antisera (#395). Antibodies were electrophoresed in a pH 4 - 7 gradient and blotted onto PVDF membranes as described in the text. Figure (A) shows the proteins on PVDF stained with a colloidal gold dye. Figure (B) is a Western blot using monoclonal 1.14 of the same transfer. The control lane on the left of Figure A shows the relative molecular weights of heavy and light chain. Eight heavy chain and five light chain electropherotypes were identified.

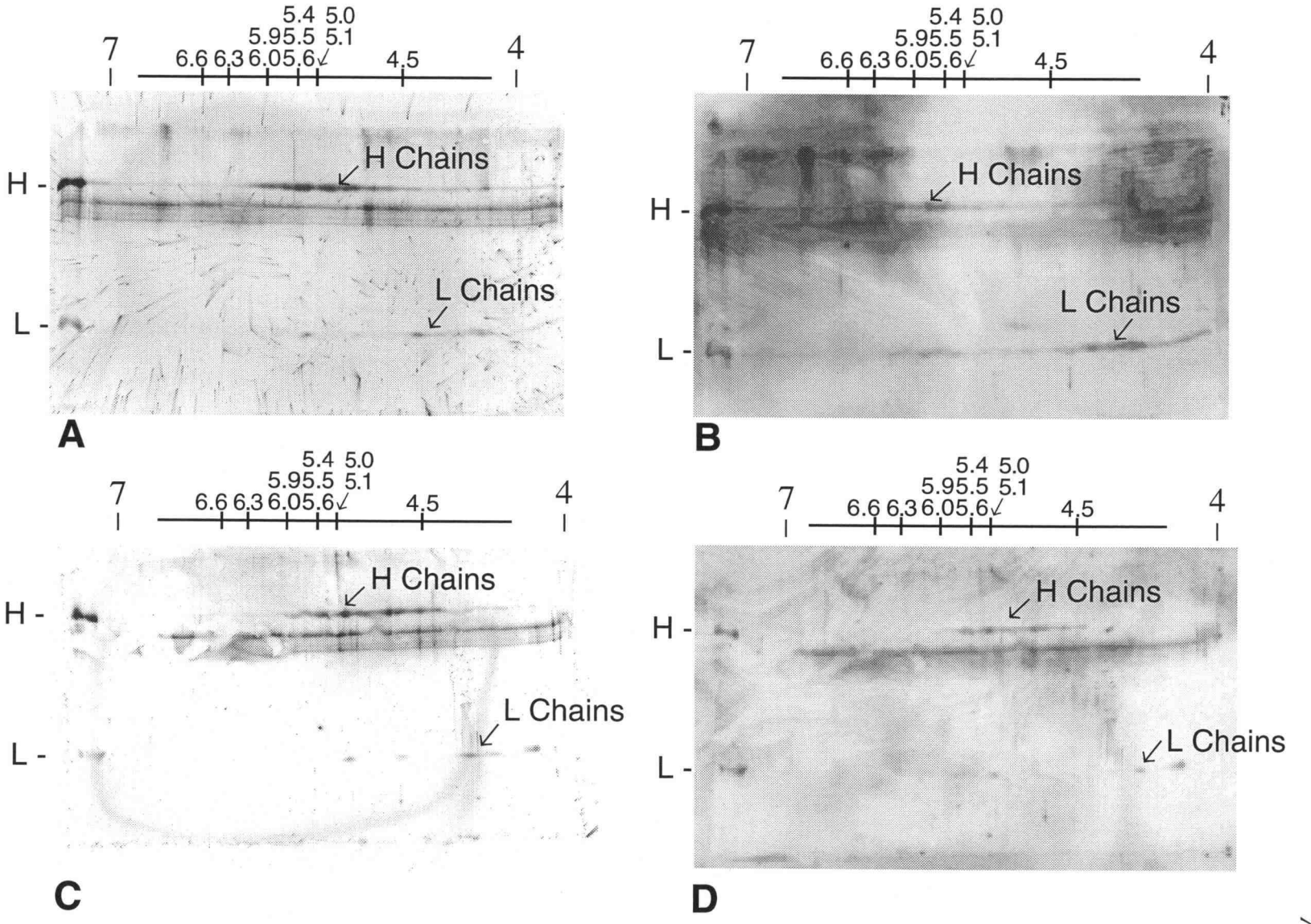
rainbow trout heavy chain epitopes. A panel of anti-trout monoclonal antibodies were developed, each binding an epitope on rainbow trout heavy chain. A standard immunopurified antibody preparation (#395) was electrophoresed and immunoblotted as previously described. Each of the purified monoclonal antibodies was used to detect immunoglobulin heavy chains by immunoblotting. One monoclonal antibody (12F8) had significant cross-reactivity with a light chain epitope (Figures 8A and 8B). Another monoclonal (9H12) showed minor cross-reactivity with light chain (Figures 8C and 8D). The sensitivity of the assay was sufficient to detect even minor H chains (those present at very low concentrations) using all of the monoclonals. There appeared to be only one isotype as defined by binding of each of the monoclonals; no one monoclonal bound a subset of the heavy chains. There was no advantage to using any of the newly developed monoclonal antibodies over the 1.14 antibody. Cross reactivity with light chains was inconsequential in establishing the ELISA; the only requirement was for recognition of all clonotypes. Thus, the 1.14 monoclonal was used in all future ELISA protocols to detect rainbow trout Ig.

Plate Antigen Saturation

TNP-BSA ELISA plate saturation was determined using the protocol described in Muñoz et al., 1986. A plot of absorbance at 405 nm vs TNP-BSA coating concentration (Figure 9) yielded the theoretical saturation

Figure 8. Staining of rainbow trout antibodies by two representative monoclonal antibodies, 12F8 and 9H12. Trout anti-TNP preparation #395 was electrophoresed as described in the text. Heavy and light chain protein spots were stained with colloidal gold (A and C). Monoclonal antibody 12F8 (B) showed significant binding to rainbow trout light chain as determined by Western blot, while 9H12 (D) showed lower binding activity by Western blot for the same antibody preparation.

Figure 8



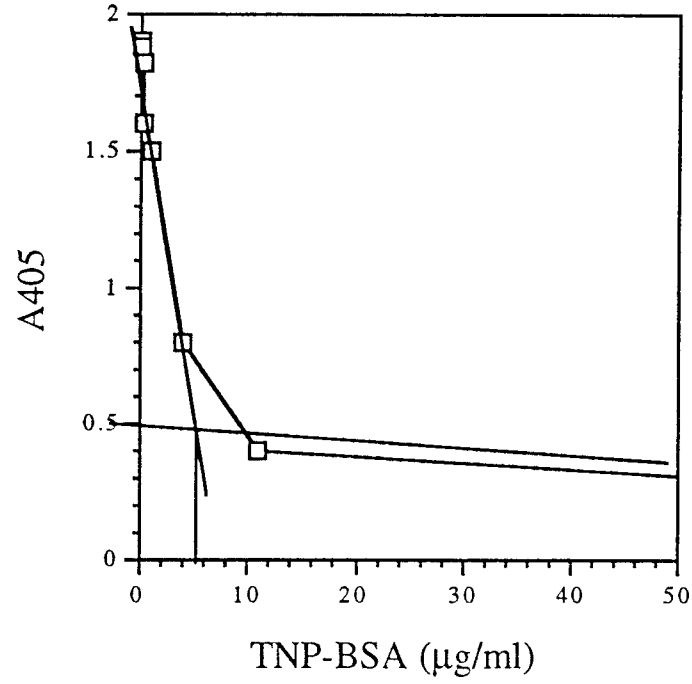


Figure 9. Determination of surface saturating antigen concentration by peroxidase saturation. Plates were coated using serial dilutions of TNP-BSA. Non-occupied sites were determined by peroxidase saturation. Regression lines for decreasing and constant OD readings were calculated and surface-saturating antigen concentration was calculated as the intersection between both regression lines. Technique from Muñoz et al., 1986.

concentration, approximately 6 µg/ml. This value assisted in the determination of the upper limit of plate coating concentration. Actual coating concentrations were determined for each set of assay conditions (early vs late bleed analyses, mouse vs trout antibody analyses, etc.) A practical upper limit for plate coating antigen concentration was found to be 50 µg/ml, as determined by checkerboard titration of antigen with positive control antibody.

Typical Affinity Profiles: Fine Resolution of Antibody Subpopulations

The microtiter plate affinity ELISA yields valuable information about the presence or absence of particular affinity subpopulations within a sample. Two sera might have similar average affinity constants (aKs), but be composed of completely different antibody affinity subpopulations. Sera and supernatant sample affinity distributions were analyzed by plotting each antibody subpopulation as a percentage of the total. A typical affinity profile from an immune serum is shown in Figure 10.

Determination of Plate Coating Antigen Concentrations

Optimization of the plate coating antigen concentration is essential to extract all of the information contained in an affinity analysis. If the coating antigen concentrations are too high to distinguish high affinity clone populations, the resulting distribution profile resembles Figure 11A. From

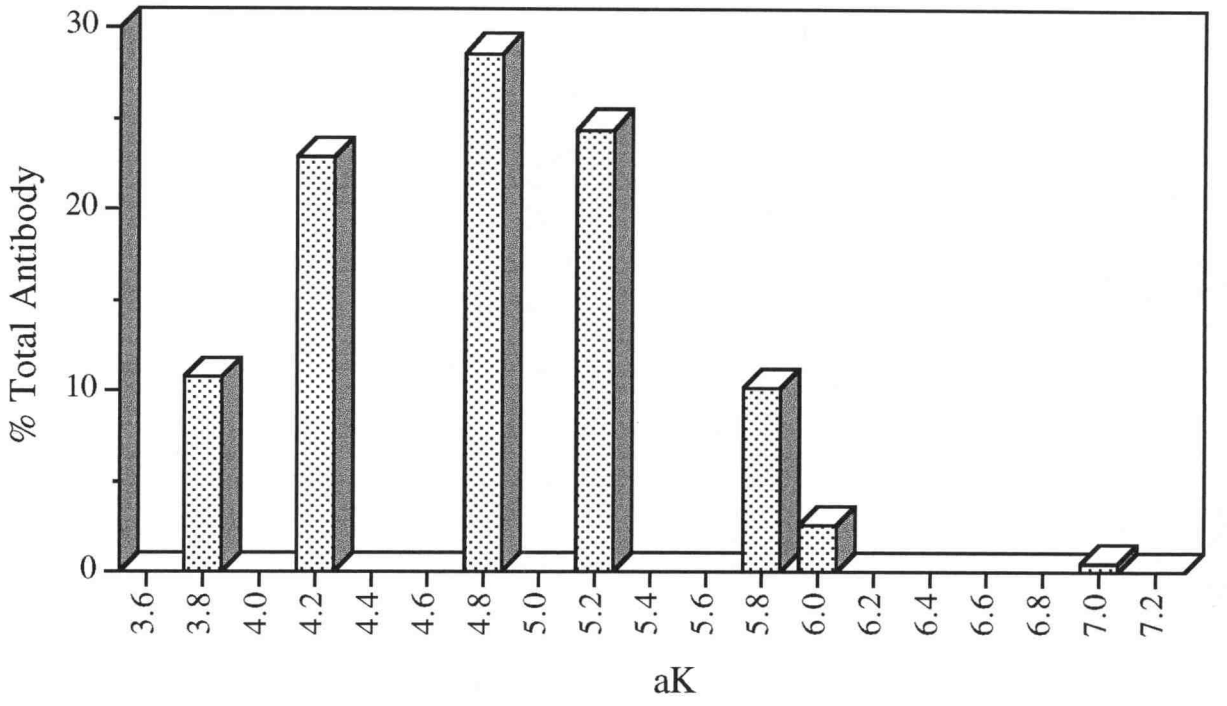


Figure 10. Typical antibody affinity distribution from a rainbow trout anti-TNP serum. The affinity ELISA subdivides the mixture of antibodies in serum into discrete subpopulations. Subpopulation affinities (abscissa) are inversely proportional to the concentration of free haptenic inhibitor required to block 50% of antibody binding in each row of the assay. Subpopulation percentages (ordinate) are proportional to the decrease in absorbance due to loss of (lower affinity) antibody binding at progressively decreasing coating antigen concentrations.

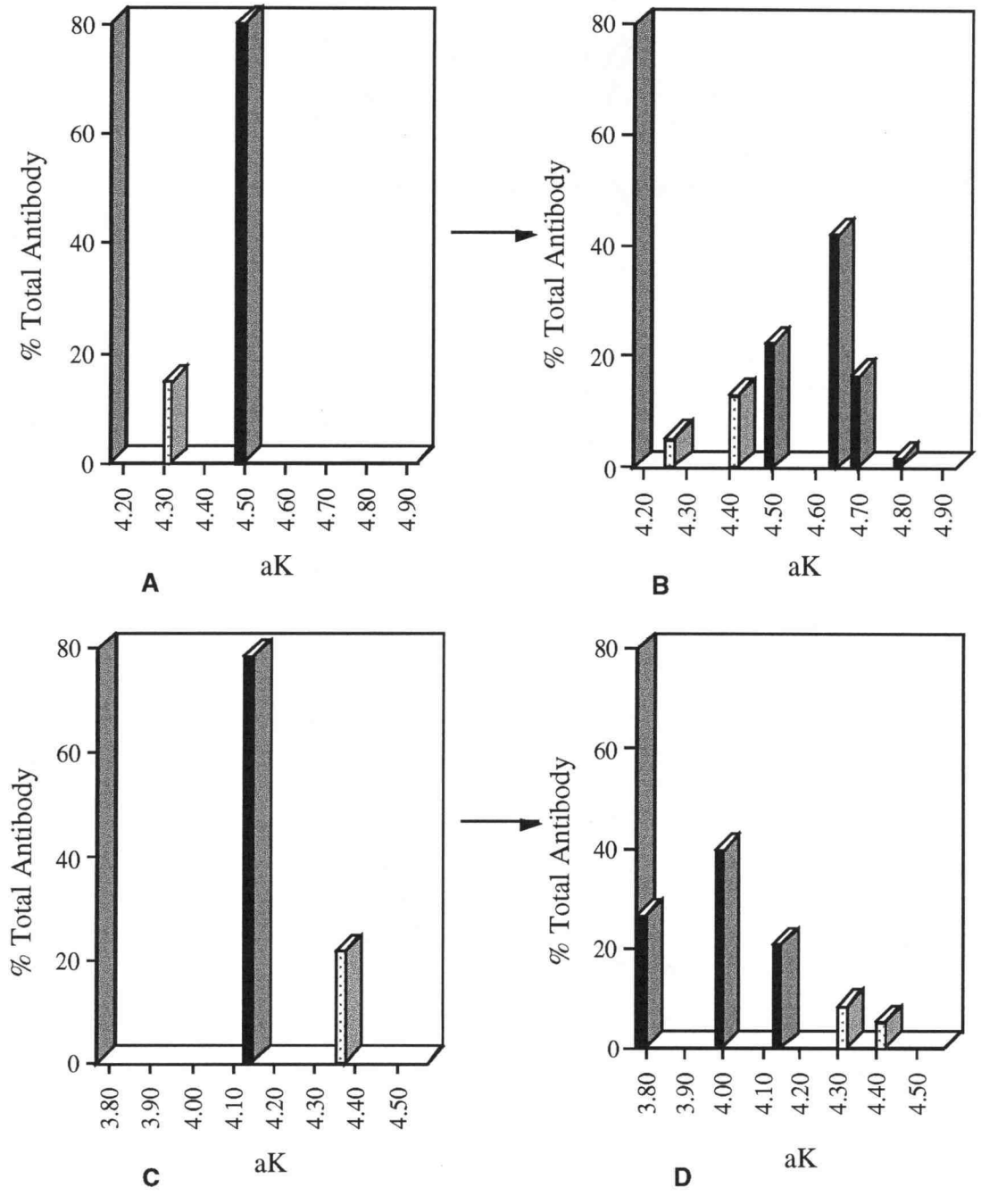


Figure 11. Effects of improper plate antigen coating concentrations. The result of a high affinity serum analyzed on a plate in which coating antigen concentrations are too high is shown in 11A. The same serum analyzed on a plate with reduced coating antigen concentrations and/or increased dilution rates is shown in (B). A low affinity serum analyzed on a plate with too little coating antigen (C) will generate a more finely resolved affinity profile if analyzed on a plate containing higher concentrations of coating antigen and/or a lower dilution rate (D).

this graph, one can determine only that approximately 80% of the antibodies have affinities above 4.5 (solid bars). Applying a lower concentration of plate coating antigen to the initial row and/or increasing the serial dilution scheme of coating antigen reveals more detailed information about the highest 80% of the antibody affinities in the population. Figure 11B shows the resulting distribution in which the highest 80% of the antibody population is dissected into four smaller subpopulations. Care must be taken, however, in analyzing the significance of small changes. Figure 11C shows the reverse situation in which low initial plate coating antigen concentrations restrict low affinity antibodies from binding. Increasing plate coating antigen concentrations and adjusting the serial dilution scheme accordingly resolves the lowest affinity antibodies into a series of smaller, discrete affinity subpopulations (Figure 11D).

Assay Repeatability

Confidence in assay repeatability was determined by examining repeated affinity measurements on antibodies from immune sera. The sera were generated from Oregon-reared trout receiving one injection of 100 μg TNP-KLH in Freund's complete adjuvant. Sera were selected from various time points during the immune response. Groups of sera were analyzed by up to six different investigators on different days using common (shared) stock reagents (TNP-BSA, TNP-LYS, biotinylated 1.14, SA-HRPO, BSA/TTBS).

Two analyses were performed. In the first set of experiments, 16 sera were given to a panel of investigators, each of whom determined the affinity profile and weighted average affinity for each serum. The mean and variance for the calculated average affinities from each serum are shown in the first half of Table II. Alternatively, 15 individual sera were analyzed either in duplicate or triplicate. Means and variances were tabulated for these repeats and are shown in the right half of Table II. Group variance for the analysis of an individual serum ranged from 0.0 to 0.14, while individual repeats had a slightly tighter clustering of values, deviating from 0.0 to 0.07.

Trivial assay conditions have a small effect on the antibody subpopulations which bind to each row of the ELISA plate; thus, the calculated affinity of the population bound in any given row may deviate slightly from assay to assay. Plotting the distribution, however, is sufficient to highlight the existence of clonal populations absent in other samples. A sample triplicate analysis is shown in Figure 12.

Alternate Ways of Expressing Antibody Affinity Data

Affinity subpopulations falling within 0.5 aK units were grouped to conveniently summarize the affinity composition of an entire serum antibody population. Figure 13 shows this analysis applied to the distribution profiles from Figure 12.

Table II. Repeat analysis of sera.

Group Analysis [1]			Individual Repeats [2]		
Sample	n	K [3]	Sample	n	K [3]
1	5	4.89 ± 0.10	1	2	4.88 ± 0.03
2	5	4.93 ± 0.02	2	3	5.47 ± 0.01
3	5	5.22 ± 0.08	3	3	4.78 ± 0.01
4	6	5.02 ± 0.14	4	3	4.81 ± 0.01
5	5	5.49 ± 0.02	5	3	5.02 ± 0.01
6	5	5.67 ± 0.07	6	3	4.98 ± 0.01
7	4	4.88 ± 0	7	2	4.90 ± 0.01
8	5	5.13 ± 0.02	8	2	4.77 ± 0.01
9	5	4.65 ± 0.01	9	3	5.58 ± 0.01
10	4	4.62 ± 0.01	10	3	5.66 ± 0
11	6	4.77 ± 0.01	11	2	5.28 ± 0.07
12	6	6.03 ± 0	12	3	5.24 ± 0.03
13	6	5.95 ± 0	13	2	4.50 ± 0
14	5	5.09 ± 0	14	3	5.72 ± 0.01
15	6	5.58 ± 0.03	15	3	5.56 ± 0.06
16	4	4.80 ± 0.01			

[1] Serum samples were analyzed by n different investigators. Group analysis sample numbers are independent of individual repeats.

[2] Sera were analyzed in duplicate or triplicate.

[3] Weighted average affinity ± variance.

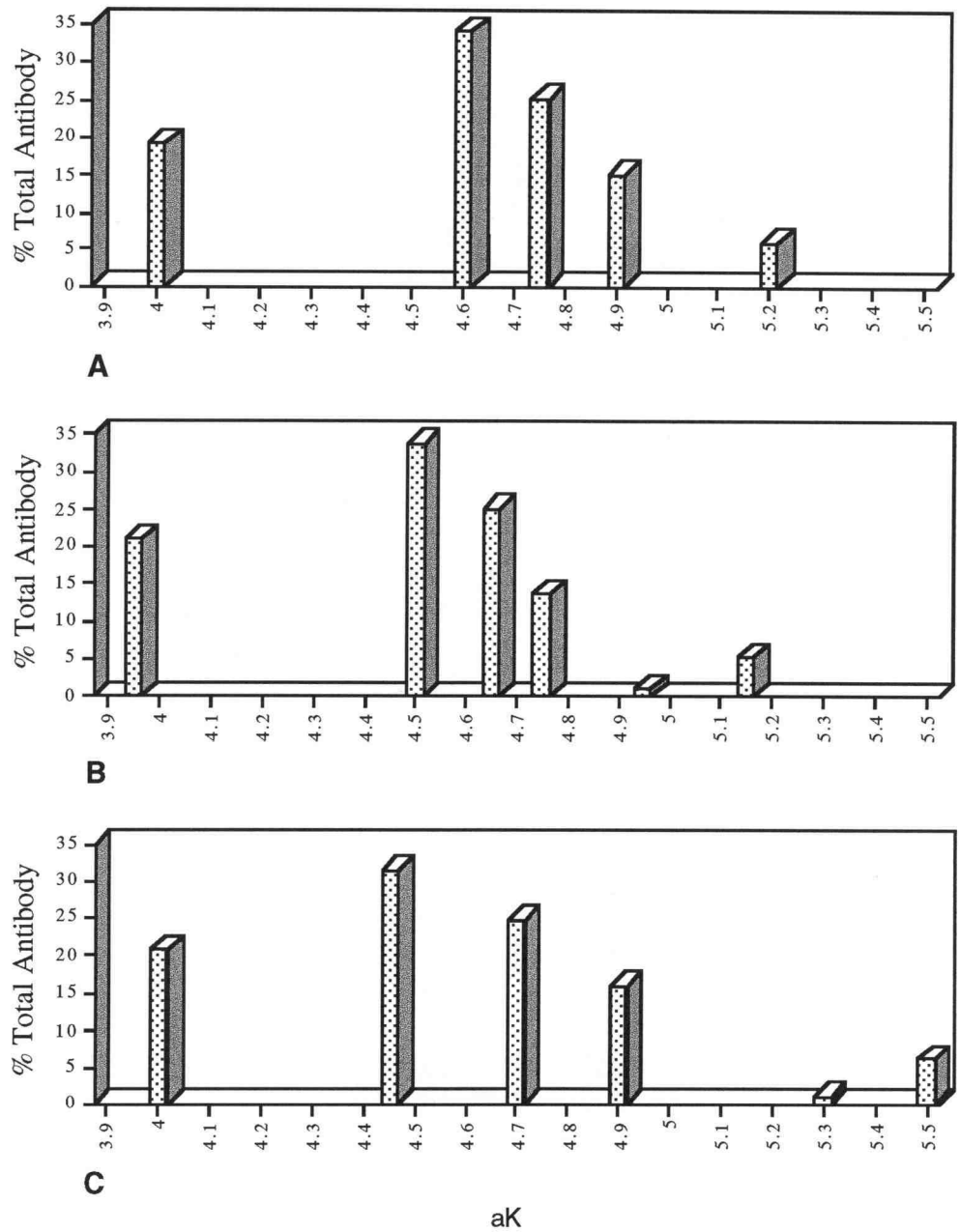


Figure 12. ELISA affinity analyses (A, B, C) of a trout anti-TNP serum. Triplicate analyses of trout immune sera were performed in an effort to determine the repeatability of the assay. Bars denote the percentage of the antibody population (ordinate) represented by each affinity subpopulation (abscissa).

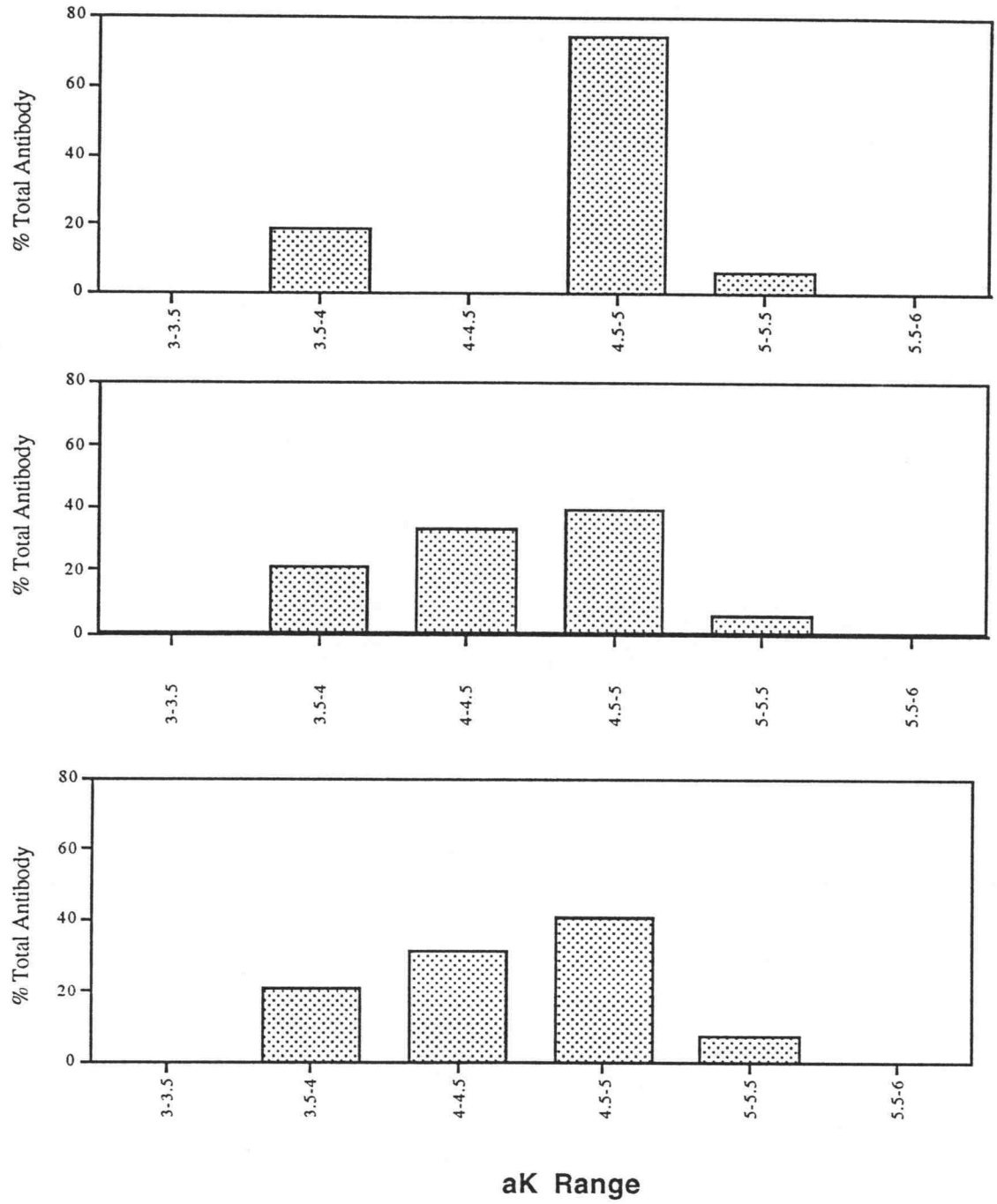


Figure 13. Histograms depicting the percentage of total antibody within arbitrary aK ranges. Apparent affinity constants from within sera were grouped into arbitrary 0.5 aK units. The percent of the total antibody within each aK range was plotted. The triplicate analyses from Fig. 12 (A, B, C) are re-analyzed by this method.

Each serum has an intrinsic binding activity (units/μl) which was calculated by ELISA prior to running the affinity analysis to normalize the amount of anti-TNP activity used in each assay. Thus, each subpopulation represents a percentage of the total activity for that sample. Maximum information is gleaned from a plot containing information about the distribution of both antibody binding activity within a sample and the breakdown of affinity subpopulations. These parameters are plotted three-dimensionally in Figure 14. In this example, repeated bleeds from an immunized trout were followed over time and analyzed for both titer and affinity distribution. The figure clearly shows both an increase in titer between the prebleed and the first bleed at week 9 demonstrated by the logarithmic increase in bar height. There is also an increase in affinity, demonstrated by the relative locations of affinity subpopulations along the X-axis.

Assessment of Affinity Maturation in Rainbow Trout

Affinity Distributions During Primary and Secondary Responses to TNP-KLH

The affinity ELISA was used to monitor antibody affinity changes over time after immunization (affinity maturation) in repeated bleed sera from individual trout. Sera from trout were collected prior to immunization, at the peak of the primary response, week 9, and during and after the secondary

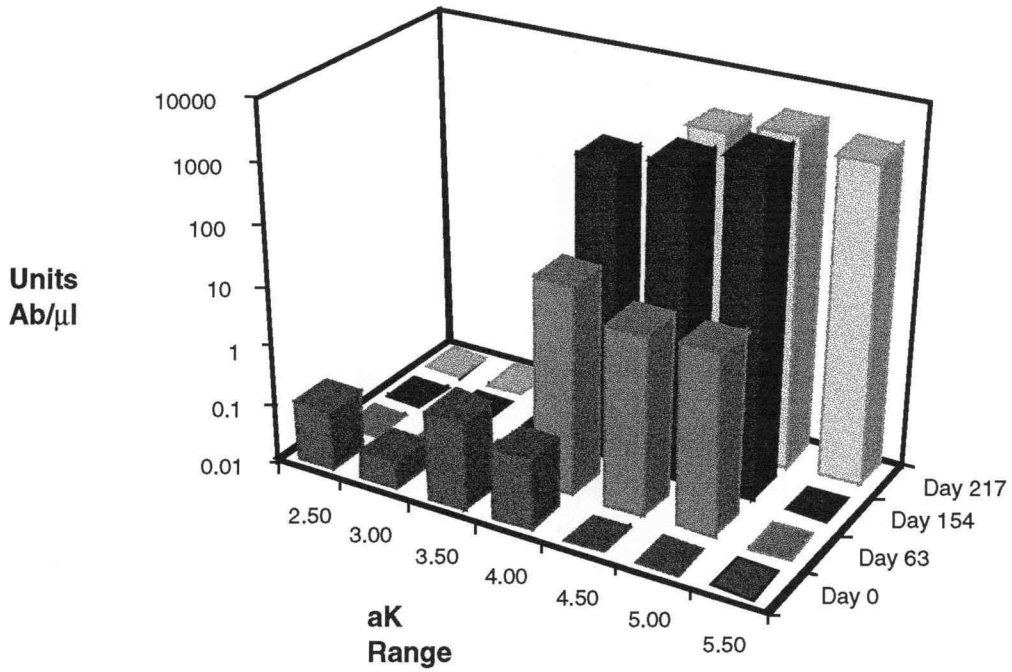


Figure 14. Three dimensional histogram analysis. Three dimensional histograms conveniently summarize both the titer and affinity distribution of repeated bleeds. Bar height (z-axis) represents units of antibody activity possessing the indicated affinity range (x-axis).

response, weeks 22 and 31 as described in the Materials and Methods section. Serum antibody levels and affinity distributions were determined as described above. The data were plotted as described in the previous section to show the relationships between time, affinity, and titer. Six representative distribution analyses are shown in Figures 15A-15F. Affinities in prebleeds ranged from $10^{2.5}$ to $10^{3.5}$ liters mole^{-1}. This was consistent among fish. By nine weeks post immunization, titers had increased significantly, roughly 100 to 10,000-fold over prebleed levels. Similarly, antibodies with affinities 10 to 100 times greater than those in prebleed sera appeared at week 9. Antibody affinity increases slowed considerably after week 9. There was little if any further increase in average affinity two weeks post secondary immunization (week 22) or late in the secondary response (week 31). Some trout even demonstrated a small decrease in average affinity during the secondary response (Figures 15B and 15D).

Early Resolution of Affinity Maturation (3 week)

The majority of the increase in antibody affinity clearly occurred within the first nine weeks post immunization. An experiment was conducted in which enough serum was collected from each of nine immunized rainbow trout to analyze the appearance of high affinity clones early after immunization (within the first three weeks). Immune trout produced antibodies with affinities greater than 10^4 L mole^{-1} as early as three weeks post

Figure 15. Demonstration of affinity maturation of the rainbow trout anti-TNP response. The distribution of antibody affinities for six representative rainbow trout at four time points during the antibody response is shown (A-F). All trout exhibited affinity subpopulations at 9 weeks post immunization which were not present in prebleed sera.

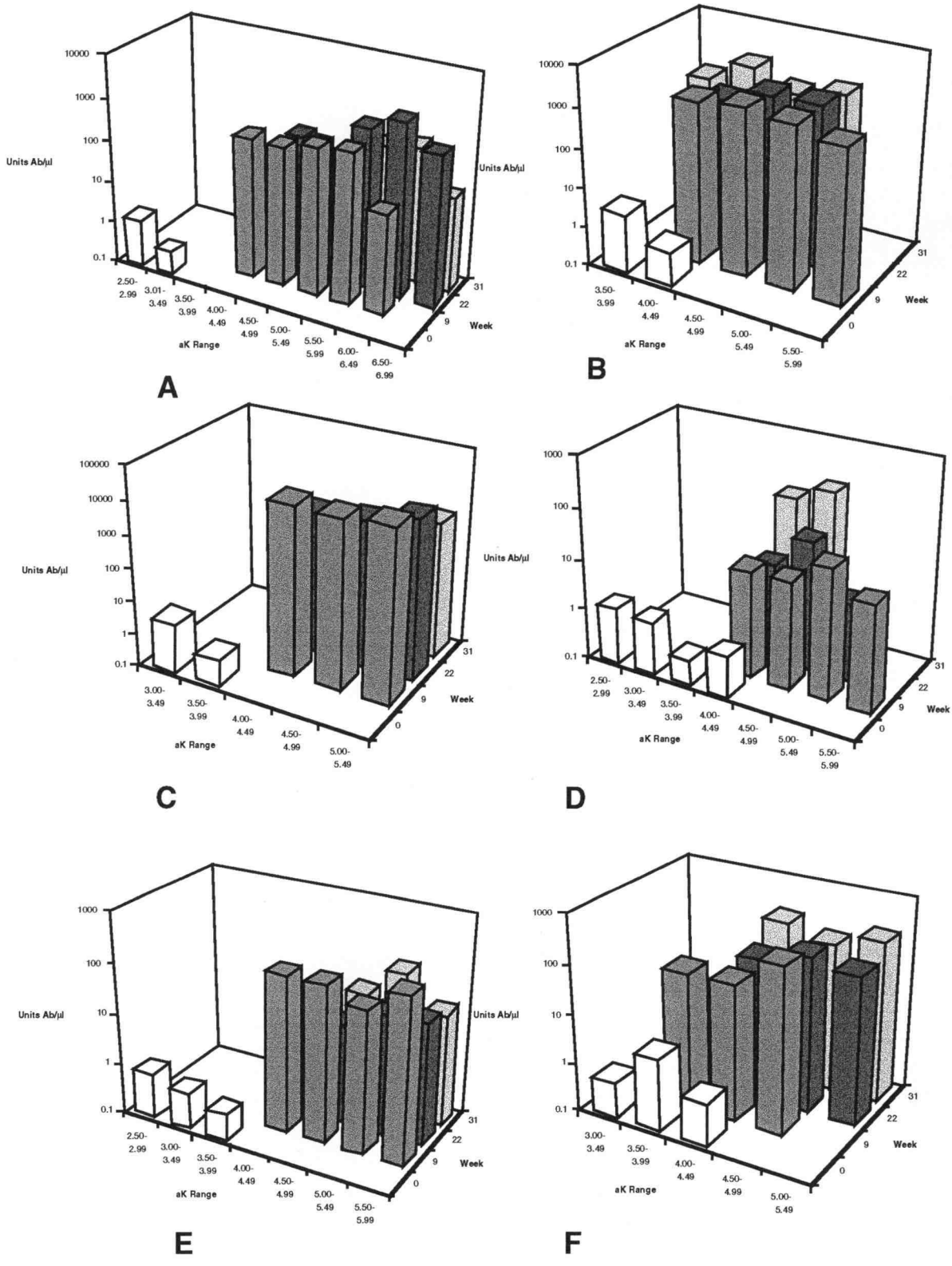

Units Ab/µl
10000
1000
100
10
1
0.1
2.50-2.99
3.01-3.49
3.50-3.99
4.00-4.49
4.50-4.99
5.00-5.49
5.50-5.99
6.00-6.49
6.50-6.99
aK Range
Week
0
9
22
31
A
3.50-3.99
4.00-4.49
4.50-4.99
5.00-5.49
5.50-5.99
B
100000
3.00-3.49
C
2.50-2.99
D
E
F

Figure 15

immunization (Figures 16A-I). A sham (PBS/Freund's emulsification) immunized trout showed no increase in antibody affinity over time (Figure 16J). Group average affinities were calculated for both time points. These are summarized in Table III.

Application of the Affinity Assay to a Pathologic Situation: AFB_1 Immunotoxicity

Four groups of rainbow trout were exposed embryonically to graded doses of aflatoxin B_1 or a carrier control. Subsets of each aflatoxin treatment group were immunized with either a T-dependent form of antigen (TNP-KLH) or a T-independent form of antigen (TNP-LPS). Prior to immunization, and at weeks 9, 22, and 31 post immunization, serum samples were obtained for antibody affinity analyses. The average affinity for each trout serum was determined using the affinity ELISA (not shown). Mean group affinities were compared by ANOVA. Table IV summarizes mean group affinities ± standard errors of the mean. Differences in mean group affinities were reported as significant when $p \leq 0.05$. The only two groups differing significantly in group affinity were the 1 ppm TNP-KLH immunized group and the control TNP-KLH group at the earliest bleed, week 9 ($p=0.011$). By week 22, the control group produced affinities indistinguishable from all aflatoxin treatment group affinities. This "retardation" of affinity maturation was seen only in the T-dependent response to antigen. All secondary

Figure 16. Affinity distributions at three weeks post immunization in trout immunized with TNP-KLH in reund's Complete Adjuvant. Affinity distributions were assessed prior to immunization and three weeks post immunization in 9 normal trout (A-I) and one control trout (J), immunized with PBS in Freund's adjuvant. Bars represent the percent of total antibody within a serum sample at the indicated aK at week 0 (■) and week 3 (▯).

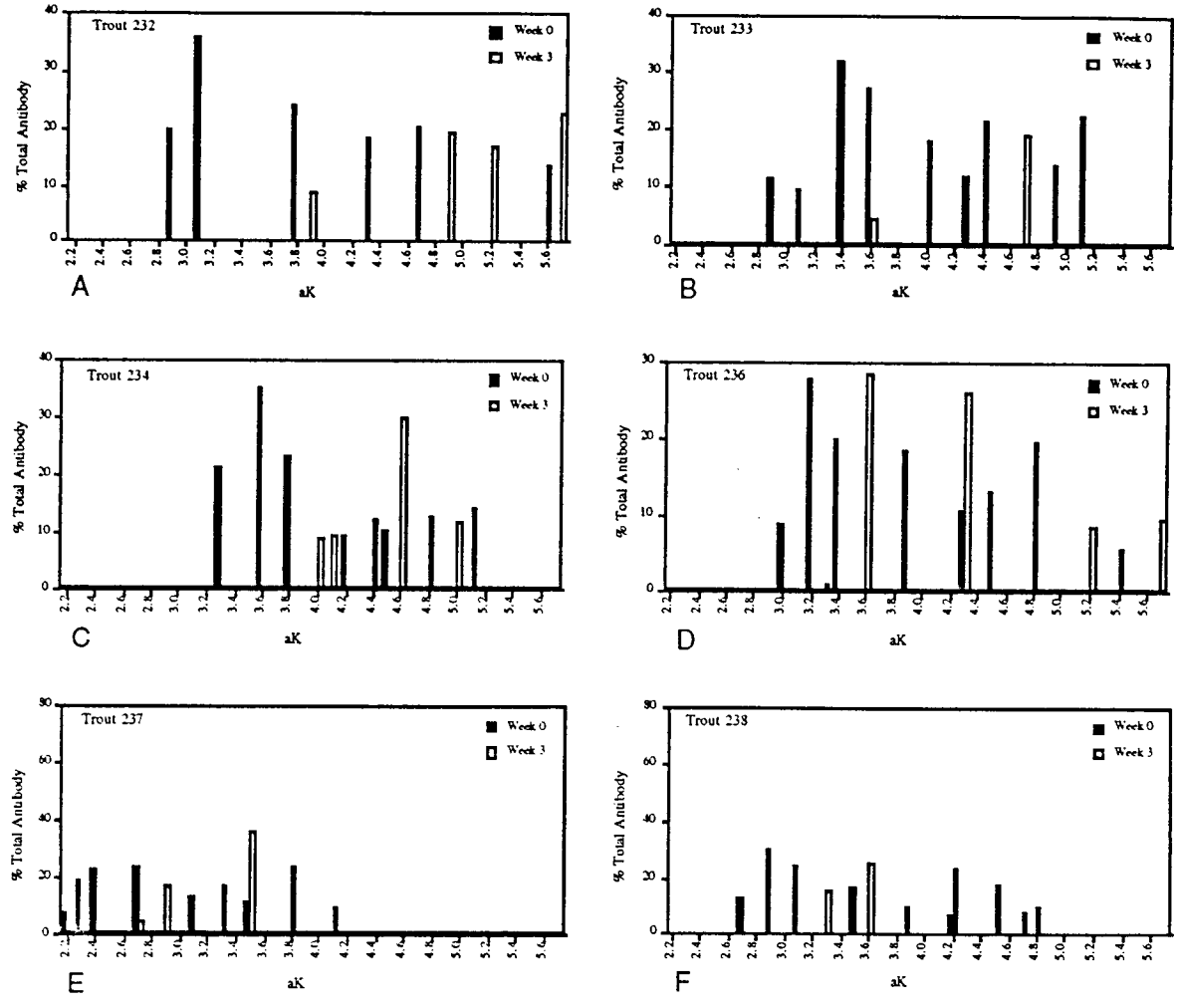

Trout 232
Week 0
Week 3
% Total Antibody
aK
A
Trout 233
B
Trout 234
C
Trout 236
D
Trout 237
E
Trout 238
F

Figure 16

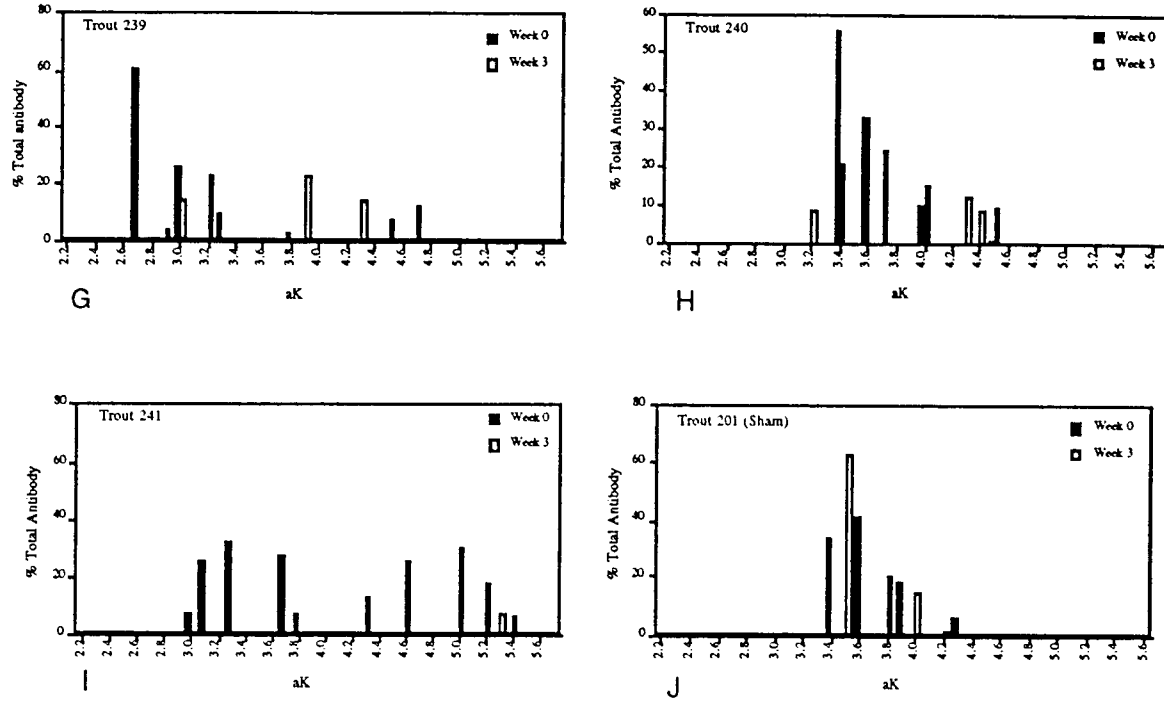


Figure 16 (cont'd)

Table III. Trout afffinities during the first three weeks post immunization.

Time [1]	n	Affinity [2]
Week 0	9	3.50 ± 0.067
Week 3	9	4.53 ± 0.12 [3]

1 Weeks post immunization
2 Group mean average affinity ± std. error of the mean
3 Significant difference (p=<0.0001, Chi-square) between week 3 and prebleed average affinity

Table IV. The effect of aflatoxin exposure on rainbow trout primary and secondary antibody response affinities.

Time [1]	Immunogen [2]	n	AFB_1 [3]	Group K [4]
9	TNP-KLH	12	Control	5.30 ± 0.085 [5]
	TNP-KLH	8	1 ppm	4.89 ± 0.123 [5]
	TNP-LPS	6	Control	4.94 ± 0.125
	TNP-LPS	7	1 ppm	4.86 ± 0.156
22	TNP-KLH	10	Control	5.45 ± 0.066
	TNP-KLH	8	1 ppm	5.23 ± 0.140
	TNP-LPS	6	Control	5.09 ± 0.079
	TNP-LPS	6	1 ppm	5.00 ± 0.202
31 [6]	TNP-KLH	11	Control	5.52 ± 0.072
	TNP-KLH	7	1 ppm	5.39 ± 0.171
	TNP-LPS	6	Control	4.96 ± 0.141
	TNP-LPS	7	1 ppm	5.25 ± 0.096

[1] Weeks post 1° immunization
[2] 50 μg (i.p.) in Freund's adjuvant
[3] 60 min. bath exposure at the eyed egg (embryo) stage
[4] Mean weighted average affinity ± std. error of the mean
[5] Significant difference (p=0.011) between 1 ppm and control
[6] 11 weeks post 2° immunization

response groups (weeks 22 and 31, Table IV) produced antibodies with affinities indistinguishable from each other by ELISA. Average affinities for control animals were in agreement with earlier studies on non-aflatoxin treated fish, both for the kinetics of the response and the measured average affinities (not shown).

The Effect of Immunogen Concentration on Antibody Affinity

Introduction

Consistent with the theory of antigen selection of high affinity B cells (the basis of affinity maturation) is the hypothesis that diminishing concentrations of antigen should elicit higher affinity antibodies. However, demonstration of this was deemed impractical *in vivo.*, as such experiments would require a statistical analysis of the effect of varying immunogen dose on large groups of rainbow trout. Moreover, *in vitro* demonstration of such a mechanism is theoretically possible, and should provide a better controlled system, free from interference from lymphocyte trafficking.

The Effects of Exogenous TNP-LPS on Anti-TNP Titer

Leukocyte cultures were stimulated with serial dilutions of TNP-LPS for 9 days. After a 9 day incubation, anti-TNP activity in the culture supernatants was determined. Two supernatants were selected for re-analysis

in the presence of serial dilutions of exogenous TNP-LPS. TNP-LPS in culture competed with plate-bound TNP-BSA for supernatant antibody. The titer determined at each inhibitory concentration of TNP-LPS is plotted in Figure 17.

This experiment simulated the effect of leaving antigen in culture wells during leukocyte stimulation. Significant inhibition of the calculated titer was seen between 10 and 1000 μg/ml TNP-LPS. Because some experiments required culture levels of TNP-LPS exceeding 1.0 μg/ml, all leukocyte cultures were pulsed with antigen (or mitogen, LPS) for 24 hours, the pulse was removed, and cultures were refed with fresh medium without antigenic or mitogenic stimulation for the remainder of the 9 day incubation period. In this way, the inducing antigen would not interfere with the affinity analysis of the resulting culture supernatants.

In Vitro Dose Response Curves

Dose Response by ELISA

Six replicate experiments were conducted to determine the dose responses for antigenic (TNP-LPS) or mitogenic (LPS) stimulation of peripheral blood leukocytes (PBL) and anterior kidneys cells (AK). Two representative curves are presented for each experiment. The PBL TNP-LPS anti-TNP response peaked at 1.0 μg/ml (Figure 18A), while the LPS anti-TNP

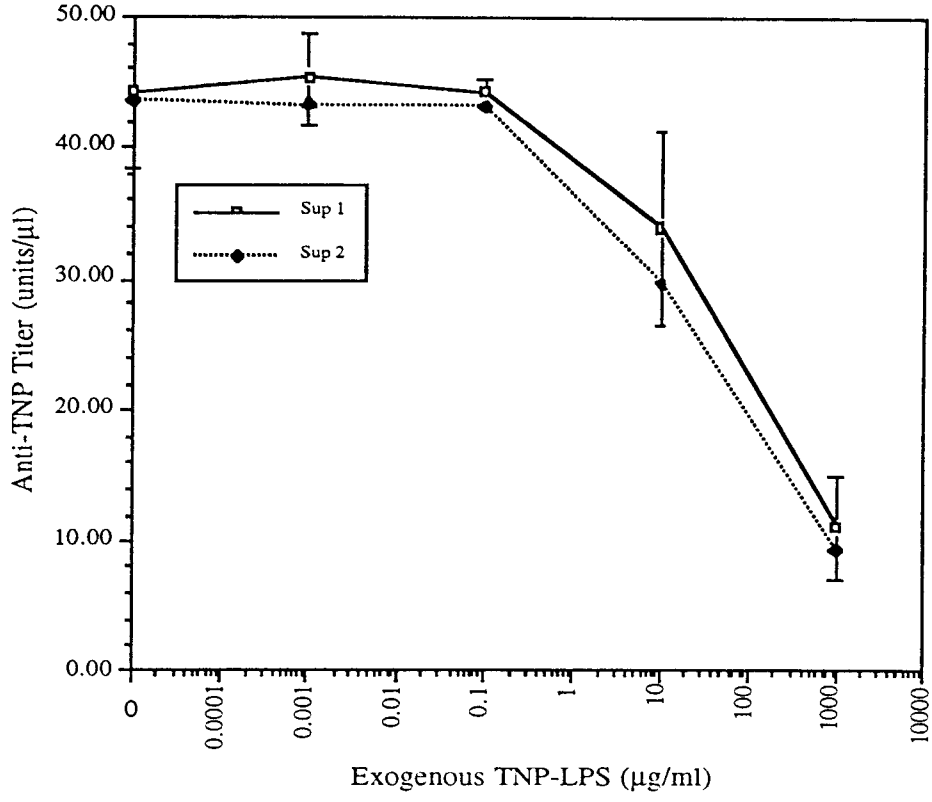


Figure 17. The effect of exogenous TNP-LPS on measurement of anti-TNP titer in leukocyte culture supernatants. Two anti-TNP supernatants from peripheral blook leukocyte cultures were spiked with 100-fold serially decreasing dilutions of TNP-LPS, with subsequent determination of anti-TNP titer. Duplicate titrations were performed on two anti-TNP supernatants at each inhibitor concentration. Vertical bars represent one standard deviation about the mean. TNP-LPS added to supernatants (presumably binding supernatant antibody) significantly affected the measurement of antibody titer above 1 µg/ml ($p<0.01$, ANOVA).

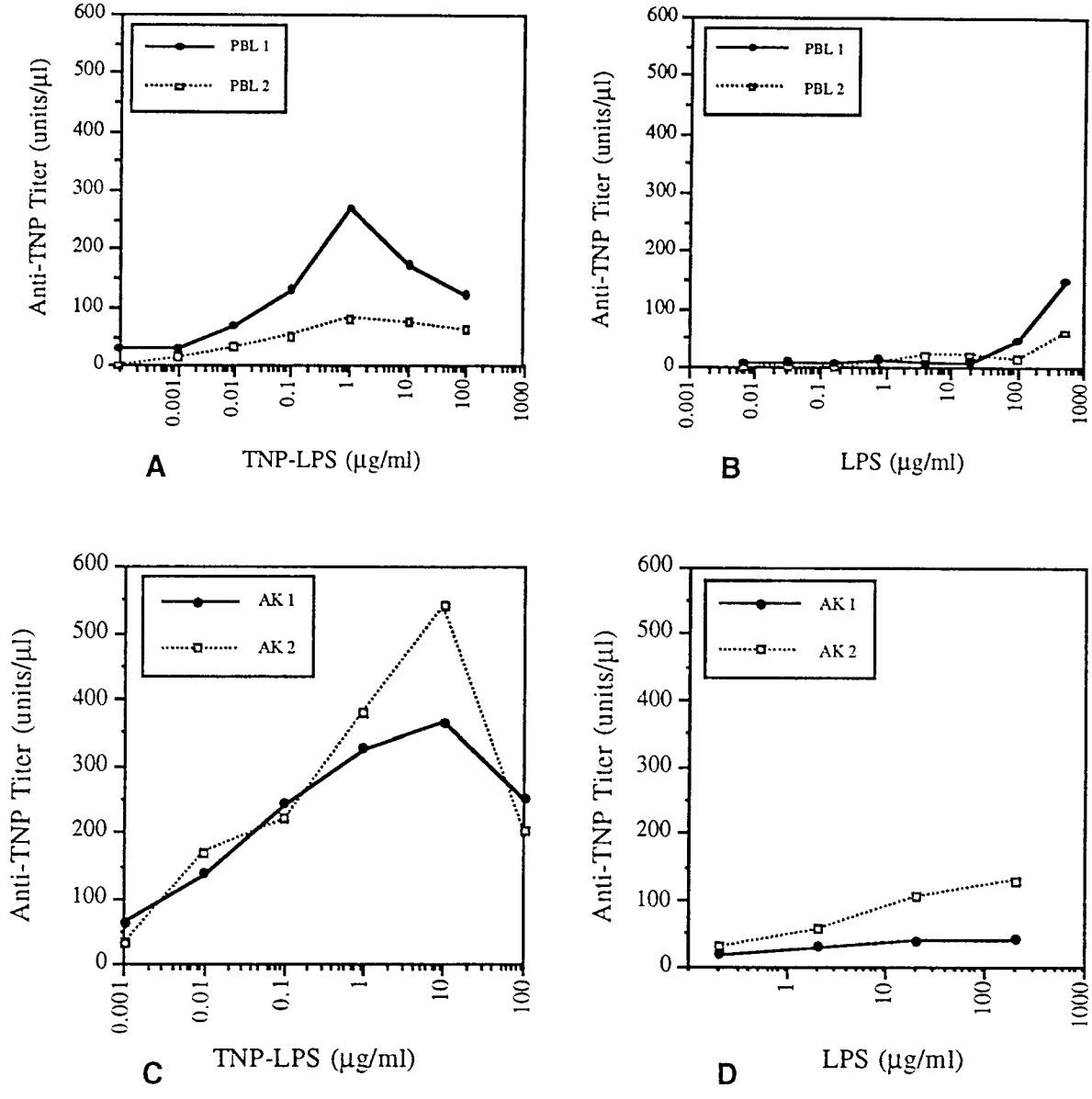


Figure 18. PBL and AK dose response curves. Cultured rainbow trout peripheral blood (A,B) and anterior kidney (C,D) leukocytes were stimulated with serially decreasing doses of TNP-LPS or LPS in duplicate cultures as described in the text. Anti-TNP titers (units of activity/µl) were determined by ELISA and plotted.

response continued to rise past the highest treatment concentration, 500 μg/ml (Figure 18B). Anterior kidney cells responded to antigenic stimulation with a maximum titer at 10 μg/ml (Figure 18C), and continued to increase in titer beyond the highest LPS treatment, 200 μg/ml (Figure 19D). All titers were obtained by combining the supernatants from a minimum of eight wells in order to obtain adequate supernatant volumes. Doses of TNP-LPS below 0.01 μg/ml and of LPS below 4 μg/ml failed to consistently stimulate enough anti-TNP antibody production for supernatant affinity analyses. Thus, these stimulatory doses were attempted only when enough cells were available to yield large enough supernatant volumes to compensate for low titers.

Dose response by the enumeration of plaque forming cells (PFC)

Cells were harvested 9 days after initial antigenic stimulation for determination of the number of anti-TNP producing cells via the Jerne hemolytic plaque assay. The PFC assay independently verified the dose response determined by ELISA. The number of plaques at each antigen dose is plotted in Figure 19. The plaque forming response was biphasic, showing a peak at 1.0 μg/ml and another at 100 μg/ml. It is possible that the high dose peak was due to non-specific, mitogenic stimulation of cells by the LPS moiety of TNP-LPS (as described in Irwin, 1987), but this was not studied further. Below 10 μg/ml, the TNP-LPS PFC dose response mirrored the response as determined by ELISA.

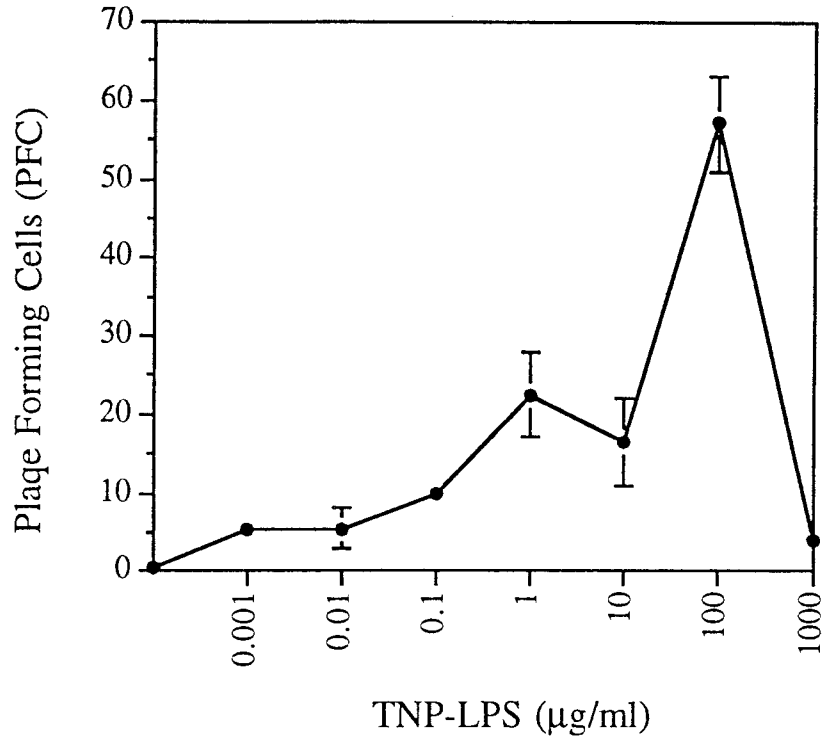


Figure 19. *In vitro* TNP-LPS plaque forming cell dose response. Plaque generation by PBL stimulated with various doses of TNP-LPS were enumerated in duplicate cultures. Vertical bars indicate standard error about the mean.

Dose Dependent Production of High Affinity Antibodies *In Vitro*

Duplicate PBL and AK cultures were pulsed with serial dilutions of antigen (TNP-LPS) or mitogen (LPS). Supernatants were harvested after 9 days. PBL cultures stimulated with between 0.001 and 1.0 μg/ml contained antibodies with affinities significantly higher (typically $10^{4.7}$ - $10^{4.9}$ L mole^{-1}) than those in high dose (>1 μg/ml) wells (Figure 20). Anterior kidney leukocytes showed a similar response pattern to TNP-LPS (Figure 21), producing antibodies of statistically higher affinity in culture wells stimulated with 0.1 μg/ml or less than in cultures stimulated with ≥1 μg/ml. Both PBL and anterior kidney leukocytes responded to all four mitogenic (LPS) doses by producing antibodies of similar affinity (Figures 22 and 23). The affinities produced in LPS stimulated cultures were routinely indistinguishable from the (relatively) low affinity high dose TNP-LPS culture supernatant antibodies, with average affinity constants of approximately $10^{4.4}$ L mole^{-1} in PBL cultures, and $10^{4.2}$ L mole^{-1} in anterior kidney cultures.

In Vitro Antibody Affinity Profiles

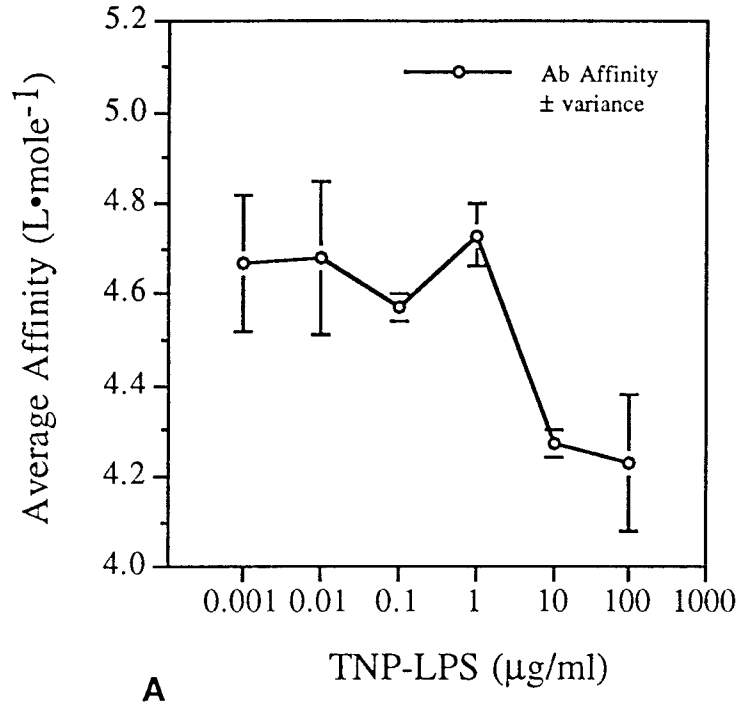
Low dose TNP-LPS stimulation led to the appearance and detection of antibodies of higher affinity than were present in high dose stimulated cultures. Figures 24A and 25A show the gradual detection of high affinity clones in one of the six replicate experiments cited above. In these figures, one can see the participation of high affinity clones to the generation of the

Figure 20. Effect of TNP-LPS dose on peripheral blook leukocyte *in vitro* antibody affinity generation. Peripheral blook leukocyte cultures were stimulated with serially decreasing doses of TNP-LPS. Leukocytes from each trout (A,B) were split into duplicate assays. A minimum of 8 culture wells for each TNP-LPS dose were pooled for analysis. Weighted average affinities for culture supernatant antibodies at each TNP-LPS dose were determined by the affinity ELISA. Vertical bars represent the variance of duplicate cultures from an individual fish.

Figure 21. Effect of TNP-LPS dose on anterior kidney *in vitro* antibody affinity generation. Anterior kidney cultures were stimulated with serially decreasing doses of TNP-LPS. Leukocytes from each trout (A,B) were split into duplicate assays. A minimum of 8 culture wells for each TNP-LPS dose were pooled for analysis. Weighted average affinities for culture supernatant antibodies at each TNP-LPS dose were determined by the affinity ELISA. Vertical bars represent the variance of duplicate cultures from an individual fish.

Figure 22. Effect of LPS dose on peripheral blood leukocyte *in vitro* antibody affinity generation. Peripheral blood leukocyte cultures were stimulated with serially decreasing doses of LPS. Leukocytes from each trout (A,B) were split into duplicate assays. A minimum of 8 culture wells for each LPS dose were pooled for analysis. Weighted average affinities for culture supernatant antibodies at each LPS dose were determined by the affinity ELISA. Vertical bars represent the variance of duplicate cultures from an individual fish.

Figure 23. Effect of LPS dose on anterior kidney *in vitro* antibody affinity generation. Anterior kidney leukocyte cultures were stimulated with serially decreasing doses of LPS. Leukocytes from each trout (A,B) were split into duplicate assays. Weighted average affinities for culture supernatant antibodies at each LPS dose were determined by the affinity ELISA. Vertical bars represent the variance of duplicate cultures from an individual fish.


5.2
5.0
4.8
4.6
4.4
4.2
4.0
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Ab Affinity
± variance
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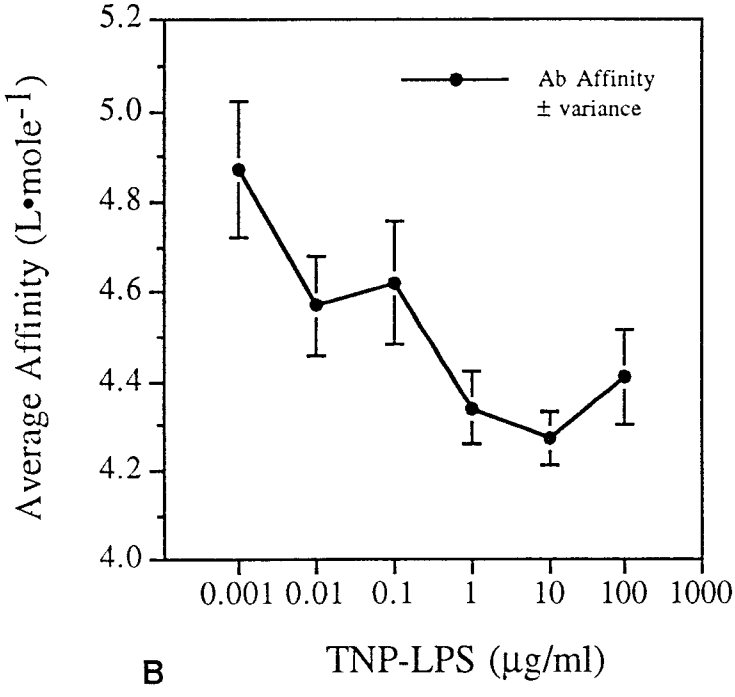
TNP-LPS (µg/ml)
A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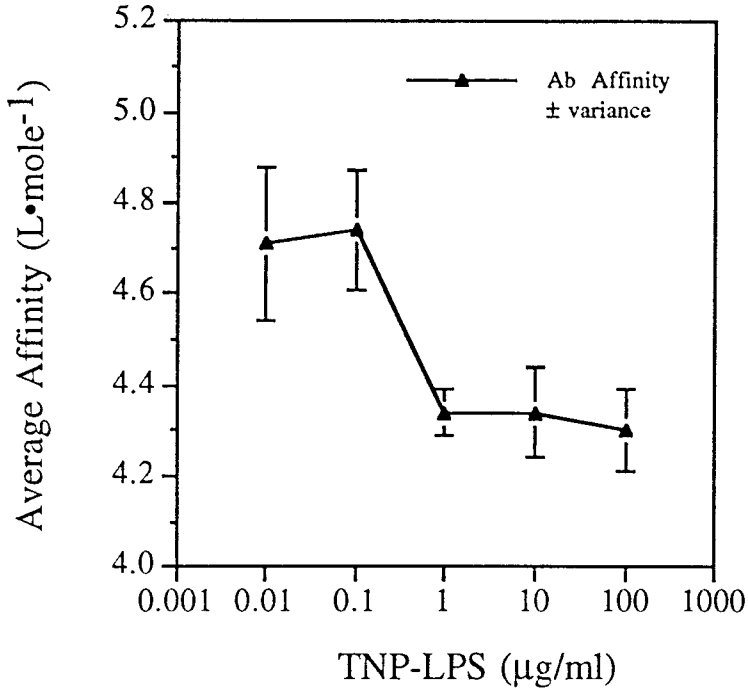
5.0
4.8
4.6
4.4
4.2
4.0
Average Affinity (L•mole-1)
Ab Affinity
± variance
0.001 0.01 0.1 1 10 100 1000
TNP-LPS (µg/ml)
B

Figure 20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Average Affinity (L•mole-1)
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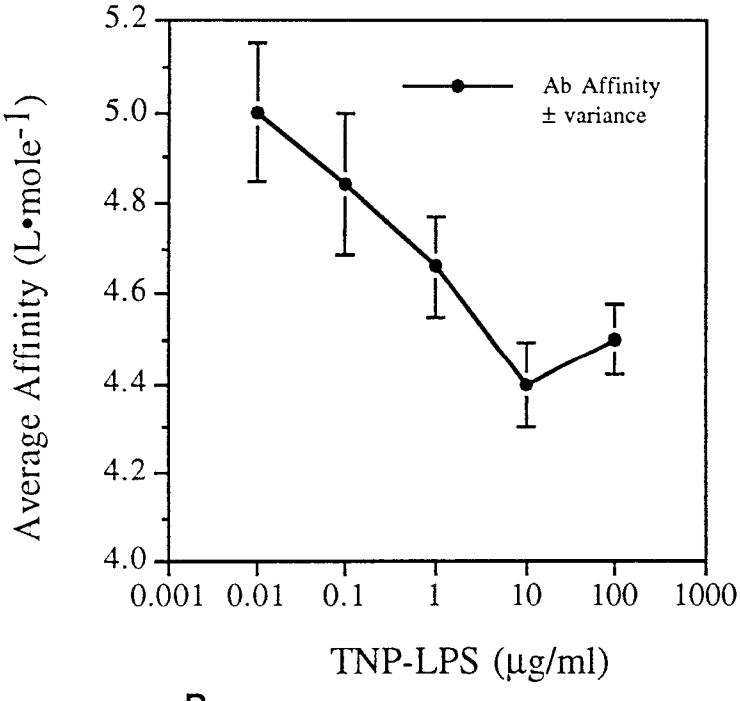
1000
TNP-LPS (µg/ml)

A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Average Affinity (L•mole-1)
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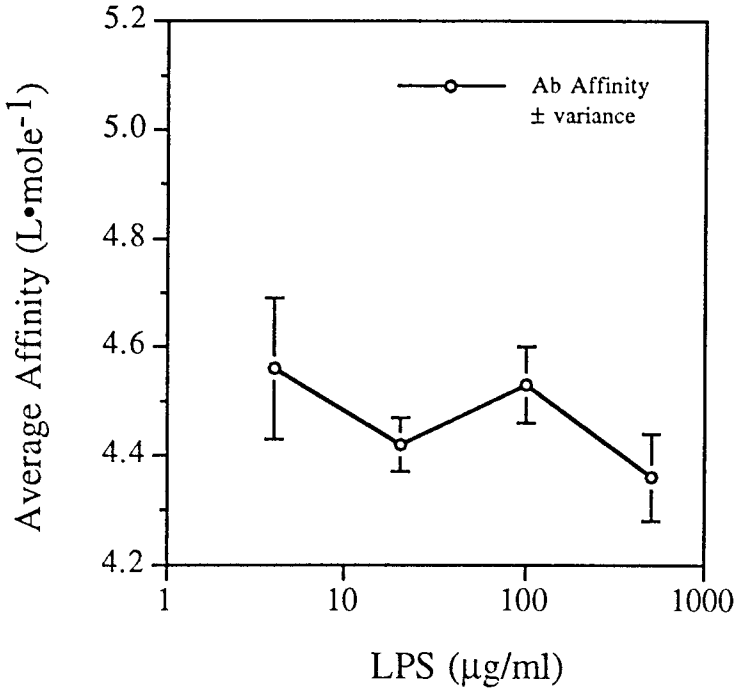
100
1000
TNP-LPS (µg/ml)

B

Figure 21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5.0
4.8
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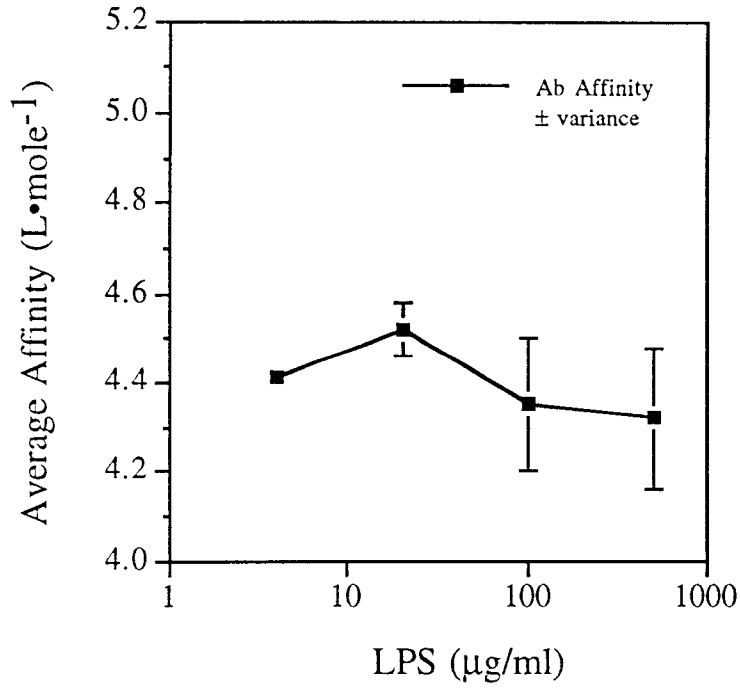
4.2
Average Affinity (L•mole-1)
Ab Affinity
± variance
1
10
100
1000
LPS (µg/ml)

A


5.2
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4.4
4.2
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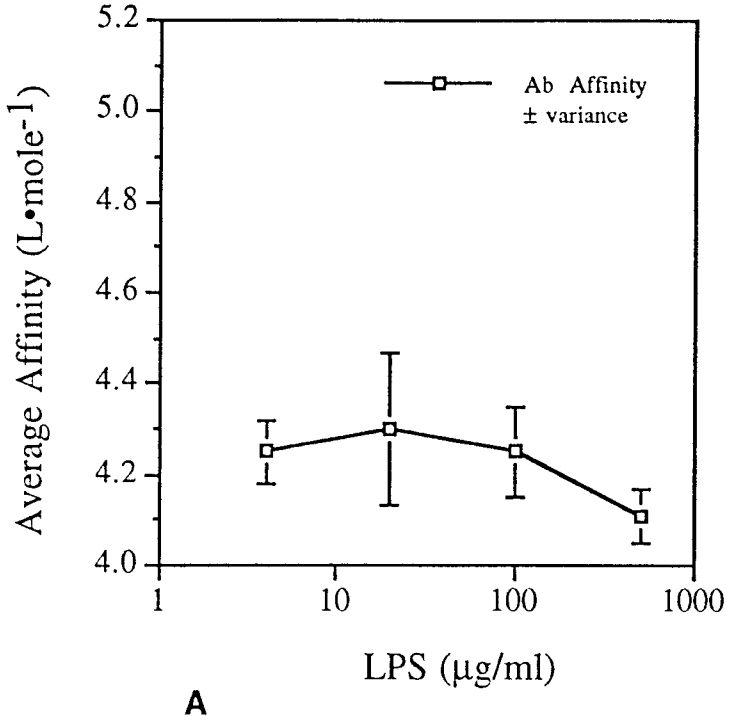
Average Affinity (L•mole-1)
Ab Affinity
± variance
1
10
100
1000
LPS (µg/ml)

B

Figure 22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5.0
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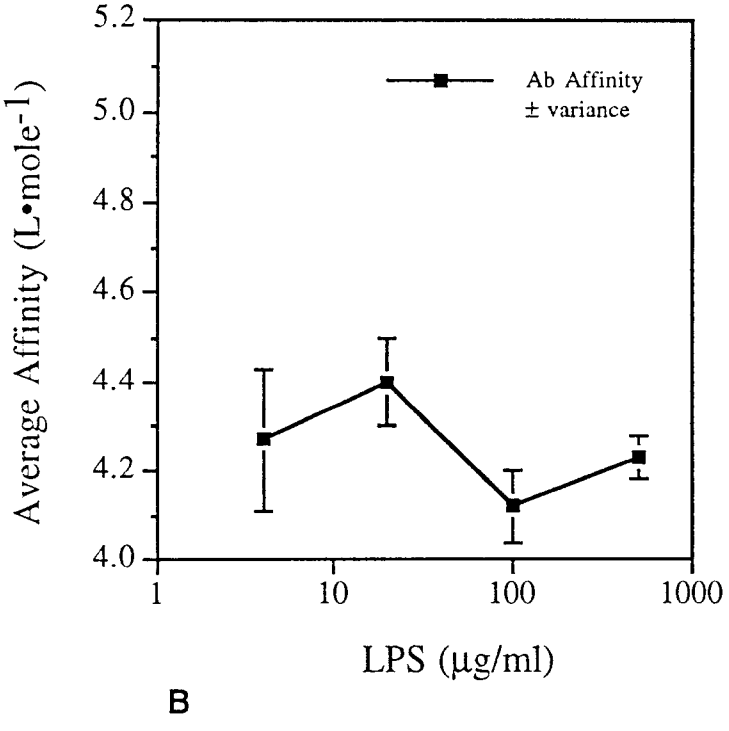
4.6
4.4
4.2
4.0
Average Affinity (L•mole-1)
Ab Affinity
± variance
1
10
100
1000
LPS (µg/ml)

A


5.2
5.0
4.8
4.6
4.4
4.2
4.0
Average Affinity (L•mole-1)
Ab Affinity
± variance
1
10
100
1000
LPS (µg/ml)

B

Figure 23

Figure 24. Representative three dimensional histogram of antibody affinities from TNP-LPS and LPS stimulated PBL cultures. The peripheral blood leukocytes from a rainbow trout were partitioned for stimulation by graded doses of TNP-LPS (A) or LPS (B). Replicate culture wells were combined and analyzed by ELISA for anti-TNP titer and affinity distribution. Antibody affinities were grouped into 1.0 aK unit ranges. Bar height (z-axis) represents units of antibody activity possing the indicated affinity range (x-axis).

Figure 25. Representative three dimensional histogram of antibody affinities from TNP-LPS and LPS stimulated anterior kidney cultures. The anterior kidney leukocytes from a rainbow trout were partitioned for stimulation by graded doses of TNP-LPS (A) or LPS (B). Replicate culture wells were combined and analyzed by ELISA for anti-TNP titer and affinity distribution. Antibody affinities were grouped into 1.0 aK unit ranges. Bar height (z-axis) represents units of antibody activity possessing the indicated affinity range (x-axis).

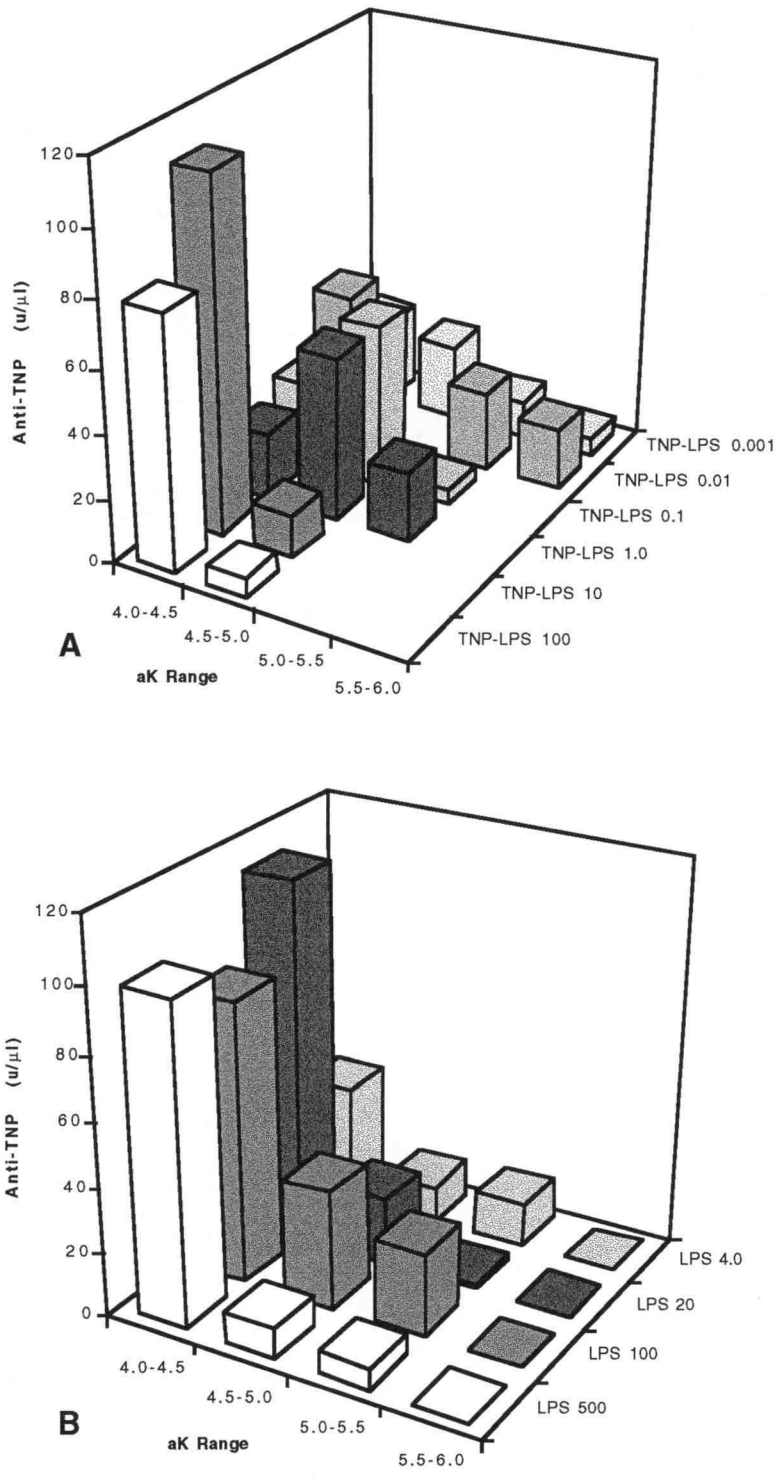

120
100
80
60
40
20
0
Anti-TNP (u/µl)
4.0-4.5
4.5-5.0
5.0-5.5
5.5-6.0
aK Range
A
TNP-LPS 0.001
TNP-LPS 0.01
TNP-LPS 0.1
TNP-LPS 1.0
TNP-LPS 10
TNP-LPS 100
120
100
80
60
40
20
0
Anti-TNP (u/µl)
4.0-4.5
4.5-5.0
5.0-5.5
5.5-6.0
aK Range
B
LPS 4.0
LPS 20
LPS 100
LPS 500

Figure 24

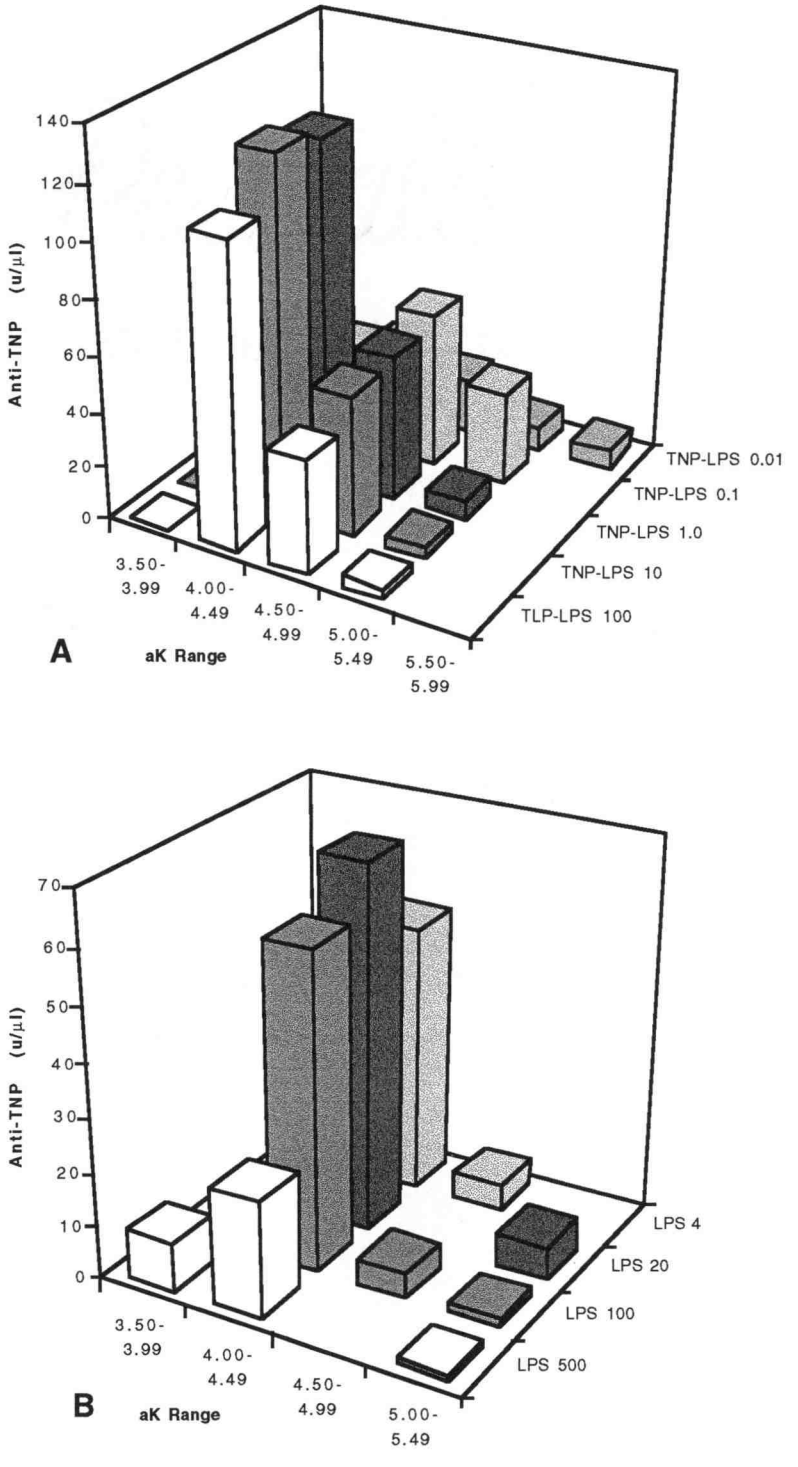

140
120
100
80
60
40
20
0
Anti-TNP (u/µl)
TNP-LPS 0.01
TNP-LPS 0.1
TNP-LPS 1.0
TNP-LPS 10
TLP-LPS 100
3.50-3.99
4.00-4.49
4.50-4.99
5.00-5.49
5.50-5.99
A
aK Range
70
60
50
40
30
20
10
0
Anti-TNP (u/µl)
LPS 4
LPS 20
LPS 100
LPS 500
3.50-3.99
4.00-4.49
4.50-4.99
5.00-5.49
B
aK Range

Figure 25

increased average affinity reported above. This was evidenced at TNP-LPS treatments of less than 0.01 μg/ml in both PBL (24A) and AK (24B). In other in vitro cultures (not shown), this sensitivity varied no more than 10-fold. The antibody responses of both PBL and AK cultures stimulated with LPS (Figures 24B and 25B, respectively) were dominated by antibodies in the range of 10^4 to $10^{4.5}$. No new affinities were detected in cultures stimulated with doses of LPS decreasing from 500 to 4 μg/ml. It was impossible to test antibodies produced in vitro at concentrations stimulating doses of LPS lower than 4 μg/ml, as there was no detectable anti-TNP titer at these concentrations.

Comparison of Mammalian Multimeric and Monomeric Antibodies With Trout Ig

Separation of Murine IgM and IgG Immunoglobulins

A Sephacryl S-300 column was prepared for the molecular weight based separation of murine IgM from IgG. The elution profiles of standard proteins are shown in Figure 26. While only two fractions separated thyroglobulin (669kd) from ß-amylase (200kd), a slightly greater difference in molecular weights between murine IgM (approximately 900kd) and IgG (approximately 160kd) was predicted to yield an even greater separation between these two molecules. As the actual elution profiles for each serum were determined by

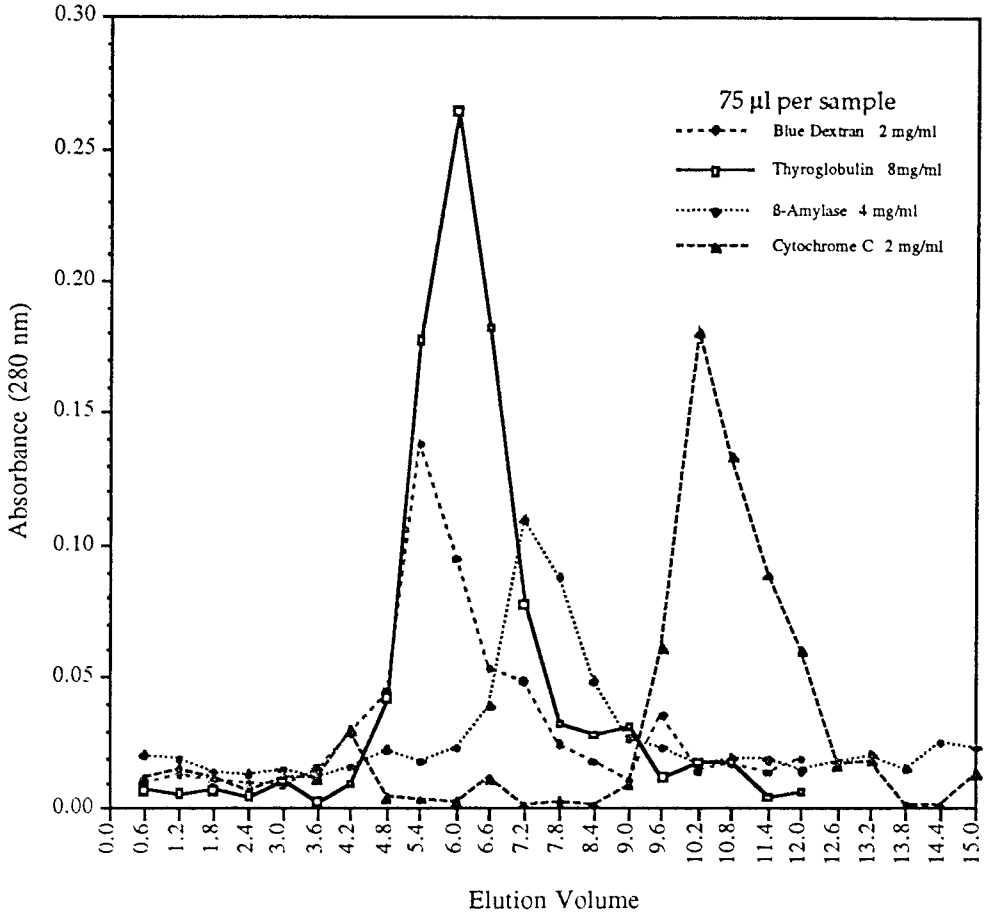


Figure 26. Chromatographic separation of standard proteins. Thyroglobulin (669 kd), ß-amylase (200 kd), and cytochrome C (12.4 kd) were separated by size exclusion on a 17 x 1.2 cm Sephacryl S-300 column. Blue dextran (2000 kd) was used to determine the void volume (56 ml).

ELISA, standard proteins were run only to verify the integrity of the column prior to separation of sample immunoglobulins.

Mice were bled prior to immunization with TNP-KLH, and at days 7 and 21 post immunization. 45 - 75 µl serum samples were collected. Serum IgM and IgG was separated from each serum sample on the basis of size by passage through the Sephacryl S-300 column. One ml fractions were collected. Titers of mouse IgM and IgG in each fraction were determined by ELISA using specific goat anti-mouse isotype reagents. Elution profiles from two representative mice (#5 and #8) are shown in Figures 27A-F. IgM and IgG peak fractions from each separation were pooled. Care was taken to select fractions from regions of the curves which minimized cross-contamination of isotypes. In Figure 27, fractions 7 and 8 were pooled from the first two bleeds to obtain IgM, while fractions 13-15 were pooled to obtain IgG. A control mouse (immunized with PBS in Freund's adjuvant) showed no increase in IgM or IgG production at week 3, nor did it show an increase in anti-TNP titer (not shown).

Comparison of Murine IgM and IgG Affinity Profiles

Pooled IgM and IgG fractions from all 11 mice were assayed by ELISA for anti-TNP activity and subsequently analyzed by the affinity ELISA to determine antibody affinity profiles. IgG prebleed anti-TNP titers were uniformly too low to be used in the affinity analysis. IgM fractions from

Figure 27. IgM and IgG elution profiles. IgM (•) and IgG (□) from the serum of each mouse at week 0 (A,D), week 1 (B,E), and week 3 (C,F) was separated on the basis of size. The elution profiles from two representative mice, #5 (A-C) and #8 (D-F) are shown. Peak fractions fractions were combined as described in the text.

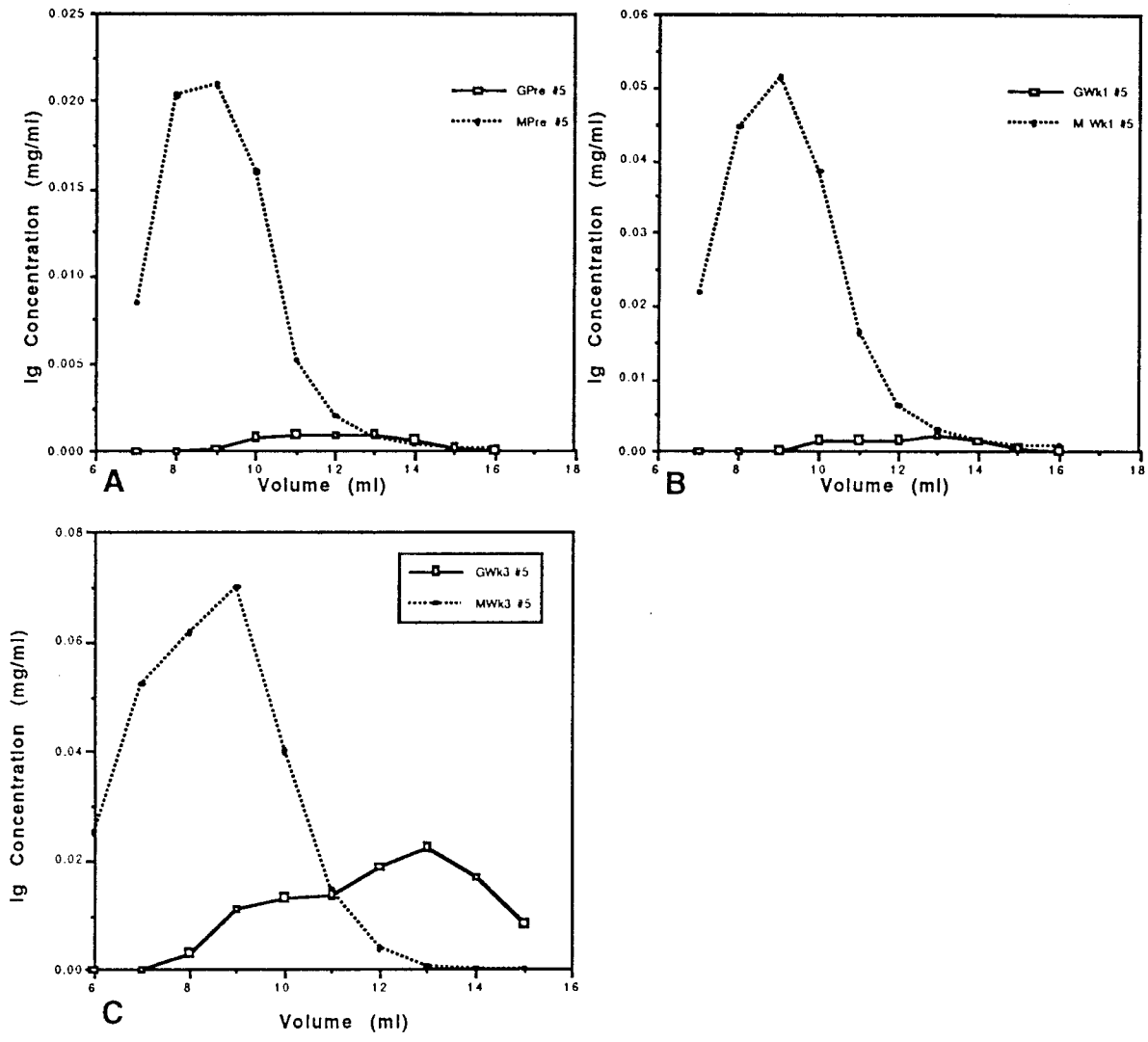

A
GPre #5
MPre #5
Ig Concentration (mg/ml)
Volume (ml)
B
GWk1 #5
M Wk1 #5
Ig Concentration (mg/ml)
Volume (ml)
C
GWk3 #5
MWk3 #5
Ig Concentration (mg/ml)
Volume (ml)

Figure 27

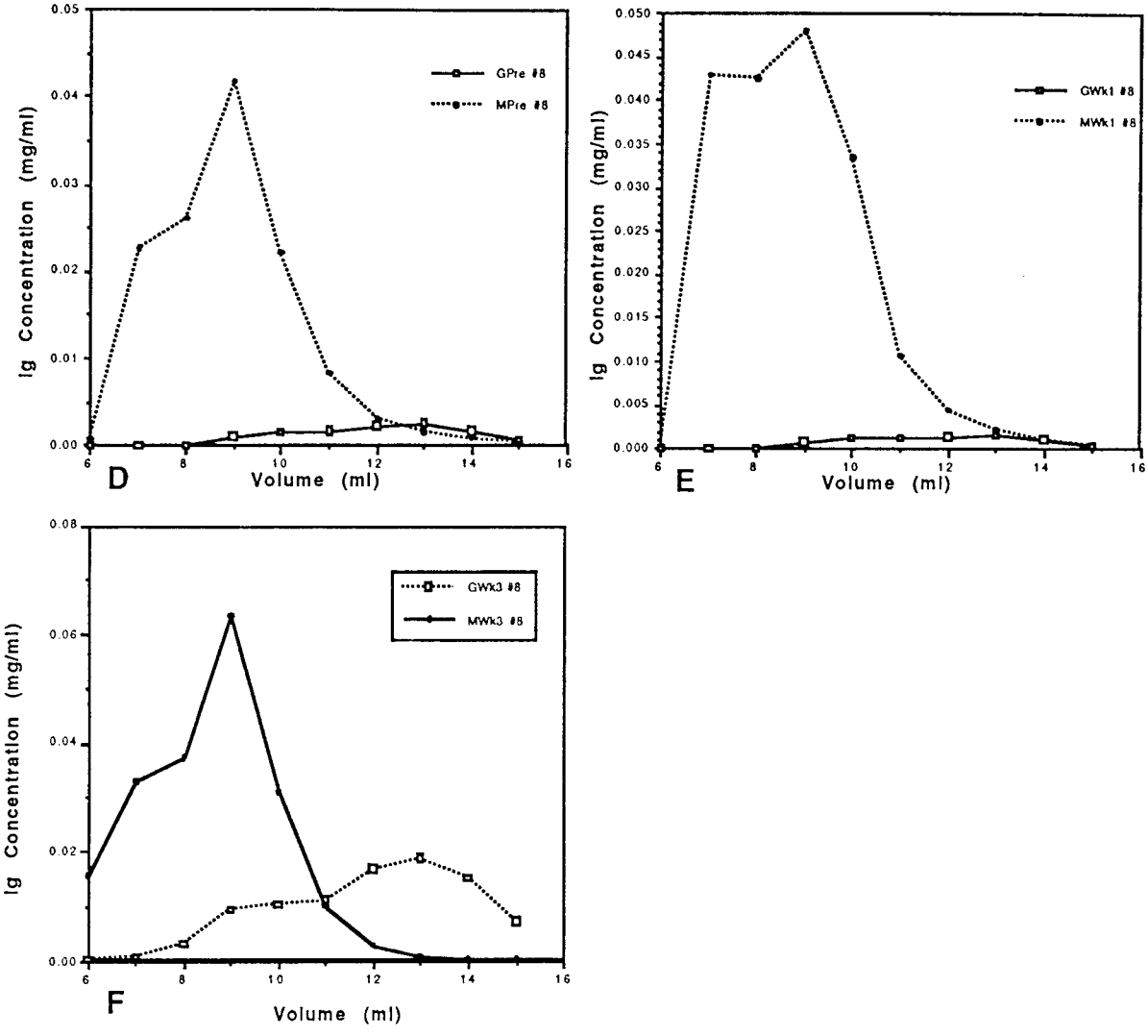


Figure 27 (cont'd)

seven of 11 mice prior to immunization had enough anti-TNP activity for affinity analyses.

Mean Group affinities for each isotype are shown in Table V. Prebleed and week 1 IgM affinities were significantly lower than week 1 IgG affinities. IgG affinities after one week of immunization had already reached an average of $10^{6.3}$ L mole^{-1}. A small, statistically significant increase in IgG affinity was observed over the next two weeks.

The increase in IgM affinity between the prebleed and week 1 bleed was small ($10^{0.4}$ L mole^{-1}) compared to the increase in affinity observed between week 1 and week 3 ($10^{1.6}$ L mole^{-1}).

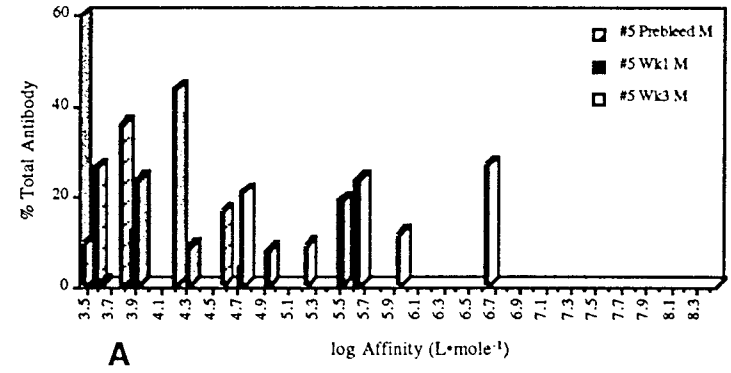
Representative IgM and IgG affinity distributions from two mice (#5 and #8) are shown in Figure 28. Figures 28A and 28C, IgM affinity profiles, clearly show the appearance of discrete antibody subpopulations (clear bars) which were absent in earlier sera. While the affinity profiles for IgG (Fig. 28B,D) reflect the small average increases in measured affinity between weeks 1 and 3, the emergence of high affinity clones which were not being elaborated at the earlier time point was less evident.

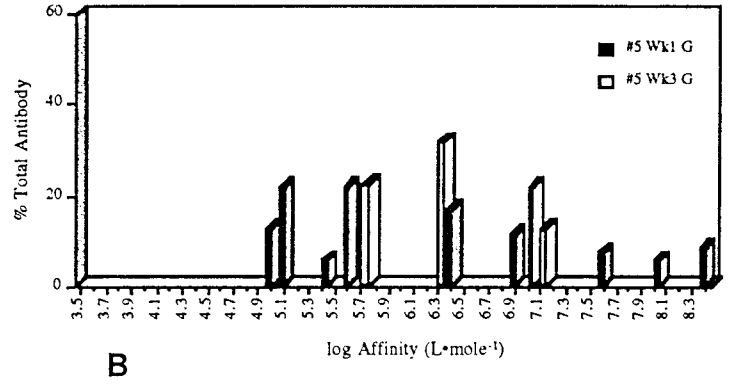
Table V. Murine IgM and IgG group mean affinities.

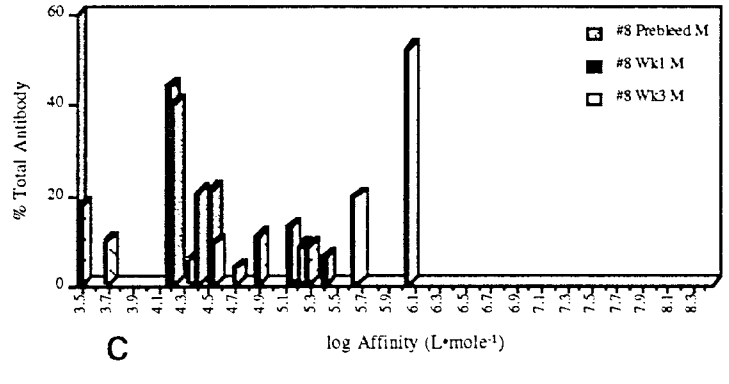
Time [1]	Murine Isotype	Group Mean Affinity [2]	Trout Affinity [3]
Prebleed	IgM	4.0 ± 0.08 [4]	3.2 ± 0.07
Prebleed	IgG	--------	
Week 1	IgM	4.4 ± 0.09 [5]	---------
Week 1	IgG	6.3 ± 0.11 [6]	
Week 3	IgM	6.0 ± 0.08 [7]	4.5 ± 0.12
Week 3	IgG	6.7 ± 0.10	

[1] Weeks post immunization with TNP-KLH
[2] Group affinity; mean ± std error of the mean
[3] From Table III (for comparison)
[4] Significantly different from IgM wk 1 ($p \leq 0.05$, ANOVA)
[5] Significantly different from IgM wk 3 ($p \leq 0.0001$, ANOVA)
[6] Significantly different from IgG wk 3 ($p \leq 0.01$, ANOVA)
[7] Significantly different from IgG wk 3 ($p \leq 0.0001$, ANOVA)

Figure 28. Time dependent affinity distributions of murine IgM and IgG. IgM and IgG fractions from all 11 mice were analyzed by the affinity ELISA for the distribution of antibody affinities. Representative affinity profiles from mouse #5 (A,B) and #8 (C,D) are shown. Bars represent the percent of total antibody at the indicated aK at week 0 (▨), week 1 (■) or week 3 (□).


#5 Prebleed M
#5 Wk1 M
#5 Wk3 M
% Total Antibody
log Affinity (L•mole-1)
A


#5 Wk1 G
#5 Wk3 G
% Total Antibody
log Affinity (L•mole-1)
B


#8 Prebleed M
#8 Wk1 M
#8 Wk3 M
% Total Antibody
log Affinity (L•mole-1)
C

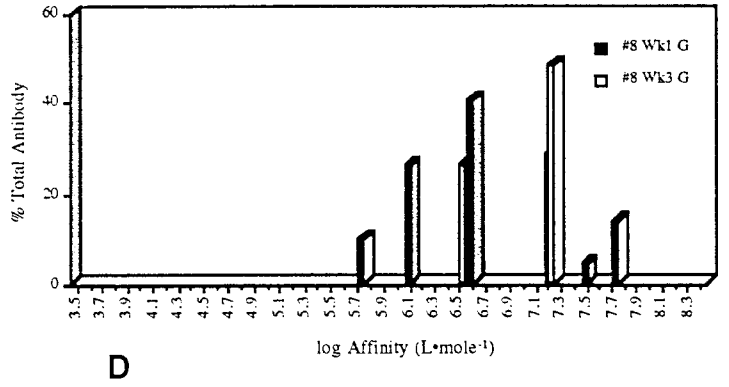

#8 Wk1 G
#8 Wk3 G
% Total Antibody
log Affinity (L•mole-1)
D

Figure 28

DISCUSSION

Thus far, little work has been devoted to the study of affinity maturation in fish. The few reports that exist suggest that affinity maturation either does not exist or is quite limited (Clem and Small, 1967; Voss et al., 1978; Mäkelä and Litman, 1980; O'Leary, 1981; Arkoosh, 1989). Since somatic mutation is integral to the development of significant affinity maturation in mammals, some have suggested that somatic mutation may not occur in lower vertebrates (Matsunaga, 1985). However, more recently, molecular evidence has been presented which demonstrates the presence of somatic mutants in peripheral blood leukocytes in sharks (Hinds-Frey et al., 1993) and *Xenopus* (Wilson et al., 1992).

What is currently required and provided by the use of the solid phase assay developed here, is the ability to dissect the affinity subpopulational structure of serum antibodies. Derivation of simple average affinity constants cannot discern the emergence of new, high affinity antibodies, only the average increase of the antibody pool. With the use of this assay, a number of features of the antibody response can be resolved. As can be seen in Figure 16, for example, the lowest anti-TNP antibody affinity groups which are present before the injection of antigen contribute nothing to the antibody pools that are present at three weeks post injection. Additionally, as the response

matures (2 weeks post secondary injection) certain individuals possess even higher affinities than those expressed in the late primary response (Fig. 15 A&F). This may be taken as strong evidence for the emergence of new, higher affinity clones. Upon encountering antigen, these clones expand, and their secreted antibody products become substantial constituents of the antibody pool. One curious finding is that not all individuals demonstrate this late shift towards production of very high affinity antibodies. Some populations exhibit no significant change after the initial burst of higher affinity antibody production (Fig. 15 C&E) while others appear to exhibit a loss of their highest affinity populations (Fig. 15 B&D). Taken together, two alternate explanations for these observed phenomena may be posed. One possibility may be that somatic mutation is occurring, but it does not necessarily occur in every individual, nor does it occur to the same degree in each individual. Alternatively, there may be no somatic mutation. If the latter is true, the initial burst of high affinity antibodies simply represents the initial antigen selection events wherein all pre-existing B cells with the highest affinity for TNP are stimulated. Their relative absence in the pre-immune sera simply indicates that previous environmental antigens were not antigenically similar to TNP. In this context, the fluctuations in the expression of the highest affinity antibodies may be due to the antigen-specific suppression of the highest affinity B cells. The ability of antigen-specific

suppressor cells to selectively suppress high affinity B cells has been demonstrated previously by Takemori and Tada (1974) in the murine system.

To date, most measurements of affinity maturation have employed equilibrium dialysis. Equilibrium dialysis measures the intrinsic binding constant of an antigen binding site. True affinity (vs. functional avidity) measurements can only be made using monoclonal antibodies. K_o measured by equilibrium dialysis is simply a uniform way of ascribing an affinity value to a heterogeneous population of antibodies of various affinities by determining the equilibrium constant when half the sites are bound. Thus, it is not directly comparable to average affinity. Furthermore, such measurements do not yield any information about the distribution of affinities within heterogeneous populations of antibodies. Using equilibrium dialysis (or the other classical forms of affinity measurement) one can not distinguish between affinity shifts due to the increase in clone size of a prominent pre-existing antibody affinity pool or an increase due to the proliferation of new, high affinity clones during an immune response.

Using the affinity ELISA, it has now been documented that rainbow trout serum antibodies increase in average affinity over time. It was shown that three weeks after immunization with the T-dependent antigen TNP-KLH, rainbow trout anti-TNP antibody affinities increased from an average of $10^{3.3}$ L mole^{-1} to $10^{4.2}$ L mole-1, a difference of nearly ten-fold (Table III). Later in the primary response at week 9, average affinity rose to $10^{5.3}$ L mole^{-1}

(Table IV), yet another ten fold increase. Secondary response average affinities at weeks 22 and 31 ($10^{5.5}$ L mole^{-1}) were not significantly different from those measured 9 weeks into the primary response.

Previous reports examining trout antibody affinity over time reported no maturation of antibody affinity (O'Leary, 1981; Arkoosh, 1989). The immunization schemes and analytical procedures utilized, however, may have been insufficient to make such determinations. First, O'Leary (1981) immunized rainbow trout (*O. mykiss)* with relatively high doses of TNP-BSA (1-2 mg). The group average K_o measured 6 to 37 weeks after immunization using fluorescence quenching was 9.3×10^4 M^{-1} to 2.3×10^5 M^{-1}. Using a modified Farr technique, he determined group average antibody affinities during this same time period decreased from 1.9×10^5 M^{-1} (week 6) to 1.2×10^{-5} M^{-1} (week 31). These K_o values are very similar to the affinities reported here by solid phase ELISA, for all sera collected past nine weeks post immunization. Antibody affinities measured three weeks post immunization were ten-fold lower than those measured at week 9 and ten-fold lower than those measured at week 6 by O'Leary.

The sensitivity of the ELISA made it possible to measure antibody affinities in pre-immune sera as well as sera taken three weeks after immunization. As serum samples were often exhausted in determining these measurements even by use of the ELISA, it is likely that insufficient antibody was present at earlier times to measure affinities using the methods

employed by O'Leary (although absolute confirmation of this is not possible). Thus, it is possible that while early increases in antibody affinity may have occurred, sampling times did not permit distinctions between treatment group affinities to be made.

Arkoosh (1989) used a form of the affinity ELISA employing one plate coating antigen concentration to measure antibody affinity. In her study, rainbow trout were immunized with 100 μg TNP-KLH. Again, the earliest time point at which serum antibody affinities were determined was 6 weeks post immunization. No significant shifts in affinity were measured in either the primary response up to 20 weeks post primary immunization, or during the 17 weeks subsequent to secondary immunization. There was a gradual shift in measured affinity between week 6 and week 20, the significance of which may have been masked by significant variability in the average affinities of individual trout. Finally, estimates of affinity were significantly lower in her experiments than those reported here (from a low of approximately 10^2 L mole^{-1} at 13 weeks post primary immunization to a high of approximately 10^4 L mole^{-1} two weeks post secondary immunization). This is not an insignificant result. Arkoosh used only one coating antigen concentration (1.0 μg/ml) with which to conduct a hapten inhibition analysis. As can be seen from these studies (Fig. 4), this antigen concentration is sufficient to capture even the lowest affinity antibodies. Therefore, Arkoosh was likely measuring the average of the lowest affinity antibodies over time.

It is also likely, therefore, that this design did not permit the detection of late-appearing, high affinity antibodies.

A definite increase in the average affinity produced in rainbow trout immune sera over time has now been documented. Though the response to a defined antigen (TNP) was described, it still remained to be shown that the increase in affinity was due to antigen driven selection of high affinity B cell clones. In vivo dose response experiments were deemed impractical. It was also deemed unnecessary for the following reasons: in vivo antibody generation studies are subject to the confounding effects of cell recruitment from peripheral sources, differential distribution of antigen within tissues, and organ dependent regulatory mechanisms. To show that antibody affinity generation was subject to selective stimulation of high affinity clones by decreasing concentrations of antigen, an in vitro antigen stimulation assay was developed.

It was determined that pulsing of antigen cultures was necessary (Fig. 17), due to the inhibitory effects of large concentrations of antigen in culture. It was assumed that under these conditions, free antibody was being bound by antigen, and made unavailable for titration or affinity determinations.

It was shown *in vitro* that rainbow trout leukocytes will proliferate and produce antibody to TNP in response to antigenic stimulation by TNP-LPS in a dose dependent manner (Figs. 18 and 19). Furthermore, the antibody produced increased significantly in affinity with decreasing antigenic dose

(Figs. 20 through 23). High doses of TNP-LPS (above 1 μg/ml for both PBL and AK cells) led to a rapid drop in the affinity of the antibodies produced in culture, from an average affinity of $10^{4.7}$-$10^{4.9}$ L mole^{-1} to an average of approximately $10^{4.2}$-$10^{4.3}$ L mole^{-1}. At high concentrations of TNP-LPS, in the absence of competition for antigen, it is likely that the decrease in average affinity was due to the expansion all anti-TNP clones, regardless of antigen receptor affinity. This hypothesis was substantiated by polyclonal stimulation of leukocytes with LPS. The average affinities of the anti-TNP antibodies in polyclonally-stimulated cultures approximated those in high dose TNP-LPS cultures (Figs. 22 and 23). Three dimensional histograms of the antibody subpopulations in TNP-LPS stimulated cultures (Figs. 24A and 25A) revealed that differences in antibody affinity were generated by the appearance of previously absent or undetectable clones (particularly below 0.1 μg/ml). This same form of analysis revealed that in both PBL and AK leukocyte cultures, affinity distributions were approximately constant over all LPS concentrations tested (Figs. 24B and 25B). The model for (mammalian) affinity maturation in the absence of the input of cell factors proposed by Eisen and Siskind (1964) suggested that in vivo dose dependence was due to the time dependent clearing of antigen and the concomitant preferential stimulation of high affinity B cells by progressively decreasing concentrations of antigen. It has now been shown in vitro that this model may (at least partially) explain affinity maturation in vivo in rainbow trout.

The sensitivity of the affinity ELISA permitted studying the long term immunotoxicological effects of embryonic exposure to aflatoxin B_1. Aflatoxins are potent hepatocarcinogens produced by certain toxigenic strains of *Aspergillus flavus* and *A. parasiticus*. Acute aflatoxicosis (usually acquired via oral exposure through contaminated feed) has been shown to cause hepatocarcinomas in a variety of animal species (reviewed in Bailey, 1994). Its immunosuppressive effects have also been studied in mice (Reddy and Sharma, 1989), rats (Rainbow et al., 1994), hamsters (Pier, et al., 1977), chickens (Dietert et al., 1985; Kadian et al, 1988; Ghosh et al., 1990; Scott et al., 1991), cattle (Bodine, 1984) and swine (Panangala et al., 1986). The primary observed dysfunctions were T-cell mediated, notably delayed cutaneous hypersensitivity, lymphoblastogenesis, and altered leukocyte migration. It is now known that metabolic products of aflatoxin, particularly the highly reactive 8,9-epoxide, aflatoxicol, are responsible for many of the carcinogenic effects of aflatoxin exposure (Bailey, 1994). Studies by Arkoosh (1987) revealed that embryonic exposure of rainbow trout to aflatoxin B_1 led to a long term alteration in the ability to produce a normal antibody response. The two primary features of this dysfunction were, 1) a reduced capacity to produce a secondary antibody response to the T-dependent antigen TNP-KLH, and 2) a decrease in relative affinities (K_{rel}) for heterologs of the test hapten TNP-LYS (Arkoosh, 1987). Based on these studies, it was postulated that either embryonic exposure to aflatoxin (or its metabolites) caused a direct B cell

dysfunction, or that a helper T cell dysfunction was operative. Much of the literature regarding the immunotoxic effects of aflatoxin exposure in a variety of animal species cites predominantly T cell dysfunctions (Pier et al., 1977; Reddy and Sharma, 1989).

The results in Table IV show a significant delay in the appearance of high affinity antibody populations ($>10^5$ L mole^{-1}) in trout exposed embryonically to the highest experimental dose of aflatoxin (1 ppm) vs. control trout. This phenomenon affected only the T-dependent response to TNP-KLH antigen. Affinity responses developed normally in all TNP-LPS immunized animals. A plausible explanation for this result is that upon embryonic exposure, a lymphocytic population of cells responsible for providing T cell help was damaged. Other possible explanations, such as direct B cell or suppressor T cell damage are unlikely, as no effect was observed in the T-independent response.

Finally, because of the physical similarity between rainbow trout Ig and mammalian IgM (leading many in the field to refer to trout Ig as IgM), a study was undertaken to determine if rainbow trout anti-TNP antibody affinities more closely resembled that of mammalian IgM than IgG. It was postulated that the isotype switch in the murine system might be associated with the greater increase of affinity found in mice, and that the early IgM anti-TNP antibodies might possess affinities resembling those expressed by rainbow trout. It was determined that three weeks post immunization, both murine

IgM and IgG anti-TNP were of significantly higher average affinity than rainbow trout anti-TNP (Table V). Though there was not enough murine IgG anti-TNP to measure pre-immunization affinities (presumably, cross reactive environmental antigens were stimulating primarily IgM anti-TNP responses, if any), the earliest measured IgG anti-TNP affinities were significantly higher than those measured in rainbow trout at both the same time period and later in the response. However, the earliest measured murine IgM anti-TNP antibodies had affinities similar to those measured three weeks post immunization in trout. These IgM affinities eventually increased in affinity to levels which were not achieved in rainbow trout. Thus, though structurally somewhat similar to murine IgM, trout anti-TNP affinity maturation does not appear to resemble that of either murine IgM or IgG.

SUMMARY AND CONCLUSION

Described, herein, is a the establishment of a novel microtiter plate affinity ELISA for use in determining the spectrum of affinities within a heterogeneous population of antibodies. The system employs graded concentrations of haptenic TNP-LYS to inhibit the binding of a antibody to solid phase antigen (TNP-BSA). Though the assay is described here only for use with the trinitrophenyl hapten, it should quite easily be adapted for use with the study of most hapten-antibody interactions, provided a plate bound (protein conjugated) form of the antigen can be generated. Furthermore, this system allows comparison of antibody affinities from different species, specific for the same hapten, to be directly compared, provided species-specific detection reagents are available.

Using the affinity ELISA, we have described for the first time affinity maturation in the rainbow trout immune response. We have shown *in vitro* that one mechanism for the generation of increased antibody affinity, antigen specific stimulation of high affinity clones by decreasing amount of antibody, functions in rainbow trout as it does in mammalian systems. A logical next step would be the study of the effects of somatic mutation of antibody diversity and affinity. The assay provided the means to rapidly measure antibody affinity in large numbers of animals in both the murine and piscine systems. Using this technology it was found that murine and piscine affinity maturation differ significantly. Finally, small shifts in the antibody affinity

response due to the immunotoxicological effects of embryonic aflatoxin B_1 exposure were described using this assay.

In conclusion, a simple, rapid, and reliable technique now exists for the study of antibody affinity at a level not previously available.

BIBLIOGRAPHY

Acton, R.T., P.F. Weinheimer, S.J. Hall, W. Niedermeier, E. Shelton, and J.C. Bennett (1970). Tetrameric immune macroglobulins in three orders of bony fishes. Proc. Nat. Acad. Sci. USA 68(1):107-111.

Allen, D., A. Cumano, R. Dildrop, C. Kocks, K. Rajewsky, N. Rajewsky, J. Roes, F. Sablitzky and M. Siekevitz (1987). Timing, genetic requirements and functional consequences of somatic hypermutation during B-cell development. Immunol. Rev. 96:5-22.

Alt, F.W., G.D. Yancopoulos, T.K. Blackwell, C. Wood, E. Thomas, M. Boss R. Coffman, N. Rosenberg, S. Tonegawa, D. Baltimore (1984). Ordered rearrangement of immunoglobulin heavy chain variable region segments. EMBO J. 3:1209-1219.

Amemiya, C.T. and G.W. Litman (1990). Complete nucleotide sequence of an immunoglobulin heavy-chain gene and analysis of immunoglobulin gene organization in a primitive teleost species. Proc. Natl. Acad. Sci. USA 87:811-815.

Anderson, M., C. Amemiya, C. Luer, R. Litman, J. Rast, Y. Niimura, G. Litman (1994). Complete genomic sequence and patterns of transcription of a member of an unusual family of closely related, chromosomally dispersed Ig gene clusters in *Raja*. Int. Immunol. 6:1661-1670.

Andersson, B. (1970). Studies on the regulation of avidity at the level of the single antibody-forming cell. J. Exp. Med. 132:77-88.

Andersson, E., and T. Matsunaga (1993). Complete cDNA sequence of a rainbow trout IgM gene and evolution of vertebrate IgM constant domains. Immunogenetics 38:243-250.

Arkoosh, M. (1989). Development of immunological memory in rainbow trout (*Oncorhynchus mykiss*) and aflatoxin B_1 modulation of the response. Doctoral Thesis. Oregon State University, Corvallis, Oregon. 176 numb. leaves.

Arkoosh, M.R. and S.L. Kaattari (1987). The effect of early aflatoxin B_1 exposure on *in vivo* and *in vitro* antibody responses in rainbow trout, *Salmo gairdneri*. J. Fish Biol. 31:12-22 (Supp. A).

Arkoosh, M. and S.L. Kaattari (1990). Quantitation of fish antibody to a specific antigen by an enzyme linked immunosorbent assay (ELISA). In, Techniques in Fish Immunology, Vol. 1, p15. J.S. Stolen, T.C. Fletcher, D.P. Anderson, B.S. Roberson, and W.B.v. Muiswinkel, eds. SOS Publications, Fair Haven, NJ.

Bailey, G.S. (1994). Role of aflatoxin-DNA adducts in the cancer process. In, The Toxicology of Aflatoxins: Human health, veterinary, and agricultural significance. Academic Press, Inc.

Bengtén, E., S. Strömberg, L. Pilström (1994). Immunoglobulin VH regions in Atlantic cod (*Gadus morhua* L.): Their diversity and relationship to VH families from other species. Dev. Comp. Immunol. 18:109-122.

Berek, C. And C. Milstein (1988). The dynamic nature of the antibody repertoire. Immunol. Rev. 105:2-26.

Berzofsky, J.A., G. Hicks, J. Fedorko, and J. Minna (1980). Properties of monoclonal antibodies specific for determinants of a protein antigen, myoglobin. J. Biol. Chem. 255:11188-11191.

Bodine, A.B., S.F. Fisher, S. Gangjee (1984). Effect of aflatoxin B_1 and major metabolites on phytohemagglutinin-stimulated lymphoblastogenesis of bovine lymphocytes. J. Dairy Sci. 67:110-114.

Bruderer, U., M. Deusinger, U. Schürch, and A.B. Lang (1992). Analyses of affinity distributions within polyclonal populations of antigen-specific antibodies. J. Immunol. Methods 151:157-164.

Butler, J. E., Feldbush, T. L., McGivern, P. L., and Stewart, N. (1978). The enzyme linked immunosorbent assay (ELISA): A measure of antibody concentration or affinity? Immunochemistry 15:131-136.

Claflin, L. And B. Merchant (1972). Restricted maturation of antibody-binding characteristics for hapten-specific IgM-plaque-forming cells in mice. Cell. Immunol. 5:209-220.

Claflin, J.L., J. Berry, D. Flaherty, W. Dunnick (1987). Somatic evolution of diversity among anti-phosphocholine antibodies induced with *Proteus moganii*. J. Immunol. 138:3060-3068.

Clem, L.W. And P.A. Small (1967). Phylogeny of immunoglobulin structure and function. I. Immunoglobulins of lemon shark. J. Exp. Med. 125:893-920.

Coico, R.F., B.S. Bhogal, G.J. Thorbecke (1983). Relationship of germinal centers in lymphoid tissue to immunologic memory with lymph node cells fractionated according to their receptors for peanut agglutinin. J. Immunol. 131:2254-2257.

Cossarini-Dunier, M., F.-X. Desvaux and M. Dorson (1986). Variability in humoral responses to DNP-KLH of rainbow trout (*Salmo gairdneri*). Comparison of antibody kinetics and immunoglobulins spectrotypes between normal trouts and trouts obtained by gynogenesis or self-fertilization. Dev. Comp. Immunol. 10:207-217.

Cross, G.A.M. (1990). Cellular and genetic aspects of antigenic variation in trypanosomes. Annu. Rev. Immunol. 8:83-110.

Daggfeldt, A., E. Bengtén, L. Pilström (1993). A cluster type organization of the loci of the immunoglobulin light chain in Atlantic cod (*Gadus morhua* L.) and rainbow trout (*Oncorhynchus mykiss* Walbaum) indicated by nucleotide sequences of cDNAs and hybridization analysis. Immunogenetics 38:199-209.

DeLisi, C. and B. Goldstein (1974). On the mechanism of hemolytic plaque inhibition. Immunochemistry 11:661-665.

Desvaux, F.-X., M. Cossarini-Dunier, S. Chilmonzcyk, and J. Charlemagne (1987). Antibody diversity in trouts obtained by gynogenesis or self-fertilization. Comparative analysis of the heavy chain spectrotypes. Dev. Comp. Immunol. 11:577-584.

Devey, M.E. (1986). The biological and pathological significance of antibody affinity. In, Immunoglobulins in Health and Disease, P. 57. M.A.H. French, ed. MTP Press, Boston.

Dietert, R.R., M.A. Qureshi, U.C. Nanna, S.E. Bloom (1985). Embryonic exposure to aflatoxin-B_1: Mutagenicity and influence on development and immunity. Environ. Mutagen. 7:715-725.

Doria, G., G. D'Agostaro and A. Poretti (1978). Age-dependent variations of antibody avidity. Immunology 35:735-739.

Dreyer, W.J. And J.C. Bennett (1965). The molecular basis of antibody formation. Proc. Nat. Acad. Sci. USA 54:864-869.

Eisen, H.N. and F. Karush (1949). The interaction of purified antibody with homologous hapten. Antibody valence and binding constant. J. Am. Chem. Soc. 71:363-364.

Eisen, H.N. and G.W. Siskind (1964). Variations in affinities of antibodies during the immune response. Biochem. 3:996-1008.

Epstein, S.J., P. Doty, and W.C. Boyd (1956). A therodynamic study of hapten-antibody association. J. Am. Chem. Soc. 78:3306-3315.

Farr, R.S. (1958). A quantitative immunochemical measure of the primary interaction between I*BSA and antibody. J. Inf. Dis. 103:239-262.

Feibig, H., R. Gruhn, H. Ambrosius (1977). Studies on the control of IgM antibody synthesis. III. Preferential formation of anti-DNP antibodies of high functional affinity in the course of the immune response in carp. Immunochemistry 14: 721-726.

Fernandez, C. And G. MÖller (1991). The influence of T cells on the Immunoglobulin repertoire and the affinity maturation of the immune response against dextran B512 in C57BL/6 mice. Scand. J. Immunol. 33:307-317.

Foung, S., D.T. Sasaki, F.C. Grumet, E.G. Engleman (1982). Production of functional human T-T hybridomas in selection medium lacking aminopterin and thymidine. Proc. Natl. Acad. Sci. USA 79:7484-7488.

Friguet, B., A.F. Chaffotte, L. Djavadi-Ohaniance, and M.E. Goldberg (1985). Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. J. Immunol. Methods 77:305-319.

Gershon, R.K. and W.E. Paul (1971). Effect of thymus-derived lymphocytes on amount and affinity of anti-hapten antibody. J. Immunol. 106:872-874.

Ghaffari, S.H. and C.J. Lobb (1992). Organization of immunoglobulin heavy chain constant and joining region genes in the channel catfish. Mol. Immunol. 29:151-159.

Ghosh, R.C., H.V.S. Chauhan, S. Roy (1990). Immunosuppression in broilers under experimental aflatoxicosis. Br. Vet. J. 146:457-462.

Goidl, E.A., Innes, J.B., Weksler, M.E. (1976). Immunological studies of aging. II. Loss of IgG and high avidity plaque forming cells and increased suppressor cell activity in aging mice. J. Exp. Med. 144: 1037-1048.

Goidl, E.A., J. Klass, and G.W. Siskind (1976). Ontogeny of B-lymphocyte function. II. Ability of endotoxin to increase the heterogeneity of affinity of the immune response of B-lymphocytes from fetal mice. J. Exp. Med. 143:1503-1520.

Goidl, E.A. and G.W. Siskind (1974). Ontogeny of B-lymphocyte function. I. Restricted heterogeneity of the antibody response of B lymphocytes from neonatal and fetal mice. J. Exp. Med. 140:1285-1302.

Goidl, E.A., W.E. Paul, G.W. Siskind, B. Benacerraf (1968). The effect of antigen dose and time after immunization on the amount and affinity of anti-hapten antibody. J. Immunol. 100(2):371-375.

Gray, D., Chassoux, D., MacLennan, I., and Bazin, H. (1985). Selective depression of thymus-independent anti-DNP antibody responses induced by adult but not neonatal splenectomy. Clin. Exp. Immunol. 60:78-86.

Griffiths, G.M., C. Berek, M. Kaartinen, C. Milstein (1984). Somatic mutation and the maturation of immune response to 2-phenyl oxazolone. Nature 312:271-275.

Hall, T.J., and C. Heckel (1988). Thiocyanate elution estimation of relative antibody affinity. J. Immunol. Methods 115:153-154.

Hansen, J., J.A. Leong, and S. Kaattari (1994). Complete nucleotide sequence of a rainbow trout cDNA encoding a membrane-bound form of immunoglobulin heavy chain. Mol. Immunol. 31:499-501.

Håvarstein, L.S., P.M. Aasjord, S. Ness, and C. Endresen (1988). Purification and partial characterization of an IgM-like serum immunoglobulin from Atlantic salmon (*Salmo salar*). Dev. Comp. Immunol. 12: 773-785.

Hinds-Frey, K.R., H. Nishikata, R.T. Litman, G.W. Litman (1993). Somatic variation precedes extensive diversificaion of germline sequences and combinatorial joining in the evolution of immunoglobulin heavy chain diversity. J. Exp. Med. 178:815-824.

Hordvik, I., A.M. Voie, J. Gilette, R. Male, C. Ndresen (1992). Cloning and sequence analysis of two isotypic IgM heavy chain genes from Atlantic salmon *Salmo salar* L. Eur. J. Immunol. 22:2957-2962.

Hornick, C.L. and F. Karush (1972). Antibody affinity - III. The role of multivalence. Immunochemistry 9:325-340.

Huchet, R. And M. Feldmann (1973). Studies on antibody affinity in mice. Eur. J. Immunol. 3:49-55.

Irwin, M.J. (1987) B cell heterogeneity in salmonids. Doctoral thesis. Oregon State University, Corvallis, Oregon. 138 numb. leaves.

Jacob, J., G. Kelsoe, K. Rajewsky, U. Weiss (1991). Intraclonal generation of antibody mutants in germinal centres. Nature 354:389-392.

Jerne, N.K., A.A. Nordin, and C. Henry (1963). The agar plaque technique for recognizing antibody producing cells. In, Cell Bound Antibodies. B. Amos and H. Koprowski, eds. Wistar Institute Press, Philadelphia, Pa.

Jones, C.L., R.A. Macdonald, C.S. Hosking, D.M. Roberton (1987). Estimating the relative avidity of mucosal IgA for antigen. J. Immunol. Methods 105:111-117.

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

Kadian, S.K., D.P. Monga, M.C. Goel (1988). Effect of aflatoxin B_1 on the delayed hypersensitivity and phagocytic activity of reticuloendothelial systems in chickens. Mycopathol. 104:33-36.

Karush, F. (1959). Properties of papain-digested purified anti-hapten antibody. Fed. Proc. 18:577.

Karush, F. (1962). Immunologic specificity and molecular structure. Adv. Immun. 2:1-40.

Kim, Y.T., T.P. Werblin, and G.W. Siskind (1974). Distribution of antibody affinities II. Fractionation of antibody with respect to its hapten binding affinity. Immunochemistry 11:685-690.

Kimball, J.W. (1972). Maturation of the immune response to type III pneumococcal polysaccharide. Immunochemistry 9:1169-1184.

Knudsen, K.A. (1984). Proteins transferred to nitrocellulose for use as immunogens. Anal. Biochem. 147:285-288.

Kokubu, F., K. Hinds, R. Litman, M.J. Shamblott, G. Litman (1987). Extensive families of constant region genes in a phylogenetically primitive vertebrate indicate an additional level of immunoglobulin complexity. Proc. Natl. Acad. Sci. USA 84:5868-5872.

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

Lee, M.A., E. Bengtén, A. Daggfeldt, A.S. Rytting, L. Pilström (1993). Characterisation of rainbow trout cDNAs encoding a secreted and membrane-bound Ig heavy chain and the genomic intron upstream of the first constant exon. Mol. Immunol. 30:641-648.

Lerner, R.A., Benkovic S.J., Schultz, P.G. (1991). At the crossroads of chemistry and immunology: catalytic antibodies. Science 251: 659-667.

Levy, N.S., U.V. Malipiero, S.G. Lebecque, P.J. Gearhart (1989). Early onset of somatic mutation in immunoglobulin VH genes during the primary immune response. J. Exp. Med. 169:2007-2019.

Linton, P.-J., A. Rudie, and N.R. Klinman (1991). Tolerance susceptibility of newly generating memory B cells. J. Immunol. 146:4099-4104.

Lobb, C.J. and M.O.J. Olson (1988). Immunoglobulin heavy chain isotypes in a teleost fish. J. Immunol. 141(4):1236-1245.

Lowry, O.H., N.J. Rosenbrough, A.L. Farr, R.J. Randall (1951). Protein measurements with the folin phenol reagent. J. Biol. Chem. 193:265-276.

Macdonald, R.A., C.S. Hosking, C. Jones (1988). The measurement of relative antibody affinity by ELISA using thiocyanate elution. J. Immunol. Methods 106:191-194.

MacLennan, I.C.M. and D. Gray (1986). Antigen-driven seletion of virgin and memory B cells. Immunol. Rev. 91:61-85.

Maizels, N. J.C. Lau, P.R. Blier, and A. Bothwell (1988). The T-cell independent antigen, NP-ficoll, primes for a high affinity IgM anti-NP response. Mol. Immunol. 25:1277-1282.

Mäkelä, O. And G.W. Litman (1980). Lack of heterogeneity in antibodies of a phylogenetically primitive shark. Nature 287:639-640.

Marshall-Clarke, S and J.L.H. Playfair (1975). Age dependent changes in the relative affinity of anti-dinitrophenyl antibodies in mice. Immunology 29:477-486.

Matsunaga, T., T. Chen, V. Törmänen (1990). Characterization of a complete immunoglobulin heavy chain variable region germ-line gene of rainbow trout. Proc. Natl. Acad. Sci. USA 87:7767-7771.

Matsunaga, T. (1985). Evolution of antibody repertoire - somatic mutation as a late comer. Dev. Comp. Immunol. 9:585-596.

McKean, D, K. Huppi, M. Bell, L. Staudt, W. Gerhard, and M. Weigert (1984). Generation of antibody diversity in the immune response of BALB/c mice to influenza virus hemagglutinin. Proc. Natl. Acad. Sci. USA 81:3180-3184.

Mukkur, T.K.S. (1980). Thermodynamics of hapten-antibody interactions. Trends Biochem. 5:72-74.

Mulcahy, G., E. Reid, R.D. Dimarchi, C. Gale, T.R. Doel (1992). Maturation of functional antibody affinity in animals immunised with synthetic foot-and-mouth disease virus. Research in Veterinary Science 52:133-140.

Muñoz, C., A. Nieto, A. Gayá, J. Martínez, and J. Vives (1986). New experimental criteria for optimization of solid-phase antigen concentration and stability in ELISA. J. Immunol. Methods 94:137-144.

Munson, P.J. And D. Rodbard (1980). LIGAND: a versatile computerized approach for characterization of ligand-binding systems. Anal. Biochem. 107:220-239.

Nieto, A., A. Gaya, M. Jansa, C. Moreno, J. Vives (1984). Direct measurement of antibody affinity distribution by hapten-inhibition enzyme immunoassay. Mol. Immunol. 21:537-543.

Nieuwenhuis, P. and D. Opstelten (1984). Functional anatomy of germinal centers. Am. J. Anat. 170:421-435.

O'Leary, P.J. (1981). A partial characterization of high and low molecular weight immunoglobulin in rainbow trout (*Salmo gairdneri*). Doctoral thesis. Oregon State University, Corvallis, Oregon. 72 numb. leaves.

Opstelten, D. & D. G. Osmond (1983). Pre-B cells in mouse bone marrow: Immunofluorescence stathmokinetic studies of the proliferation of cytoplasmic μ-chain bearing cells in normal mice. J. Immunol. 131:2635-2640.

Owen, J.J., D.E. Wright, S. Habu, M.C. Raff, and M.D. Cooper, (1977). Studies on the generation of B lymphocytes in fetal liver and bone marrow. J. Immunol. 118:2067 2072.

Panangala, V.S., J.J. Giambrone, U.L. Diener, N.D. Davis, F.J. Hoerr, A. Mitra, R.D. Schultz, G.R. Wilt (1986). Effects of aflatoxin on the growth performance and immune responses of weanling swine. Am. J. Vet. Res. 47:2062-2067.

Peterson (1983). Determination of total protein. Methods in Enzymology 91:95-119.

Pier, A.C., R.E. Fichtner, S.J. Cysewski (1977). Effects of aflatoxin on the cellular immune system. An. Neur. Alim. 31:781-788.

Pinckard, R.N. (1978). Equilibrium dialysis and preparation of hapten conjugates. In: Handbook of experimental immunology. 17.1-17.23. Vol, 1st ed. Oxford: Blackwells. D.M. Weir ed.

Polanec, J. I. Seppälä, S. Rousseau, and K. Hedman (1994). Evaluation of protein denaturing immunoassays for avidity of imunoglobulin G to rubella virus. J. Clin. Lab. Anal. 8:16-21.

Pulendran, B., K.G.C. Smith, and G.J.V. Nossal (1995). Soluble antigen can impede affinity maturation and the germinal center reaction but enhance extrafollicular immunoglobulin production. J. Immunol. 155:1141-1150.

Pullen, G.R., G.M. Fitzgerald, and C.S. Hosking (1986). Antibody avidity determination by ELISA using thiocyanate elution. J. Immunol. Methods 86:83-87.

Rainbow, L., S.M. Maxwell, and R.G. Hendrickse (1994). Ultrastructural changes in murine lymphocytes induced by aflatoxin B_1. Mycopathologia 125:33-39.

Rath, S., C.M. Stanley, M.W. Steward (1988). An inhibition enzyme immunoassay for estimating relative antibody affinity and affinity heterogeneity. J. Immunol. Methods 106:245-249.

Reddy, R.V. and R.P. Sharma (1989). Effects of aflatoxin B_1 on murine lymphocytic functions. Toxicology 54:31-44.

Rodbard, D., Munson, P.J., Thakur, A.K. (1980). Quantitative characterization of hormone receptors. Cancer 46: 2907-2918.

Roman, T. and J. Charlemagne (1994). The immunoglobulin repertoire of the rainbow trout (*Oncorhynchus mykiss*): Definition of nine IgH-V families. Immunogenetics 40:210-216.

Sarvas, H. and O. Mäkelä (1970). Haptenated bacteriophage in the assay of antibody quantity and affinity: maturation of an immune response. Immunochemistry 7:933-943.

Scatchard, G. (1949). The attractions of proteins for small molecules and ions. Ann. NY Acad. Sci. 51:660-672.

Schilling, J., B. Clevinger, J.M. Davie, L. Hood (1980). Amino acid sequence of homogeneous antibodies to dextran and DNA rearrangements in heavy chain V-region gene segments. Nature 283:35-40.

Schultz, P.G. (1988). The interplay between chemistry and biology in the design of enzymatic catalysts. Science 240:426-433.

Scott, T.R., S.M. Rowland, R.S. Rodgers, A.B. Bodine (1991). Genetic selection for aflatoxin B_1 resistance influences chicken T-cell and thymocyte proliferation. Dev. Comp. Immunol. 15:383-391.

Sennhauser, F.H., R.A. Macdonald, D.M. Roberton, and C.S. Hosking (1989). Comparison of concentration and avidity of specific antibodies to *E. coli* in breast milk and serum. Immunology 66:349-397.

Shimizu, A., N. Takahashi, Y. Yaoita, T. Honjo (1982). Organization of the constant region gene family of the mouse immunoglobulin heavy chain. Cell 28:499-506.

Shlomchik, M.J., A. Marshak-Rothstein, C.B. Wolfowicz (1987). The role of clonal selection and somatic mutation in autoimmunity. Nature 328:805-811.

Siskind, G.W. and B. Benacerraf (1969). Cell selection by antigen in the immune response. Adv. Immun. 10:1-47.

Siskind, G.W., P. Dunn, and J.G. Walker (1968). Studies on the control of antibody synthesis. II. Effect of antigen dose and of suppression by passive antibody on the affinity of antibody synthesized. J. Exp. Med. 127:55-66.

Staudt, L.M., and M.J. Lenardo (1991). Immunoglobulin gene transcription. Annu. Rev. Immunol. 9:373-398.

Steward, M.W. (1986). Handbook of experimental immunology. Vol. I, Chap. 25, D.M. Weir, ed. (Blackwell Scientific Publications).

Stupp, Y., Yoshida, T., Paul, W.E. (1969). Determination of antibody-hapten equilibrium constants by an ammonium sulfate precipitation technique. J. Immunol. 103(3):625-627.

Suenaga, R. and N.I. Abdou (1993). Cationic and high affinity serum IgG anti-dsDNA antibodies in active lupus nephritis. Clin. Exp. Immunol. 94:418-422.

Takemori, T. And T. Tada (1974). Selective roles of thymus-derived lymphocytes in the antibody response. II. Preferential suppression of high-affinity antibody-forming cells by carrier-primed suppressor T cells. J. Exp. Med. 140:253-266.

Tew, J.G., M.H. Kosco, G.F. Burton, A.K. Szakal (1990). Follicular dendritic cells as accessory cells. Immunol. Rev. 117:185-211.

Thakur, A.K., Jaffe, M.L., Rodbard, D. (1980). Graphical analysis of ligand-binding systems: evaluation by Monte Carlo studies. Anal. Biochem. 107:279-295.

Tonegawa, S. (1983). Somatic generation of antibody diversity. Nature 302:575-581.

Velick, S.F., Parker, C.W., and Eisen, H.N. (1960). Excitation energy transfer and the quantitative study of the antibody hapten reaction. Biochemistry 46:1470-1482.

Voss, E.W., W.J. Groberg and J.L. Fryer (1978). Binding affinity of tetrameric coho salmon Ig anti-hapten antibodies. Immunochem. 15:459-464.

Wabl, M., P.D. Burrows, A. Von Gabain, and C. Steinberg (1985). Hypermutation at the immunoglobulin heavy chain locus in a pre-B-cell line. Proc. Natl. Acad. Sci. USA 82(2):479-482.

Wilson, M., E. Hsu, A. Marcuz, M. Courtet, L. DuPasquier, C. Steinberg (1992). What limits affinity maturation in *Xenopus* - The rate of somatic mutation or the ability to select mutants? EMBO J. 11:4337-4347.

Wilson, M.R. and G.W. Warr (1992). Fish immunoglobulins and the genes that encode them. Annu. Rev. Fish Dis. 2:201-221.

APPENDICES

APPENDIX I: BUFFERS AND REAGENTS

I. Cacodylate buffer (0.28 M)

44.8 gm Cacodylic acid (Sigma, St. Louis, MO)

9 pellets NaOH

Dissolve in one liter of distilled water. Adjust to pH 7.0 with dropwise addition of 2.0N HCl. Filter sterilize through 0.22 μm and store at 4°C. Prepared as described by Rittenberg and Amkraut (1966).

II. Phosphate buffered saline (PBS)

III. Borate buffered saline (BBS)

6.28 g boric acid (Sigma, St. Louis, MO)

9.54 g $Na_2B_4O_7 \bullet 10H_2O$ (sodium tetraborate)

8.5 g NaCl (sodium chloride)

Dissolve in one liter of deionized water.

Prepared as described in Garvey et al. (1977)

IV. Benzocaine (10% stock solution)

10 g ethyl p-aminobenzoate (Sigm, St. Louis, MO)

90 ml ethanol

Mix thoroughly. Store at RT. Dilute 1:1000 (approx 5 ml to 5 L) to use.

V. ELISA coating fuffer

0.795 g Na_2CO_3

1.465 g $NaHCO_3$

QS to 500 ml with ddH_2O. PH to 9.6. Store at 4°C.

VI. Tween-tris buffered saline (TTBS)

1. 121.4g Trizma Base
2. 8.18 g EDTA
3. 174.0 g NaCl

Dissolve in 3 L ddH_2O. Add 50.0 ml conc. HCl to bring pH near to 8.0 (adjust pH with HCl as necessary). Add 20 ml Tween 20 (polysorbitan monolaureate). QS to 20.0 L.

VII. ABTS substrate solution

1. Citrate buffer

   0.2 g Citric Acid

   100 ml ddH_2O.

   PH to 4.0. Store 4°C.

2. 0.1 g ABTS (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) in 10.0 ml ddH_2O.
3. Hydrogen peroxide (H_2O_2) 30%.

Combine 9.6 ml citrate buffer with 400 µl ABTS stock and 7µl H_2O_2 for each ELISA plate to be developed.

VIII. 3M Tris-HCl, pH 8.8 or 6.8

36.3 g Trizma base. QS to 100 ml with ddH_2O. PH to 8.8 or 6.8 with conc. HCl.

IX. Acrylamide/bis-acrylamide stock (30% Total, 36.5:1)

29.2 g Acrylamide powder.

0.8 g Bis-acrylamide.

QS to 100 ml w/ddH_2O. Filter through Whatman filter paper or 0.22 µm cellulose acetate filter. Store at 4°C.

X. 10% SDS stock

10.0 g SDS. QS to 100 ml w/ddH_2O. Filter through Whatman filter paper or through 0.22 µm.

XI. 10% Triton X-100

QS 5.0 ml Triton X-100 to 50.0 ml with ddH_2O.

XII. Sample dye (1st dimension)

5.7 g Urea (9.5M)

2.0 ml 10% Triton X-100 stock (2%)

0.5 ml 2-mercaptoethanol (5%)

0.5 ml ampholyte (Total 2% if 40% stocks are available).

Dilute to 10.0 ml w/ddH_2O in a warm water bath. Aliquot 0.5 ml in Ependorff tubes and store at -70°C until used.

XIII. Sample overlay buffer (1st dimension)

5.41 g Urea (9.0M)

0.250 ml Ampholytes (1%)

500 µl Bromophenol Blue (0.05% w/v stock in ddH_2O).

QS to 10.0 ml w/ddH_2O. Filter through 0.22 µm. Aliquot into 0.5 ml Ependorff tubes. Store at -70°C.

XIV. IEF Upper Chamber Buffer (Catholyte, 20 mM NaOH)

Dissolve 0.2 g NaOH in 250 ml ddH_2O. Degas 15 min.

XV. IEF Lower Chamber Buffer (Anolyte, 10mM H_3PO_4)

Dilute 1.36 ml concentrated H_3PO_4 in 2L ddH_2O. Degas 15 min.

XVI. Sample dye (2nd dimension)

0.15 g Trizma base

0.4 g SDS

1.0 ml 2-mercaptoethanol

2.0 ml Glycerol

7.0 ml ddH_2O

0.02 g Bromophenol blue

QS to 10.0 ml. Filter through 0.22 µm. Aliquot 0.5 ml in Ependorff tubes. Store at -70°C.

XVII. First dimension acrylamide stock

10.0 ml 30% Total, 36.5:1 acrylamide stock (IX above)

0.086g bis-acrylamide

XVIII. IEF tube gel

2.75 g Urea

0.667 1st Dimension acrylamide stock (XVII above)

1.0 ml 10% (v/v) Triton X-100

0.25 ml (40%) Ampholytes

0.985 ml ddH_2O

Degas 15 min

16 µl Ammonium persulfate (0.1 g/ml ddH_2O)

16 µl TEMED

XIX. SDS-PAGE separating gel (12% acrylamide)

4.6 ml ddH_2O

1.25 ml 3M Tris-HCl, pH 8.8 (VIII above)

4.0 ml Acrylamide stock (30% Total, 36.5:1; IX above)

Degas 15 minutes

100 µl 10% SDS in ddH_2O

50 µl Ammonium persulfate (0.1 g/ml ddH_2O)

5 µl TEMED

XX. Stacking Gel

8.2 ml ddH_2O

0.4 ml 3M Tris-HCl, pH 6.8 (VIII above)

1.25 ml Acrylamide stock (30% Total, 36.5:1; IX above)

Degas 15 minutes

100 µl 10% SDS in ddH_2O

60 µl Ammonium persulfate (0.1 g/ml ddH_2O)

6 µl TEMED

XXI. Laemli gel running buffer

14.4 g Glycine

3.03 g Trizma base

10.0 ml 10% SDS stock

QS to 1.0 L. PH to 8.9 with HCl if necessary. Store at 4°C.

XXII. Transblot buffer

43.2 g Glycine

9.09 g Trizma base

600 ml Methanol

QS to 3.0 L with ddH_2O

APPENDIX II: SAMPLE AFFINITY ELISA CALCULATIONS

The following example demonstrates how ELISA data is converted into affinity values and antibody population distributions, weighted mean serum affinity values are calculated, and comparisons of antibody responses over time are made. Table I records the raw data for a typical affinity-based ELISA. The serum was derived from bleeding a rainbow trout immunized six weeks previously with TNP-KLH. If there is a limited amount of a particular serum, duplicate values may be omited, or the number of antigen concentrations may be reduced. Be advised that doing so may reduce the reliability of the measurements.

TABLE I (Raw data)

	TNP-lysine [M]											
	0		8×10^{-7}		4×10^{-6}		2×10^{-5}		1×10^{-4}		5×10^{-4}	
	1	2	3	4	5	6	7	8	9	10	11	12
A	.082	.084	.084	.079	.074	.077	.078	.078	.078	.071	.069	.073
B	.170	.162	.122	.121	.092	.091	.076	.076	.070	.070	.068	.073
C	.331	.316	.240	.227	.152	.155	.093	.091	.074	.071	.066	.069
D	.815	.789	.685	.638	.439	.467	.203	.203	.086	.082	.069	.073
E	1.573	1.487	1.430	1.404	1.212	1.214	.776	.801	.187	.190	.073	.074
F	2.002	1.980	1.915	1.864	1.772	1.718	1.355	1.364	.502	.486	.081	.083
G	2.190	2.089	2.046	1.951	1.890	1.889	1.589	1.563	.749	.733	.094	.095
H	2.388	2.150	2.223	2.116	1.782	2.052	1.758	1.731	.898	.854	.113	.112

In Table II the values have been transformed by averaging each duplicate and subtracting the average value for wells A1 to A12 (0.077). Any value which is below this background value is ascribed a value of 0. If the zero antigen row (A) demonstrates a substantive O.D. which is hapten inhibitable the reader is advised to disregard the entire plate and more closely examine any error that may have generated such anomolous readings.

The fraction or percentage of serum antibody within each affinity subpopulation can be ascertained prior to the calculation of the actual affinity of that population. As discussed above, the least amount of coated antigen will only bind the highest affinity antibody (Row B). As the amount of coating antigen is increased, more of the lesser affinity antibodies can be accomodated. Subtraction of the O.D. found in the antigen only column (1-2) of a previous row (e.g. A from B or B from C) renders an O.D. value that is proportional to the antibody additionally bound by the increase in coating antigen. Thus, subtracting B 1^2 (0.089) from C 1^2 (.246) gives an O.D. proportional to the additional antibody that is bound by increasing the coating antigen from .0122 μg/ml to a concentration of .048 ug/ml. This additional antibody is of lower affinity than that bound by the .0122 μg /ml concentration. Dividing these values by the maximum O.D. seen in H 1^2 (2.192) gives the percent antibody additionally bound by the incremental increase in coating antigen. Sample calculations are shown in Table III. The

range of coating antigens may be shifted if there is a substantial skewing of percentages to either end of the coating antigen concentration range. Such skewing would result in an inability to resolve fine differences in either the high or low affinity subpopulations.

TABLE II (Average values - background)

	TNP-lysine [M]					
	0	**8×10^{-7}**	**4×10^{-6}**	**2×10^{-5}**	**1×10^{-4}**	**5×10^{-4}**
	1-2	3-4	5-6	7-8	9-10	11-12
A	-	-	-	-	-	-
B	.089	.044	.014	0	0	0
C	.246	.156	.076	.015	0	0
D	.725	.584	.376	.126	.007	0
E	1.453	1.340	1.136	.711	.111	0
F	1.915	1.812	1.668	1.282	.417	.005
G	2.062	1.914	1.812	1.499	.664	.017
H	2.192	2.092	1.840	1.667	.819	.035

Table III. Calculation of Antibody Subpopulation Sizes

$$\text{Highest affinity (\%)} = \frac{\text{Row B 1\^2}}{\text{Row H1\^2}} \times 100 = \frac{.089}{2.192} \times 100 = \mathbf{4.1\%}$$

$$\text{2nd highest (\%)} = \frac{(\text{Row C 1\^2}) - (\text{Row B 1\^2})}{(\text{Row H 1\^2})} \times 100 = \frac{.246 - .089}{2.192} \times 100 = \mathbf{7.2\%}$$

3rd highest (%) = $\frac{\text{(Row D 1\^2) - (Row C 1\^2)}}{\text{(Row H 1\^2)}} \times 100 = \frac{.725 - .246}{2.192} \times 100 = \mathbf{21.8\%}$

4th highest (%) = $\frac{\text{(Row E 1\^2) - (Row D 1\^2)}}{\text{(Row H 1\^2)}} \times 100 = \frac{1.453 - .725}{2.192} \times 100 = \mathbf{33.2\%}$

5th highest (%) = $\frac{\text{(Row F 1\^2) - (Row E 1\^2)}}{\text{(Row H 1\^2)}} \times 100 = \frac{1.915 - 1.453}{2.192} \times 100 = \mathbf{21.1\%}$

6th highest (%) = $\frac{\text{(Row G 1\^2) - (Row F 1\^2)}}{\text{(Row H 1\^2)}} \times 100 = \frac{2.062 - 1.915}{2.192} \times 100 = \mathbf{6.7\%}$

7th highest (%) (Lowest affinity) = $\frac{\text{(Row H 1\^2) - (Row G 1\^2)}}{\text{(Row H 1\^2)}} \times 100 = \frac{2.192 - 2.062}{2.192} \times 100 = \mathbf{5.9\%}$

Wells 3-12 of each row represent a binding inhibition study using free hapten to inhibit the population of antibody bound by the antigen concentration coated in each row. The logarithm of the reciprocal of the concentration of hapten required to inhibit 50% of the O.D. found in 1^2 of each row is considered to be proportional to the affinity constant for the subpopulation of antibodies bound by that concentration of antigen. This concentration can be determined by first converting the O.D. values in each row into percentages of the maximum O.D. found in column 1^2 of that row. Calculations for only two rows (B and H) are shown below in Table IV. These Rows would represent the highest (B) and the lowest (H) affinity antibodies.

Table IV. Calculation of Antibody Subpopulation Affinities

Inhibition Values for the Highest Affinity Antibody

Maximum OD	=	B 1^2 (.089) x 100 B 1^2 (.089)	= 100%
% OD w/8x10-7M hapten	=	B 3^4 (.044) x 100 B1^2(.089)	= 49.4%
% OD w/4x10-6M hapten	=	B 5^6 (.014) x 100 B1^2(.089)	= 15.7%
% OD w/2x10-5M hapten	=	B 7^8 (0.0) x 100 B 1^2(.089)	= 0%
% OD w/1x10-4M hapten	=	B 9^10 (0.0) x 100 B 1^2(.089)	= 0%
% OD w/5x10-4M hapten	=	B 11^12 (0.0) x 100 B 1^2(.089)	= 0%

Table V. Inhibition Values for the Lowest Affinity Antibody

Maximum OD	=	H 1^2 (2.192) x 100 / H 1^2 (2.192)	= 100%
% OD w / 8x10-7M hapten =		H 3^4 (2.092) x 100 / H 1^2(2.192)	= 95.4%
% OD w / 4x10-6M hapten =		H 5^6 (1.840) x 100 / H 1^2(2.192)	= 83.7%
% OD w / 2x10-5M hapten =		H 7^8 (1.667) x 100 / H 1^2 (2.192)	= 76.0%
% OD w / 1x10-4M hapten =		H 9^10 (.819) x 100 / H 1^2 (2.192)	= 37.4%
% OD w / 5x10-4M hapten =		H 11^12 (0.035) x 100 / H 1^2 (2.192)	= 1.2%

These percentages can now be plotted and an [H]50 value for each antibody subpopulation determined graphically as depicted in figure 1, or mathematically (Banowetz, 1987; Arkoosh, 1989).

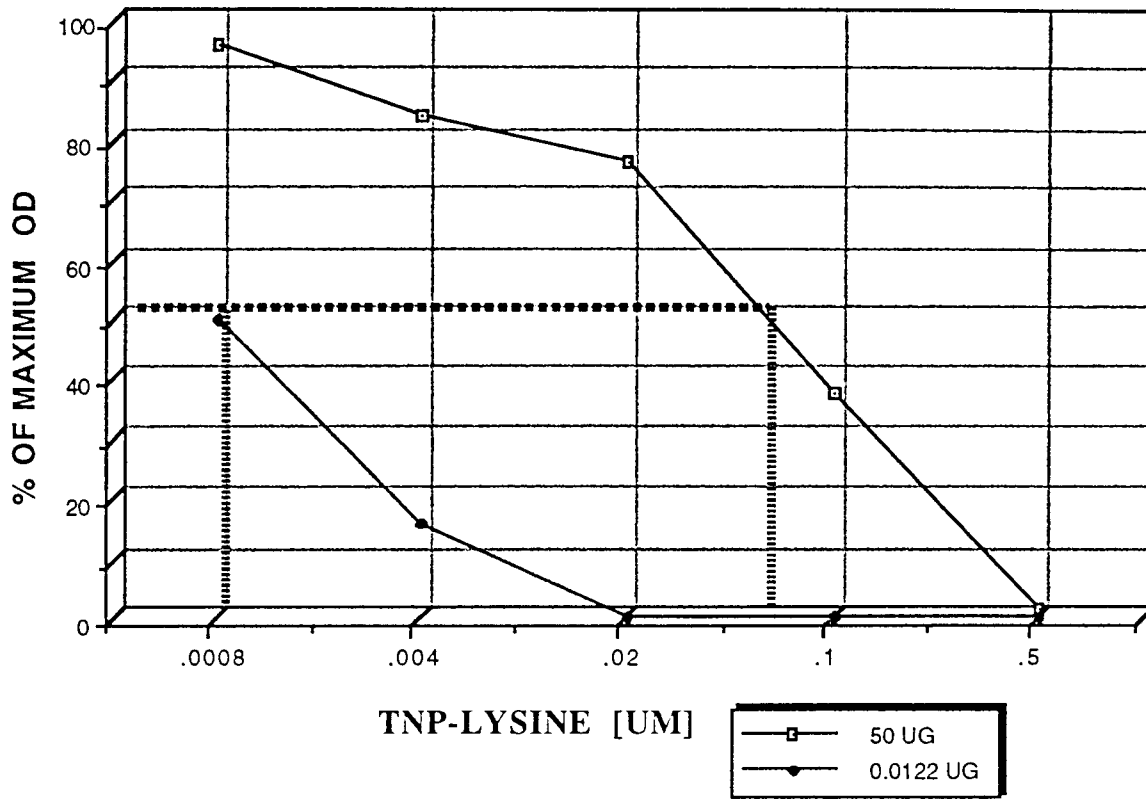


Figure 1 $[H]_{50}$ analysis of serum anti-TNP antibody bound by two concentrations of TNP-BSA. Data used in this figure were derived from Table V. $[H]_{50}$ for 0.0122 µg/ml TNP-BSA was 7.94 x 10^{-7} M giving an aK of 6.10 for the corresponding antibodies. $[H]_{50}$ for 50 µg/ml TNP-BSA was 5.89 x 10^{-3} M giving an aK of 4.23.

The two plots in figure 1 represent the inhibition curves for the highest affinity antibodies (0.0122 µg of coated antigen/ml) and the lowest affinity antibodies (50 µg of coated antigen/ml). Such analysis is performed for each antigen coating concentration. An accumulated table of values for

aKs and relative proportions of each affinity subpopulation may be derived (Table VI). Thus the $[H]_{50}$ values are converted to affinity constants (aK) using the following formula:

$$aK = \log\ (1 / [H]_{50})$$

where $[H]_{50}$ = the concentration of hapten in moles/liter which results in 50% inhibition of the maximum O.D. (Column 1-2)

Affinity values for the remaining subpopulations (Rows C - G) may be determined and their values checked against the values given in Table V. Upon determining the aK values and the proportion of the total antibody residing within each subpopulaion, a weighted average affinity value may be calculated for the serum (Table VI). Such values facilitate the rapid, or relatively uncomplicated comparison of various sera. For example, the weighted mean affinity value for the 6 week antiserum was 4.88 (Table VI), but that of the 14 week antiserum from the same fish was 6.00, indicating affinity maturation.

Table VI. Proportions and Affinities of Antibody Subpopulations

[TNP-BSA]		aK	%	P	aK x P
50	μg/ml	4.23	5.9	.059	0.249
12.5	"	4.31	6.7	.067	0.289
3.12	"	4.44	21.1	.211	0.937
0.78	"	4.72	33.2	.332	1.567
0.19	"	5.36	21.8	.228	1.168
0.048	"	5.81	7.2	.072	0.418
0.0122	"	6.10	4.05	.041	0.247
		Σ (aK x P) [weighted average]		=	4.88