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Immunological memory has been shown to occur unequivocally in mammals. However, controversy exists regarding the capability of fish to elicite a memory response. This study demonstrates that memory does occur in the Shasta strain of rainbow trout, *Oncorhynchus mykiss*.

Trout injected with a T-dependent antigen, trinitrophenylated keyhole-limpet hemocyanin, (TNP-KLH) (100 µg/fish) were able to produce a primary serum antibody response to TNP. This was demonstrated by a quantitative ELISA specifically developed for this purpose. After a second injection of antigen (20 µg/fish), an increase in antibody titer developed to TNP along with a shorter lag period, as is characteristic of a mammalian secondary response.

The antibodies produced to TNP were further analyzed by two different ELISAs, avidity and inhibition ELISAs. Avidity

measurements, which reflects both the effects of affinity and valency on the strength of antibody binding, demonstrated no change in avidity during the primary or secondary serum antibody response. The inhibition ELISA it was possible to demonstrate subtle changes in the fine specificity of the antibody populations by using a sufficiently diverse battery of TNP-lysine analogues.

In vivo priming of trout with TNP-KLH produced cells which were capable of producing a heightened in vitro plaque-forming cell response to TNP-LPS, a T-independent antigen, and TNP-KLH a T-dependent antigen. This is similar to what has been reported in mammals and demonstrates that fish are capable of expressing memory to T-independent and T-dependent forms of antigen.

The modulation of the memory response was also monitored after a short embryonic exposure of aflatoxin B_1 in order to determine if this environmental toxicant could affect the primary and/or memory antibody response to TNP. Neither in vivo nor in vitro primary responses were affected by the mycotoxin, but the ability to produce a memory response was eliminated. A change in avidity due to aflatoxin exposure was not witnessed, but the fine specificities of the antibodies were altered as was evidenced by examination of the relative affinities for the analogues of TNP.

Development of Immunological Memory in Rainbow Trout (Oncorhynchus mykiss) and Aflatoxin B_1 Modulation of the Response

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IN LOVING MEMORY OF DADDY AND ANNE

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF TABLES

Development of Immunological Memory in Rainbow Trout (Oncorhynchus mykiss) and Aflatoxin B_1 Modulation of the Response

INTRODUCTION

This study was undertaken to characterize the development of immunological memory in rainbow trout (Oncorhynchus mykiss). The analysis of memory in fish is crucial from a comparative standpoint in that it would provide for a better understanding of the phylogeny of the immune system as well as benefit the field of fish vaccination. A more complete working knowledge of the generation of immune memory in fish is essential to the development of efficacious vaccines.

In mammals, memory is defined by several parameters including: 1. an increase in antibody titer after a second exposure to the antigen (Eisen, 1980), 2. an enhanced secondary response upon challenge with an immunogenic or non-immunogenic concentration of antigen (Tittle, 1978), 3. a shorter lag period relative to the primary response (Eisen, 1980) and 4. isotype switching events (Baker and and Stashak, 1969; Barthold et al., 1974; Braley-Mullen, 1974; Braley-Mullen, 1975). Also, affinity maturation (Paul et al., 1967), which is the ability to induce antibodies with higher affinity over time, occurs in mammals. Somatic mutation in mammals is credited for the generation of high

affinity clones (Kochs and Rajewsky, 1989) and can be detected by a shift in the fine specificities of the antibodies. Many of these parameters were examined, including the possibility that antigen might induce an increase in B cell precursors and their clone sizes.

Aflatoxin B_1, a mycotoxin, has been found to be a very potent toxin, immunosuppressive agent and hepatic carcinogen in a variety of animals (Bailey et al., 1984; Casarett and Doulls, 1986). Previous studies have demonstrated that the Shasta strain of rainbow trout is an exceptionally sensitive and economical model for the study of aflatoxin-induced hepatocellular carcinomas (Hendricks et al., 1980; Sinnhuber et al., 1977). Investigations concerning the immunological effects of aflatoxin-exposure, however, have been limited to mammals (Pier et al., 1977; Richard et al., 1983) and birds (Thaxton, et al., 1974). The effects of aflatoxin on the antibody response, and in particular, memory, in trout was examined.

LITERATURE REVIEW

MEMORY B CELL DEVELOPMENT IN MAMMALS

Early theories of immunological memory, had their origins in the context of studies concerning small pox. As early as the ninth century an Arab physician, Razi, realized that if an individual survived an infection of small pox, he/she would become immune to the disease. Razi believed that the "moisture" in the blood was responsible for small pox. Once an individual was infected, their skin would erupt in sores. These sores would release the "moisture" in the blood. If the person recovered, he/she would not be susceptible to the disease again because the "moisture" in the blood was exhausted by the initial infection. It was also his contention that, the young suffered far more from the disease than adults, because youths' blood contained more "moisture" than the blood of adults. This theory was termed an expulsion theory. Many other incorrect but interesting theories were postulated and grouped as either expulsion, depletion, distension or retention theories. All of these theories had one point in common in their postulates; the infected host acts as a passive participant in the disease (Silverstein and Bialasiewicz, 1980).

In the nineteenth century, theories began to incorporate the idea of the infected host actively participating in the response against the disease (Silverstein, 1979). One of the major theories developed by immunologists at this time was whether acquired

immunity was best explained by cellular or humoral mechanisms. The cellularists, led by Eli Metchnikoff, felt that the macrophage was responsible for acquired immunity, while the humoralists, led by Robert Koch, felt that only soluble substances of the blood could fight disease. During the early part of the twentieth century it was felt that the antibody was the key to understanding all immunological questions. It appeared that the cellularists had lost the battle. Immunologists, such as Erhlich and Landsteiner took a chemical approach in their understanding of antibodies. Meanwhile, studies on the cellular aspect of the immune response waned for the next fifty years. However, in the early 1960's questions were being posed that only cellular immunology could answer. Once more, the cellular aspects of immunology became an important subject of immunology.

MEMORY WITH T-DEPENDENT AND T-INDEPENDENT FORMS OF ANTIGEN

Ovary and Benaceraff (1963) demonstrated *in vivo* an important concept known as the carrier effect. They determined that a mouse primed to dinitrophenyl-bovine gammaglobulin (DNP-BγG) must receive a second injection of the original hapten conjugated to the original carrier in order for an increased antibody titer to develop to the hapten (DNP). Mice receiving a secondary injection of DNP conjugated to ovalbumin (DNP-OVA) rather than BγG were unable to produce this increase in antibody

titer. Therefore, it was apparent that for an increase in antibody titer to occur, memory must develop to both hapten and carrier.

An in vivo method was also used by Mitchison (1971) in his classic experiment, to determine if the carrier effect could be transferred strictly by cells. To demonstrate this he performed adoptive cell transfers of mouse spleen cells. Spleen cells from mice primed with NIP-CγG (4-hydroxy-5-iodo-3-nitro-phenylacetyl-chicken gammaglobulin) were injected into a lethally irradiated recipient. Irradiation was used to inactivate the recipient's lymphocytes (Hood et al., 1985). The recipient was given a second injection of NIP-CγG or of the same hapten conjugated to a different carrier and the animals' sera were than titered. Only the recipient receiving an injection of the original priming hapten-carrier conjugate was able to produce an increased antibody titer against the hapten. Raff (1970) determined that it was the T lymphocyte that was being primed by the carrier. This would then necessitate that the same carrier must be attached to the hapten upon rechallenge. Memory, in the classical sense, is elicited by priming with the T-dependent form of an antigen (Andersson, 1971 and Braley-Mullen, 1974). It was thus found that memory is induced by a class of antigens, those composed of protein carriers, which require T cells (T-dependent) (Braley-Mullen, 1975; Lerman et al., 1975; Umetsu et al., 1979; Feldbush, 1980). T-independent (T-I) antigens, polysaccharides and lipopolysaccharides, do not require T cells for a response, however, they are incapable of inducing memory (Andersson and Blomgren,

1971, and Braley-Mullen, 1974). Researchers using various in vivo protocols, have demonstrated the ability of a T-dependent (T-D) antigen primed animal to produce a memory response upon reexposure either to a T-D or T-I form of the antigen (Braley-Mullen, 1975; Umetsu et al., 1979). This was originally demonstrated at the cellular level using a polysaccharide antigen (T-I) (Braley-Mullen, 1975). Mice were first injected with a T-dependent antigen, pneumococcal polysaccharide conjugated to horse red blood cells (SIII-HRBC). Conjugation of a polysaccharide to a T-dependent antigen confers T-dependency. After the secondary in vivo challenge with the T-D form of antigen, SIII-HRBC, or the T-I form of antigen, SIII, an IgG memory response was detected as determined by the plaque forming cell assay. The plaque forming cell assay was developed by Jerne (1963) for the enumeration of antibody producing cells. Another study involved adoptive transfer of spleen cells from mice primed in vivo to a T-D antigen, TNP conjugated to keyhole limpet hemocyanin (TNP-KLH) (Umetsu et al., 1979). These experiments also demonstrated that not only was TNP-KLH able to generate an IgG memory response but also TNP-ficoll, a T-I form of the antigen, was able to do so. This was, again, determined by the use of the plaque forming cell assay. The secondary responses produced by both TNP-ficoll and TNP-KLH were more heterogeneous in their antibodies, greater in absolute magnitude and of higher average affinity than were the primary responses to these antigens. Thus, T-D antigens have the ability to prime a B cell population to later produce a memory IgG

response upon reexposure to either the T-D or a T-I forms of antigen. This phenomenon was also demonstrated by analysis of serum antibody responses (Braley-Mullen 1975; Rennick et al., 1983). Rennick et al. (1983) employed the adoptive transfer of murine spleen cells primed to tobacco mosaic virus protein (TMVP), a T-D antigen, in one study. Once these primed cells were transferred to an irradiated recipient, the animals were stimulated with either TMVP or a decapeptide of TMVP conjugated to succinylated human gammaglobulin (decapeptide SHγG), a T-D form of TMVP, or to a T-independent form of TMVP, decapeptide-Brucella abortus (BA). Sera collected from mice receiving decapeptide-BA as a secondary challenge were able to produce an antibody response to the decapeptide as determined by a radioimmunoassay. Decapeptide-BA, however, did not produce a serum antibody response in animals which had not been previously exposed to the antigen. Sera collected from mice which were injected with cells that had been exposed twice to a T-D form of the antigen, also produced an excellent titer against the decapeptide.

Hemagglutinin techniques have also been used to demonstrate the generation of an IgG memory response which was developed by priming with the T-D antigen SIII conjugated to sheep red blood cells (SIII-SRBC) and subsequent induction with a secondary injection of either SIII-SRBC (T-D) or SIII (T-I) (Braley-Mullen, 1975). Serum collected from mice given a secondary injection of SIII-SRBC were able to produce an IgG $\log_2$

hemagluttinin titer of approximately six, while during the primary response was always less than two. Mice given a secondary injection of SIII were also able to produce an increased IgG hemagglutinin titer during the secondary response which was greater than that observed in the primary response.

Crucial to the evolution of the field of cellular immunology was the development, by Mishell and Dutton (1967), of a method for culturing and immunizing dissociated murine spleen cells in vitro. Initially, they were able to induce antibody responses to red blood cells from various sources (sheep, goat, burro, cattle, horse, pig and rabbit). Using the plaque forming cell assay method, it was possible to determine the number of cells in the spleen that were actively producing antibodies to the stimulating antigen. This in vitro technique paralleled the kinetics of the response, the antigen dose, and the magnitude of the response observed in vivo. This indicated that similar or identical factors were at work in both systems. Originally, however, the use of this technique to elicit IgG memory responses was hampered by the fact that multiple injections were required. (North and Askonas, 1976; Roman and Rittenberg, 1976). In an animal, only a single injection is required prior to the elicitation of an IgG response. The problem associated with the study of memory cells in vitro after multiple injections was discussed by Tittle and Rittenberg (1978). They suggested that multiple injections actually makes the study of resting memory cells impossible. They argue that this procedure gives rise to B cells that have already been activated and are undergoing the

maturation process in vitro, regardless of the addition of antigen. They reasoned that a more realistic situation would be one where resting memory B cells could be specifically activated in vitro by antigen. To overcome this difficulty they developed a method which was comparable to the induction of an in vivo memory B cell response in that it did not require multiple in vivo boostings. They were able to demonstrate that resting murine spleen cells which had been previously primed in vivo to a T-D antigen were able to produce an increased plaque forming cell (PFC) response in vitro, when 2-mercaptoethanol was incorporated in their culture system. Further, they were also able to demonstrate the same phenomenon in vitro that Braley-Mullen (1975) and Umetsu et al. (1979) had demonstrated in vivo. That is, they produced an IgG memory response to the T-independent form of the T-dependent antigen used for priming. Thus, it is apparent from both in vivo and in vitro experiments that a T-dependent antigen can stimulate a population of cells which can be further stimulated by the T-dependent antigen or a T-independent form of the antigen to produce a memory response.

SUBSETS OF MEMORY B CELLS

Originally evidence accumulated which suggested that T-dependent and T-independent antigens are able to stimulate different populations of primary B cells (Playfair and Purves, 1971; Jennings and Rittenberg, 1976; Lewis and Goodman, 1977). The

possibility for such a populational division among memory B cells was also addressed (Tittle and Rittenberg, 1978; Tittle and Rittenberg, 1980; Rennick et al., 1983). Tittle and Rittenberg (1978) were the first to present evidence that the IgG memory response was generated by two different subpopulations of B lymphocytes: B1γ which responds to the T-independent form of the antigen, and B2γ which responds to the T-dependent form of antigen. Both subpopulations were produced by priming with a T-dependent antigen. The evidence which supported the existence of these two subsets were; (1) the in vitro response of IgG memory cells induced by TNP-KLH (T-dependent) and TNP-T4 (E. coli phage T4) (T-independent) were additive as determined by a plaque assay, (2) the IgG precursor frequencies of cells responding to TNP-KLH and TNP-T4 were additive and (3) by using BUdR (5-bromouridinedeoxyribose) and light, they were able to eliminate one subset with out effecting the response of the other subset. Tittle and Rittenberg (1980) also found that treatment of a T-dependent primed memory population with BUdR and light after culture with a specific form of a TI antigen (T-I1) (TNP-BA or TNP-lipopolysaccharide) eliminates both T-D and TI-2 (polysaccharide) responses. Therefore, they concluded that IgG memory B cell precursors produced to a T-D form of TNP were of two subsets-B1γ (stimulated by T-I2) and B2γ (stimulated by T-D) and a T-I1 form of the antigen was capable of stimulating both populations. Working with a different antigenic system, Rennick et al. (1983) demonstrated that the IgG memory response induced with TMVP

consists of overlapping populations of cells which respond to the T-D and TI-1 form of the antigen. This was determined by the use of adoptive transfer experiments in which the recipients of TMVP-primed cells, which were challenged simultaneously with decapeptide-BA and TMVP, did not elicit an additive antibody response as determined by an ELISA. They suggested that the population of cells responding to TNP-BA, however, was only a fraction of the cells responding to the T-dependent antigen because the response to TMVP-BA was consistently lower. Therefore, the form of the antigen used determines which subpopulations of memory B cells will become activated. Also, it is unclear whether these different subpopulations are discreet B cell lineages or whether they represent different stages of maturation within a single B cell lineage.

LIMITING DILUTION ANALYSIS

BACKGROUND

With the advent of cell culture techniques (Mishell and Dutton, 1967) and the ability to determine the precise number of responding PFC (Jerne, 1963; Cunningham and Szenberg, 1968), it was possible to develop a procedure which allowed for the estimation of the frequency of antigen-specific precursor lymphocytes as well as to quantify the progeny of these precursors (clone size). This procedure, known as limiting dilution analysis

(LDA) (Lefkovits, 1972) is used extensively in determining the number of antigen-specific immunocompentent cell precursors in B cell, T-helper, T-suppressor and T-cytotoxic cell populations (Quintans and Lefkovits, 1975; Quintans and Cosenza, 1976; Gebel et al., 1983; Good et al., 1983; Bianchi et al., 1987). Many of these initial LDA studies were dedicated to the determination of B cell precursor frequencies and their clone sizes generated to various antigens during a primary response. Lefkovits (1972) determined that the precursor frequency to sheep red blood cells (T-dependent antigen) in the spleens of NMRI/HAN mice have a range of 1.5-10.5 precursors per 10^6 spleen cells. Kettman et al. (1985) estimated an anti-SRBC precursor frequency of 1 precursor per 10^4 cells in the spleen of BDF_1 mice. This is 100-fold higher than the frequency described by Lefkovits for NMRI/HAN mice. Kettman et al., also determined the precursor frequency to TNP-Brucella abortus, a T-independent antigen to be higher (1 precursor per 5 x 10^3 cells) than that observed for the SRBC, a T-dependent antigen. A hemolytic spot test was used by both Kettman (1985) and Lefkovits (1972) for the determination of positive cultures. Quintans and Lefkovits (1973) determined the SRBC precursor frequency of B cells in the bone marrow of nude mice by using allogenic spleen cells to insure that only the B cells were limiting and that a single-hit event would occur.

The clone sizes generated by the precursor cells can also be determined if a plaque forming cell assay is used (see methods and materials). Cosenza et al. (1975) estimated that the spleen cells of

BALB/c mice immunized with rough Pneumococcus pneumoniae strain R36A have a B cell precursor frequency between 1-2.5 precursors per 10^5 cells for the phosphorylcholine epitope expressed on the organism. They were also able to determine the clone size of these precursor cells to be 9 plaque forming cells. Thus, these examples demonstrate the power of the LDA to dissect the quantitative contributions of the immune response to a complex antigen. Tripp (1988) was first to determine the precursor frequency and clone size in an ectothermic vertebrate. He used the anterior kidney cells from coho salmon (Oncorhynchus kisutch) stimulated in vitro to TNP-LPS. He determined that these fish possessed 24 precursors per 10^6 leukocytes which could generate an average clone size of 17.

LIMITING DILUTION ANALYSIS OF B CELL MEMORY POPULATIONS

It was shown that the development of memory is associated with a tremendous increase in the production of plaque forming cells following subsequent exposure to antigen, but it was not until the advent of the LDA that the mechanism of this increase in PFC could be ascertained. Brooks and Feldbush (1981) were able to determine that a mixture of popliteal lymph node cells from a rat given either a primary injection of DNP-bovine gammaglobulin (BγG) or DNP-ovalbumin (OVA) has an anti-DNP B cell precursor frequency of 1.2 precursor cells per 10^4 leukocytes with a clone size of 27. Popliteal lymph node cells, that were stimulated again

in vitro with DNP-OVA, had an increased precursor frequency of 8 precursor cells per 10^3 leukocytes and a clone size of 190. LDA has also been used in the murine system to analyze memory subpopulations from mice. Tittle (1978) examined the IgG memory precursor frequency of spleen cells from mice primed in vivo to TNP-KLH on bentonite. This was accomplished by using either TNP-T4 as the T-independent antigen and TNP-KLH as the T-dependent antigen. He found the IgG precursor frequency memory response to TNP-T4 to be nearly 5 times greater (120 precursor cells per 10^5 cells) than what was expressed for the T-dependent antigen (0.26 precursor cells per 10^5 cells). Tittle did not correlate these results with the precursor frequency obtained from an unprimed animal. However, it did indicate that priming with a T-dependent antigen induces both a T-dependent and T-independent memory pool of lymphocytes. The increase in PFC and, thus, also antibody titer is due to the expansion of antigen-specific precursor pools or populations as well as to an ability of these precursors (memory) to generate larger clones.

AFFINITY MATURATION

Association constants are used to describe the affinity of an antibody combining site for its corresponding antigen. The affinity increase seen through time during a specific antibody response in an animal, is referred to as affinity maturation. This phenomenon, initially described by Eisen and Siskind (1964) during a primary

response with antibodies generated to DNP-BγG, has been examined using a variety of haptens (Mäkelä and Karjalainen, 1977; Berek et al., 1985; Gayà et al., 1986). Haptens are used in affinity studies as well as in fine specificity analysis because they are single, small, antigenic determinants which enable them to be used in a variety of techniques not possible for larger antigens.

Maturation of the immune response to the hapten oxazolone, was demonstrated by using fluorescence quenching and equilibrium dialysis of murine monoclonal antibodies. The affinity of the antibodies to oxazolone was found to increase during the secondary response (Berek et al., 1985) as well as during the primary response (Griffiths et al., 1984). Mäkelä and Karjalainen (1977) have shown that murine antibodies to (4-hydroxy-3-nitrophenyl)acetyl (NP) also exhibit an increase in affinity during the secondary response relative to the primary response. Gayà et al. (1986) demonstrated affinity maturation with serum antibodies to the arsonate hapten during the memory response by the use of a uniquely designed ELISA.

Affinity maturation has also been demonstrated in rabbits (Eisen and Siskind, 1964, Kimball, 1972 and Werblin et al., 1973). Equilibrium dialysis was used to determine that antibody affinity, from rabbits given a single injection of DNP-BγG, increases during the primary response (Werblin et al., 1973). Antibodies from rabbits hyperimmunized with Type III pneumococcal polysaccharide also demonstrated a progressive increase in their average affinity over time as determined by equilibrium dialysis.

(Kimball, 1972). Thus, it would appear that affinity maturation occurs during a memory antibody response as well as during a primary response.

The mechanisms for controlling affinity maturation are not known although several theories have been proposed (Siskind and Benacerraf, 1969; Tada and Takemori, 1974; De Kruyff and Siskind, 1979; Steward and Steensgaard, 1983; Gayà et al., 1986; Steward et al., 1986). The most popular hypothesis is that B cells are driven to proliferate by cellular competition for limited amounts of antigen (Siskind and Benacerraf, 1969). Therefore, B cells with receptors of higher affinity outcompete the B cells which have lower affinity receptors for antigen. Also, as this antigen becomes limiting in concentration, during the immune response, only B cells with increasingly higher affinity receptors will be activated. It has, however, also been proposed (Gayà et al., 1986) that affinity maturation is not due solely to the dominance of high affinity B cell clones but also to the progressive appearance of a low percentage of high-affinity B cell clones. Other experimental results suggest that the generation of B cell clones that express high affinity antibodies are generated from preexisting B cell clones as a result of somatic mutation and further recruitment of these high affinity clones by the immunizing antigen (Allen et al., 1987; Berek and Milstein, 1987; Allen et al., 1988). Kocks and Rajewsky (1989) suggest that this theory as well as the more common theory apply to the generation of higher affinity antibodies.

T cells are also thought to be important in affecting antibody affinity. T-helper cells have been shown to be required for the production of high affinity antibodies (DeKruyff and Siskind, 1979). In this study, mice, which had been lethally irradiated and reconstituted with bone marrow cells and various concentrations of T cells, were immunized with DNP-BγG and a plaque assay was performed 3 weeks later to determine the affinity as well as the magnitude of the response (Dekruyff and Siskind, 1979). Dekruyff and Siskind (1979) found that when the number of T cells were restricted, the magnitude and the affinity of the response was lessened. However, when a sufficient number of thymus (T) cells were given, a high affinity response was produced. The affinity reached a plateau with the addition of thymus (T) cells but the magnitude of the response continued to increase.

Alternatively, T-suppressor cells have been shown to inhibit the production of high affinity antibodies (Takemori and Tada, 1974). Takemori and Tada (1974) were able to demonstrate that spleen cells and thymocytes primed with KLH, then adoptively transferred for a primary or secondary response to DNP-KLH induced a decrease in the avidity in antibodies produced during a PFC response. It was suggested that carrier-primed T-cells were able to suppress the maturation of the primary and secondary immune response.

FINE SPECIFICITY

The fine specificity of an antibody can be determined by its relative affinity (Krel) to various analogs of the immunizing hapten (Mäkelä et al., 1976). Krel is, therefore, determined by comparing the binding of an antibody population to a homologous hapten with its binding to a heterologous hapten (Pressman, 1968). Krel is useful in describing the effect a structural change in an antigen has on the binding of an antibody. By determining these Krel values for an antibody population one is actually studying the architecture of the antibody's combining site. For example, if a population of antibody molecules binds antigen A with a higher affinity than it binds to the crossreactive antigen B, it would be possible to conclude that the antibody population is more specific for antigen A than B by a factor of

$$Ka\ (A) / Ka\ (B)$$

(Berzofsky and Schechter, 1981). Ka represents the association constant of antibody for antigen. Thus, various alterations in the substituent groups can be made and the effect on antibody binding studied. For example, the removal of a nitro group or the addition of a carbonyl group may decrease or increase the affinity of binding with respect to the homologous hapten. Thus it may be determined which chemical structures are crucial for the binding of antibodies to their antigens.

Early work in Krel determinations were conducted with various methods. These included equilibrium dialysis, fluorescence quenching, competitive equilibrium dialysis and hapten inhibition of precipitation (Pressman, 1968.). More recently, RIAs (Klinman et al., 1976) and ELISAs (Chang et al., 1982) have been adapted for this purpose.

GENERATION OF DIVERSITY

Antibody diversity to an antigen may be generated in a mammal in a variety of ways (Tonegawa, 1983; Alt et al., 1987):

1. The animal's germline genetic complement possesses a large number of variable, diversity and joining gene segments that can join in a variety of alternate combinations (combinatorial diversity).
2. These genes display junctional diversity when they fuse during the recombinatorial event.
3. Somatic point mutations can occur throughout the variable region at specific points during differentiation.
4. Different combinations of heavy and light chains can occur. Of these four mechanisms only somatic mutation is associated primarily with the generation of the memory response and exclusively with T-dependent antigens.

Mutations in the immunoglobulin variable region gene are generally induced with T-dependent antigens (Griffiths et al., 1984; Berek et al., 1985; Berek et al., 1987) and not with T-independent

antigens (Maizels and Bothwell, 1985; Stenzel-Poore et al., 1988). Somatic mutation generally occurs on rearranged V, D and J DNA and their immediate flanking elements (Kim et al., 1981; Gearhart and Bogenhagen, 1983). Mutational frequencies gradually increase in antibody genes from late stages of the primary response (Griffiths, 1984), continuing through the secondary response (Berek et al., 1984; Cumano and Rajewsky, 1986) and even during the hyperimmune response (Berek et al., 1987). Contrary to earlier suggestions (Gearhart et al., 1981), somatic mutation may not be dependent on isotype switching (Rudikoff et al., 1984).

Somatic mutation allows for the adaptation of the memory response to an antigenic stimulus. These newly generated antibodies may bind to their immunizing antigens with either increased affinity or enable them to bind a structurally related antigen. This increase in antibody specificity is especially important in microbial infections where the microbes attempt to escape detection by the immune system by altering their antigenicity. With an increased range of specificities it is less likely that such a microbe will escape detection (Kocks and Rajewsky, 1989).

MEMORY B CELL DEVELOPMENT IN FISH

The development of memory has been studied in a number of fish species (Table 1). Using a modification of the in vitro culture system developed by Mishell and Dutton (1967), Miller and Clem (1984) demonstrated an in vitro memory response in catfish (Ictalurus punctatus) to a T-dependent hapten-carrier conjugate. They were able to accomplish this by first priming in vivo with the T-dependent antigen or the carrier portion of the antigen which was later used in the in vitro stimulation. This was demonstrated with a mixture of anterior kidney, spleen and peripheral blood leukocytes. A comparable culture system for rainbow trout splenocytes has been developed by Kaattari et al. (1986) for determining the primary in vitro antibody response to a T-independent antigen, TNP-LPS and a T-dependent antigen, TNP-KLH. The development of memory to these antigens was not examined.

The development of B cell memory as assessed by circulating antibody has been examined in various fish species. Dunier (1985) did not show an increase in antibody titer (as normally expected in a mammalian memory response) for rainbow trout injected with DNP-KLH or DNP-LPS. However, Tatner (1986) did demonstrate the development of a memory response in trout to the T-dependent antigen, human gammaglobulin (HγG). Trout fry were exposed to HγG twice by direct immersion in the antigen and the mean $\log_2$ of

Table 1. Characterization of immunological memory in fish.

Investigator	Species	Antigen[1]	Antibody Titer[2,3]	PFC[3,4]	Latent Period[3]	Affinity[3]
Miller & Clem, 1989	catfish	DNP-HSA or DNP-KLH		↑		
Dunier, 1985	rainbow trout	DNP-KLH or DNP-LPS	N.C.[5]			
Tatner, 1986	rainbow trout	HγG	↑[6]			
Tatner, 1986	rainbow trout	Aeromonas salmonicida	N.C.			
Trump & Hildemann, 1970	goldfish	BSA	↑		↓[7]	
Desvaux & Charlemagne, 1981	goldfish	RBC			↓	
Ambrosius & Frenzel, 1972	carp	DNP-BγG	↑		↓	
Avtalion, 1969	carp	BSA	↑		↓	
Finstead & Fichtellus, 1965	lamprey	Brucella	↑			
Clem & Sigel, 1965	lemon shark	BSA	N.C.			
	gar	BSA	N.C.			
	margate	BSA	↑			
	gray snapper	BSA	↑			
Clem & Small, 1970	giant grouper	DNP-BγG				N.C.
O'Leary, 1981	rainbow trout	TNP				N.C.
Litman et al., 1982	horned shark	azobenzenearsonate				N.C.
Mäkelä & Litman, 1980	horned shark	furyloxazolone-Brucella abortus				N.C.
Litman, 1980	horned shark	azobenzenearsonate				N.C.
Lobb, 1985	channel catfish	DNP				N.C.
Voss et al., 1978	coho	fluorescyl				N.C.

1 Antigen used for immunization.

2 Antibody titer after immunization.

3 A space represents a category which was not assayed by the investigator.

4 PFC represents plaque forming cells.

5 N.C. signifies no change in the response due to immunization.

6 Arrow (↑) signifies an increase.

7 Arrow (↓) signifies a decrease.

the antibody response was determined. In contrast to the response to this T-dependent antigen, a significant difference between the primary and memory responses was not observed when a T-independent antigen, Aeromonas salmonicida was used.

Trump and Hildemann (1970) were able to demonstrate the ability of goldfish (Carassius auratus) to produce a secondary antibody response to bovine serum albumin (BSA) in vivo as determined by a passive hemagglutination assay. The magnitude of the secondary antibody response increased and the lag period decreased in comparison to the primary response. Desvaux and Charlemagne (1981) demonstrate that a weak memory response could be generated in the goldfish by monitoring serum antibodies against the red blood cells of sheep, toad, or the horse. Although an increase in titer was not observed in the secondary response, a shorter lag period did occur. Passive hemagglutination was utilized once again with a cyprinid species, the carp. An increase in the serum antibody titer demonstrated that a memory response does develop in the carp to DNP-BγG (Ambrosius and Frenzel, 1972) and to bovine serum albumin (BSA) (Avtalion, 1969). A decreased lag period also occurred for the secondary response relative to the primary response. B cell memory has also been studied in Agnatha. Immunological memory involving circulating antibody has been demonstrated in the lamprey by the generation of a secondary antibody response to Brucella (Finstad and Fichtelius, 1965). Clem and Sigel (1965) did not detect a secondary antibody response by hemagglutination to BSA in the lemon shark

(Negaprion brevirostris) and a fresh water fish, the garfish (Lepisosteus spp.). However, they were able to elicit a secondary response in two marine teleosts, the margate (Anisotremus virginicus) and gray snapper (Lutjanus griseus).

The chondrichthyes and osteichthyes were originally considered to have only one class of immunoglobulin which resembled mammalian IgM (Warr and Marchalonis, 1982). Immunoglobulins isolated from most cartilaginous fish were either of two forms, a monomer or a pentamer. (Marchalonis and Edelman, 1965; Marchalonis and Edelman, 1966; Clem and Small, 1967). Since the monomer form is antigenically identical to the pentamer, it is considered to be IgM-like. The immunoglobulins studied in the teleosts were either monomers or tetramers and both of these forms were also antigenically identical (Sheton and Smith, 197; Trump, 1970; Acton et al., 1971; Bradshaw et al., 1971; Marchalonis, 1971). Evidence exists, however, in the teleost which suggests that there is actually more than this one IgM-like isotype. Structural studies performed on the immunoglobulin from the bile of a marine fish, sheepshead (Archosarsus probatocephalus), demonstrated the presence of a dimeric immunoglobulin. This dimer was antigenically similar to the high molecular weight immunoglobulin found in the serum but it had a different heavy chain molecular weight. Thus, it would appear that in the sheepshead there is a possibility of the existence of another isotype.

Expansion of these studies into the molecular arena has been initiated by the generation of heavy chain cDNA libraries from the catfish. Six clones encoding the catfish heavy chain genes were identified and sequenced. Sequences of the six heavy chain clones revealed one clone which was found to differ in a single base in the constant (C3) region (Ghaffari and Lobb, 1989). This is probably due to isotypic variation because in mammals, somatic mutation does not occur in the constant region (Kim et al., 1981; Gerhart and Bogenhagen, 1983). Different light chain isotypes have also been demonstrated in the catfish (Lobb et al., 1984). The light chains differ in molecular weight (i.e 26,000, 24,000 and 22,000 dalton forms). These light chains were shown to be antigenically distinct as determined by monoclonal antibody analysis. Also, peptide maps of the light chains were found to be different. Overall, the employment of molecular and monoclonal techniques to the study of fish immunogobulin has confirmed the fact that more than one isotype exists in fish.

LIMITING DILUTION ANALYSIS

In order to analyze precursor frequencies and clonal development in salmonids, limiting dilution analysis (LDA) was adapted for use in salmonids (Tripp, 1988). Tripp determined that coho salmon anterior kidney cells stimulated with TNP-LPS have an estimated precursor frequency of 24 per 10^6 leukocytes and a corresponding clone size of 17. He also determined the need for

accessory cells in the response to TNP-LPS. The accessory cells were found to be adherent cells, which were necessary to provide the interleukins necessary for the response (Tripp, 1988). Filler cells were also required for the response. The filler cells were an inactivated population of leukocytes which provide optimal density for growth of the usually very dilute responding cell population (Litman and Waldman, 1979). These represent the only studies in fish utilizing LDA. Therefore, LDA has been strictly limited to the analysis of the primary response in fish.

AFFINITY MATURATION

Affinity maturation studies of antibodies have been performed in lower vertebrates, yielding distinctly different results from those seen with mammals. A study performed with the giant grouper, Epinephelus itaira, demonstrated that an increase in antibody affinity over time to DNP-BγG does not occur after multiple injections (Clem and Small, 1970). The high and low molecular weight antibodies found in the hyperimmune sera of rainbow trout, also, do not show an increase in affinity over time to TNP as demonstrated by fluorescence quenching and equilibrium dialysis (O'Leary, 1981). The horned shark (Heterodontus francisci), a representative of the chondrichthyes, failed to produce antibodies with increased affinity to two haptens, 2-furyloxazolone (Mäkelä and Litman, 1980) and to p-azobenzenearsonate (Litman et al., 1980). The affinity of antibodies produced during a primary

and secondary antibody response to furyloxazolone, were examined. The affinity of these antibodies were characterized by the phage neutralization assay as described by Savas and Mäkelä (1970). (Litman et al., 1982). The horned shark failed to produce antibodies with increased affinity even after prolonged immunization to the para-azobenzenearsonate hapten. This lack of affinity maturation was also demonstrated in the nurse shark (Ginglymostoma cirratum). The nurse shark 18S antibody did not show an increase in affinity for DNP-KLH over a 20 month period, with repeated injections of antigen, as demonstrated by equilibrium analysis. However, the 7S antibody showed a 100-fold increase in its average association constant over this time (Voss and Sigel, 1972). The affinity of the antibodies was not examined after a single injection of antigen.

Affinity maturation studies have also been performed in other teleosts. Lobb (1985) examined the affinity of channel catfish anti-DNP antibodies and found a lack of affinity maturation during the primary antibody response. Affinity maturation also does not occur in the anti-fluorescyl antibodies of coho salmon (Voss et al., 1978). Primary and secondary antibodies were examined by fluoresence quenching and equilibrium dialysis. It is apparent, that fish do not demonstrate affinity maturation.

FINE SPECIFICITY

There is a paucity of fine specificity analyses with serum antibodies from lower vertebrates. However, Mäkelä and Litman (1980) showed that the horned shark (Heterodontus francisci) lacked variation in fine specificity to furyloxazolone as characterized by inhibition of a phage neutralization assay. This analysis was performed during the primary and secondary response at single time point during each response.

Fine specificity has been examined at the level of the antibody producing cell with splenic and anterior lymphocytes from coho salmon using the homologous hapten, TNP-lysine (Figure 1) (Irwin, 1987). Both organs appeared to demonstrate equivalent fine specificity. Although this study was not a temporal analysis of the PFC produced at different lifestages, it did describe the general structural requirements of a salmonid antibody combining site for binding to TNP. These requirements are three fold. First, for high affinity antibody binding to the TNP hapten, the hapten must possess at least two nitro groups on its benzene ring (position 2 and 4). A third nitro group (position 6) does not alter the binding. Secondly, a long hydrocarbon chain must be present on the benzene ring for good binding. Lastly, electronegative groups must be at least 3 carbon groups distal from the nitrated phenyl ring.

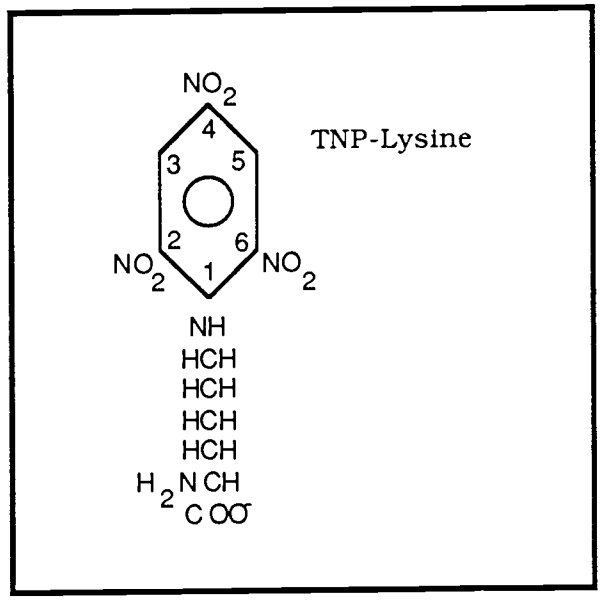


Figure 1. Structure of the homologous hapten, TNP-Lysine.

GENERATION OF DIVERSITY

A factor that may account for the restricted affinity seen in various fish species may be due to the organization of their V_H, D_H, J_H and C_H immunoglobulin gene elements. For example, in the mouse, these genes are organized with a cluster of variable gene elements followed by subsequent clusters of diversity gene elements, J elements and finally C elements. In contrast, studies performed on the shark's (Heterodontus francisci) VDJC heavy chain gene segments have shown them to be arranged as many independent clusters (Figure 2). That is, one entire complement of functional VDJC elements follows another complement of VDJC and so on (Hinds and Litman, 1986). This arrangement of genes could reduce the mechanisms by in which diversity is generated in phylogenetically lower vertebrates (Du Pasquier, 1982). For example, this arrangement should reduce the recombinatorial diversity.

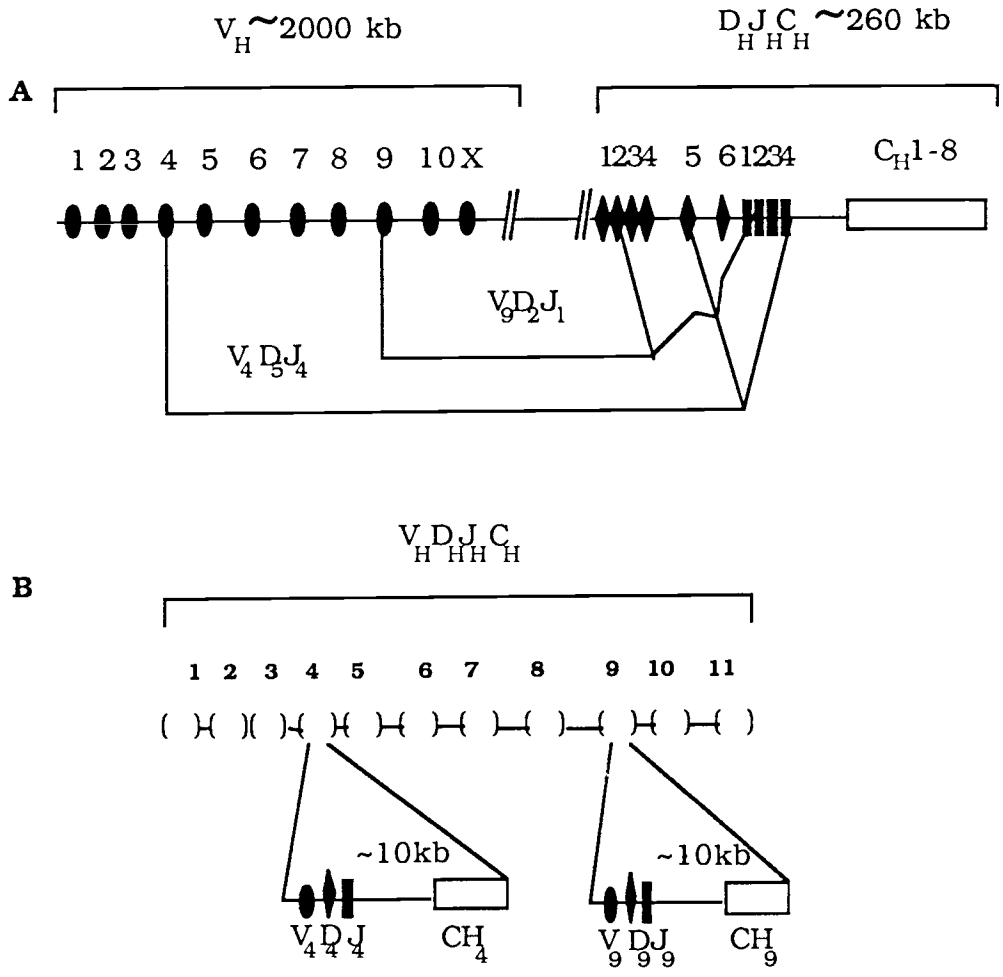


Figure 2: A schematic representation of the murine heavy chain genome (A) and of the elasmobranch genome (B). This illustrates the relationship of V_H elements (⬮), D_H elements (⧫) J_H elements(▮), and C_H elements (▭) in the murine and elasmobranch genome. Illustration from Hinds and Litman, 1986.

AFLATOXIN

Aflatoxin, a mycotoxin, has been found to be a very potent hepatic carcinogen, toxin (Bailey et al., 1984) and immunosuppressive agent (Casarett and Doulls, 1986) in a variety of animals. Also, there is circumstantial evidence from human studies which links aflatoxin exposure with the occurrence of hepatocellular carcinomas and altered host immunity (Denning, 1987).

Aflatoxin is produced from toxigenic strains of Aspergillus flavis, Aspergillus parasiticus and Penicillium puberulum (Schoental, 1967). The synthesis of aflatoxin from these molds is highly dependent upon physical conditions, such as moisture and temperature. Optimal conditions for biosynthesis of the toxin are temperatures between 25-45°C and moisture contents between 18-19.5%. Zinc is also an important element for the production of aflatoxin (Lillehoj et al., 1974). Under optimal conditions, all toxigenic strains produce aflatoxin B_1 in a variety of foods (Rao et al., 1965; Wogan, 1966). High levels of contamination are particularly found in food stuffs consumed in third world countries. However, virtually no aflatoxin is consumed in the USA or UK (Denning, 1987), probably due to a more efficient process for storing food.

Aflatoxin's effects on the immune system have been most clearly demonstrated with a variety of farm and laboratory animals. Turkeys, chickens, ducklings, cows, swine (Panangala et al., 1986), guinea pigs (Pier et al., 1977), barrows (Harvey et al.,

1988), rats (Mohapatra and Roberts, 1985), and mice (Yang et al., 1985), have all been shown to exhibit immunological impairment upon exposure to aflatoxin. These studies have primarily assessed the effects of aflatoxin on the generation of cellular immunity. Of the above mentioned group of animals, most studies have been conducted in chickens, turkeys and steers (Thaxton et al., 1974; Paul et al., 1977; Pier and Heddleston, 1980; Campbell et al., 1983; Richard et al., 1983; Bodine et al., 1984; Giambron et al., 1985; Stewart et al., 1985; Ubosi et al., 1985). Chickens and turkeys fed a crude mixture of aflatoxin demonstrate a significant decrease in their ability to produce a delayed-type hypersensitivity skin reaction in comparison to animals not fed contaminated feed. However, neither humoral immunity to Pasteurella multocida, as determined by an ELISA, nor acquired immunity to Newcastle Disease virus, as demonstrated by a hemagglutination inhibition assay, were affected (Giambron et al., 1985). Campbell et al. (1983) and Ubosi et al., (1985) demonstrated that chickens fed aflatoxin and injected with sheep red blood cells (SRBC) were able to produce an antibody response similar to chickens not exposed to aflatoxin to SRBC, as determined by agglutination. This was in contrast to the work of Thaxton et al. (1974). These investigators demonstrated that chickens fed aflatoxin show a suppressed hemagglutinin response. It has also been found that turkeys which consume aflatoxin during and after immunization to Pasteurella multocida, become deficient in their ability to develop resistance to the bacteria. However, turkeys fed aflatoxin after exposure to the

pathogen were not affected (Pier and Heddleston, 1970).

Complement titers were also found to be decreased in chickens fed aflatoxin. Complement activity, as demonstrated by using a tube complement assay (Campbell et al., 1983) and a radial hemolytic plate procedure (Stewart et al., 1985), was reduced by approximately 50% in both assays. Cattle injected with a single dose of partially purified aflatoxin have also demonstrated decreased complement activity (Thurston, 1986).

Cattle fed corn, naturally infected with aflatoxin, exhibit a decrease in their ability to produce a delayed cutaneous hypersensitivity reaction to the purified protein derivative (PPD) of Mycobacterium tuberculosis, but demonstrate no significant decrease in their ability to produce antibody to B. abortus (Richard et al., 1983). Other studies have shown that in vitro exposure to aflatoxin B_1 decreases the bovine cellular immune response as determined by a significant decrease in the mitogenic potential of peripheral blood leukocytes to respond to phytohemagglutinin, concanavalin A and pokeweed mitogen (Paul et al., 1977). Overall, most studies have concentrated on aflatoxin's ability to affect the cellular or T cell arm of the immune system with a relative paucity of studies devoted to the humoral reaction.

Over the past 20 years rainbow trout have become a very valuable experimental animal in the study of hepatocellular carcinomas induced by exposure to aflatoxin B_1. To date, the Shasta strain of rainbow trout appear to be the most sensitive animal to the carcinogenic effects of aflatoxin B1 (reviewed by

Hendrick et al., 1982). Both dietary or embryonic exposure to the carcinogen are highly effective in inducing carcinogenic effects in these animals. Although considerable studies have been conducted on the mechanisms involved in the chemical carcinogenesis of aflatoxin, little is known about it's effect on the immune response in fish.

Interest developed in using rainbow trout as a model system for cancer studies during 1957-1960, when an epizootic of liver cancer occurred in trout. The cause of the epizootic was traced back to cottonseed (Wolf and Jackson, 1963) contaminated with aflatoxin. One major reason rainbow trout were so sensitive to aflatoxin B_1 is due to the way it is metabolized in liver cells. Metabolism of aflatoxin favors the formation of a 2,3-epoxide, over the competing detoxification reactions, which then proceeds to form DNA adducts. The reactions which are known to occur in liver cells are reviewed by Bailey et al., 1984. In brief, <u>in vitro</u> studies using isolated hepatocytes have shown that the major unconjugated metabolite of aflatoxin B_1 is aflatoxicol (Bailey et al., 1982) which approaches aflatoxin B_1 in carcinogenicity (Schoenhard et al., 1981) and therefore can not be considered a detoxified product (Bailey et al., 1984). This is in contrast to the mammalian system where the major detoxified products are aflatoxin P_1, Q_1 and M_1 (Dahms and Gurtoo, 1976) which have very reduced to no carcinogenic activity. Polar metabolites of aflatoxin B_1 also differ between rainbow trout and mammals. The major polar metabolite in trout, upon examination of bile, is a

glucoronide of aflatoxicol. The aflatoxin-glutathione conjugate could account for only 13-18% of the polar metabolites. However, in rats, the major biliary polar metabolite is the aflatoxin-glutothione metabolite (Degen and Newmann, 1981). The formation of the glutothione metabolite requires the use of the 2,3 epoxide as a substrate as does the epoxide hydrase reaction. Therefore these two reactions are in direct competition for this substrate with DNA adduct formation (Bailey et al., 1984). Although the importance of the epoxide hydrase reaction is not known in trout, it may be the reduced formation of the two conjugation reactions which account for high DNA adduct formation observed in the trout (Bailey et al., 1984). This DNA alteration may be the mechanism for the initiation of carcinogenesis (Ames, 1979). However, this generalized mechanism of cellular injury or toxicity may cause more damage than carcinogenesis especially when these transformations occur in the anterior kidney which is the primary lymphoid organ of the fish (Irwin, 1987).

MATERIALS AND METHODS

ANIMALS

Four hundred Shasta strain of rainbow trout (Oncorhynchus mykiss) were supplied by Dr. Jerry Hendricks of the Department of Food Science and Technology, Oregon State University. The trout were maintained at the Oregon State University Fish Disease Laboratory, Food Toxicology and Nutrition Laboratory and The Western Fish Toxicology Station of the Corvallis Environmental Research Laboratory, all of which are located in Corvallis, Oregon. Fish pathogen-free water, used throughout these studies, was maintained at approximately 12°C. The fish were fed a daily ration of Oregon Test Diet (OTD) (Hendricks, 1982) which was kindly provided by Dr. Jerry Hendricks.

BALB/c mice were maintained by the Laboratory Animal Resource Center at Oregon State University.

WEIGHTS

Mean weights were determined for each group of fish when the plasma or serum samples were collected. After the secondary injection, individual weights were taken.

ANTIGEN PREPARATION

Trinitrophenylated-lipopolysaccharide (TNP-LPS) was prepared as described by Jacobs and Morrison (1975). Briefly, 50 mg of E. coli lipopolysaccharide (LPS) W, serotype 0111.84 (Difco, Detroit, MI) 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 of picrylsulfonic acid (Sigma, St. Louis, MO) in 2.5 ml of 0.28 M cacodylate buffer was added dropwise to a test tube containing the LPS solution. The tube containing this mixture was then foil-wrapped, and allowed to mix on a rotator mixer, (Model 151, Scientific Industries, Bohemia, NY) for 2 hours at room temperature. Upon coupling, the TNP-LPS solution was exhaustively dialyzed against three changes of 0.7 M phosphate buffered saline, PBS, pH 7.4 (see Appendix 1) with a final change against RPMI 1640 (Gibco, Grand Island, N.Y.) (see Appendix 1). The solution was then pasteurized for 30 minutes at 70°C and stored at 4°C in a sterile stoppered serum bottle.

Trinitrophenylated-keyhole limpet hemocyanin (TNP-KLH) was prepared as described by Rittenberg and Amkraut (1966). The keyhole limpet hemocyanin (KLH) was a generous gift from Dr. M. B. Rittenberg (Dept. of Microbiology and Immunology, Oregon Health Sciences University, Portland, OR.). In brief, 5 ml of KLH (14.4 mg/ml) was dialyzed against 3 changes of 0.28 M cacodylate buffer. After dialysis the KLH solution was stored in a foil covered tube. Trinitrophenylation of the KLH was accomplished by first

dissolving 16.2 mg picrylsulfonic acid (Sigma, St. Louis, MO) in 1.5 ml of 0.28 M cacodylate buffer (see Appendix 1). This solution was added dropwise to the prepared KLH. The solution was mixed on a rotator mixer for 1 hour at room temperature. The solution was then dialyzed against four one liter changes of PBS. The final dialysis was performed with RPMI-1640. The conjugate was then filter sterilized and placed in a sterile stoppered serum bottle.

Trinitrophenylated bovine serum albumin (TNP-BSA) was prepared according to Garvey et al. (1977). Briefly, 500 mg of BSA (Sigma, St. Louis, MO) was added to 10 ml of borate buffer (Appendix 1). The tube was spun at 1400 x g for 20 minutes. Trinitrophenylation was accomplished by first mixing 125 mg of picrylsulfonic acid in 2.5 ml borate acid (see Appendix 1) The BSA solution was then transferred to a 10 ml foiled covered beaker. The picrylsulfonic acid solution was added dropwise into the BSA solution under constant mixing. Stirring was continued for two hours at room temperature. The solution was transferred to a dialysis bag and dialyzed extensively against borate buffered saline (BBS) (see Appendix 1).

AFLATOXIN EXPOSURE

Rainbow trout embryos were exposed to aflatoxin B_1 as described by J. H. Hendricks et al. (1981). In brief, 200, 21 day old rainbow trout embryos were exposed to an aqueous mixture of aflatoxin B_1 for 30 minutes. The embryos were rinsed in fresh

water several times and their development was allowed to proceed as normal. Following this procedure, between 65-75% of the fish develop liver carcinomas within 12 months after exposure. Control rainbow trout were treated as above except they were not exposed to aflatoxin B_1.

IMMUNIZATION

Normal and aflatoxin exposed rainbow trout were immunized intraperitoneally (i.p.) with 100 μg of TNP-KLH emulsified in equal volumes of Freund's complete adjuvant (FCA; Difco Laboratories, Detroit, MI). The final volume injected into each fish was 0.02 ml. Where applicable, the fish were given a secondary injection, i.p., of TNP-KLH (20 μg) emulsified in an equal volume of Freund's incomplete adjuvant (FIA) at 20 weeks post primary injection. Also, at this point, a group of fish from the uninjected stocks were given a primary injection, i.p., of 100 μg of TNP-KLH emulsified in FCA. The final volume injected into each fish was 0.02 ml.

Potential for non-specific FCA contribution to the production of serum anti-TNP antibodies was determined. Thirteen rainbow trout were injected i.p. with 0.100 ml of 100 μg of TNP-KLH in FCA and 13 control rainbow trout were injected with 0.100 ml of PBS in FCA. Four fish from each group were bled eight weeks after the primary injection. Both groups of trout received a secondary injection of TNP-KLH (20 μg) in FIA in a final volume of 0.100 ml twenty weeks after the primary injection and bled 3 weeks later.

Trout used for LDA were injected intraperitoneally with 100 μg of TNP-KLH in FCA and allowed to rest for at least 1 year before use.

PLASMA AND SERA COLLECTION

Blood samples were collected from the caudal blood vessels after removal of the caudal peduncle. The blood samples were collected in heparinized micro-hematorcrit capillary tubes (VWR, Seattle, WA). Immediately after collecting the blood, one end of the tube was plugged in seal-ease (Cray Adams, Parsippany, N.J.). The sample was then spun down in a hematocrit centrifuge (International Equipment Company, Needham Heights, MA.) for 5 minutes. The hematocrit tube was broken above the cell pellet, the plasma was removed and stored in a microfuge tube at -70°C.

Sera was collected from fish greater than 20 grams. After removal of the caudal peduncle, the blood was collected in a microfuge tube, allowed to clot and centrifuged in a microfuge (Beckman Instruments, Palo Alto, CA.) for 3 minutes. The sera was removed from the clot and stored in a microfuge tube at -70°C. The plasma and serum samples were used in determining anti-TNP activity, fine specificity analysis and in the average avidity (Ka) studies.

CULTURE MEDIUM

Medium components were purchased from Whittaker M.A. Bioproducts, Walkersville, MD, unless otherwise noted. Tissue culture medium (TCM) was made according to Mishell and Dutton (1967). Briefly, 200 ml of TCM consisted of 172 ml of RPMI-1640 containing L-glutamine supplemented with 0.2% sodium bicarbonate (Sigma, St. Louis, MO), 20% fetal calf serum (FCS), 0.2 ml gentamicin sulfate (50 μg/ml), 0.1 ml of 50 μM 2-mercaptoethanol (MCB, Cincinnati, OH) in distilled water, 2.0 ml of 1.0 mg/ml guanine (Sigma, St. Louis, MO) and a 2.0 ml solution containing 1.0 mg/ml each of adenosine, uracil and cytosine (Sigma, St. Louis, MO.) in RPMI-1640. A nutritional supplement was prepared as described by Tittle and Rittenberg (1978). The nutritional supplement was made by the addition of 15.0 ml of the stock supplement to 7.5 ml FCS, 1.0 ml of guanine 1.0 (1 gm/l) and 1 ml of a mixture containing equal parts of adenosine, uracil and cytosine (1.0 gm each/l) in RPMI-1640.

The stock supplement was prepared according to the method of Tittle and Rittenberg (1978). All products were purchased from Whittaker M.A. Bioproducts, Walkerville, MD, unless otherwise stated. Fifteen μl of gentamicin sulfate (50 μg/ml) was added to 11.0 ml of RPMI-1640 (Gibco, Grand Island, NY) containing L-glutamine and supplemented with 0.2% sodium bicarbonate (Sigma, St. Louis, MO), 1.5 ml of essential amino acids (50X), 0.77 ml of nonessential amino acids (100X), 0.77 ml of dextrose (200 mg/ml)

in distilled water, and 0.77 ml L-glutamine (200 mM) was added to the RPMI-1640 mixture. The mixture was adjusted to a pH of 7.2 with 10 N NaOH and referred to as the stock supplement.

PREPARATION OF TRINITROPHENYLATED SHEEP RED BLOOD CELLS (TNP-SRBC)

Trinitrophenylated sheep red blood cells (TNP-SRBC) were prepared according to the method of Rittenberg and Pratt (1969). A picrylsulfonic acid solution was prepared by mixing 10 mg of picrylsulfonic acid (Sigma, St. Louis, MO) with 3.5 ml of 0.28 M cacodylate buffer in a foil wrapped test tube held at 12°C. To this solution 0.5 ml of packed sheep red blood cells (SRBC) washed three times in modified barbital buffer (MBB, see Appendix 1), was added dropwise. The suspension was then mixed slowly on a rotator for 15 minutes at room temperature to allow coupling of the hapten to the SRBC. The TNP-SRBC were then centrifuged at 1400 x g for 5 minutes at 4°C, the supernatant was removed and the pellet was mixed with a glycylglycine solution (3.7 mg/ 5.83 ml MBB). This preparation was centrifuged again, the supernatant removed, and the TNP-SRBC pellet diluted with MBB to a concentration of 10% (V/V) for use in the plaque assay.

ESTIMATION OF CELLULAR VIABILITY

A trypan blue solution was made by dissolving 0.4 gm of trypan blue (Sigma, St. Louis, MO) in 100 ml of saline (0.85%) at room temperature. The mixture was filtered with a 0.45 micron bottle top filter (Benton Dickinson, Oxnard, CA) to remove any undissolved trypan blue. The 0.4% solution was stored at room temperature. The trypan blue solution was added to the cells at a final dilution of 1:24 (one part trypan blue to 23 parts of the cellular suspension). A portion of this suspension was examined in a hemocytometer (VWR, Seattle, WA) and the number of live cells (clear) could be distinguished from the dead cells (blue) and enumerated.

STANDARD LYMPHOCYTE CULTURE AND PLAQUE ASSAY

Fish were sacrificed by cerebral concussion and transported on ice to the laboratory. After bleeding the fish by severing the caudal peduncle, the spleens were removed aseptically and placed in TCM on ice. A single cell suspension of each organ was obtained by aspiration of the minced spleen through a sterile, plastic 1 ml syringe. The resulting cell suspension was incubated on ice to allow organ fragments to settle. The supernatant, a single cell suspension, was then washed twice in TCM by centrifugation at 500 x g for 10 min at 17°C. Leukocytes were enumerated by a hemocytometer and cell viability determined by trypan blue

exclusion. Washed cells were adjusted to a concentration of 2 x 10^7 cells/ml in TCM. Fifty µl of these cells were added to individual wells of a 96 well, flat-bottom plate (Corning, Cambridge, MA) in triplicate and 50 µl of the appropriate dilution of antigen or TCM was then added. The plates were then placed in a plastic incubator box (C.B.S. Scientific, Del Mar, CA) under an atmosphere of 9.8% CO_2, 10.2% O_2 and 80% N_2 and incubated at 17°C. The cultures were fed on alternate days with 0.01 ml of the nutritional supplement.

The spleen cells were harvested from their individual wells after nine days of incubation, which was previously determined to be the peak day for the plaque-forming cell (PFC) response of rainbow trout lymphocytes (Irwin, 1987). Duplicate wells were added to a tube and the cells were washed once to remove the spent medium. This was performed on 3 pairs of replicated wells. After the supernatant was discarded, the pelleted cells were resuspended to a volume of 200 µl with RPMI-1640. A modification of the Cunningham plaque-forming cell assay (Cunningham and Szenberg, 1968) was performed using 100 µl of the lymphocyte suspension, 25 µl of a 10% suspension of trinitrophenylated sheep red blood cells (TNP-SRBC) and 25 µl of steelhead trout serum as a source of complement, appropriately diluted in MBB as previously described (Kaattari and Irwin, 1985). The cell suspensions were added to Cunningham slide chambers. Cunningham slide chambers consisted of two microscope slides connected by 3 pieces of double stick tape (3M, St. Paul, MN). The

slides were sealed with parafilm to prevent evaporation. The Cunningham chambers were incubated 1-3 hours at 17°C. Plaques were counted with the aid of a dissecting microscope to determine the number of PFC generated per culture.

MICROCULTURE AND PLAQUE ASSAY

Fish were sacrificed by anesthetic overdose with 10 ml stock benzocaine (see Appendix 1) in 3 liters of distilled water. Spleen cell suspensions were prepared from single fish as described above. Washed cells were adjusted to a concentration of $2x10^7$cells/ml with TCM. The antigen, TNP-LPS, was diluted to two times the desired final concentration and then mixed 1:1 with the spleen cell suspension. Ten µl of this antigen and cell mixture were added to each well of a Terasaki plate (Intermountain Scientific Corporation, Bountiful, Utah) using an eppendorf repetitive pipet (VWR, Seattle, WA). Three hundred µl of TCM was added along the interior edge of the plate to reduce evaporation. The plate was then incubated at 17°C in an incubator box under an atmosphere of 9.8% CO_2, 10.2% O_2 and 80% N_2. The cells were fed 2 µl of stock cocktail on day 2, 5, 8, 11 of culture with a Hamilton syringe (VWR, Seattle, WA). Cells were harvested from Terasaki plates by depositing the contents of each well directly into 40 µl RPMI-1640, 10 µl of a 10% suspension of TNP-SRBC and 10 µl of complement. This suspension was then added to a Cunningham chamber,

incubated and the number of plaques determined as described in the previous section.

Conventional 96 well flat bottom plates were also used to culture the cells for comparison of the two methods. The plates were placed in micro-test plate carriers (VWR, Seattle, WA) after incubation and centrifuged at 100 x g for 10 minutes at 17°C. The use of plate carriers instead of harvesting into tubes prior to centrifugation, allowed for less handling of the cells. The supernatant was removed while the cells remained at the bottom of the wells. Fifty µl of RPMI-1640 (Gibco, Grand Island N.Y.) was then added directly to each well, together with 10 µl of a 10% suspension of TNP-SRBC and 10 µl of steelhead trout serum as a source of complement, diluted in MBB. This mixture was added to a Cunningham slide chamber, incubated and the number of plaque-forming cells was determined as described.

LIMITING DILUTION ANALYSIS

Limiting dilution analysis was performed according to the method described by Lefkovits and Waldman (1979). Initially the assay was performed in standard 96 well flat bottom tissue culture plates (Corning, Cambridge, MA). These plates hold a final volume of 100 µl of cells at a concentration of $2x10^7$ cells/ml. Spleen cells were used as the responding cell population and the anterior kidney cells were used as the filler/feeder cell population (Tripp, 1988) with some modifications. An anterior kidney single cell

suspension was prepared in the same fashion described for the spleen cells.

The primary purpose of the filler cells is to maintain the overall concentration of cells at $1x10^6$ cells/ml while the responding cell concentration is incrementally decreased. The feeder cells provide various growth factors or interleukins for an optimal response of the spleen cell population. A standard requirement for the feeder/filler cell population is that they must not be able to produce antibodies. To achieve this, the anterior kidney cells were inactivated with mitomycin C (Sigma, St. Louis, MO) and ^{60}Co radiation. Mitomycin C was dissolved in TCM to a concentration of 2 mg/ml. Eight ml of TCM was added to two ml of anterior kidney cells at $2x10^7$ cells/ml. To these cells at a concentration of $4x10^6$ cells/ml, mitomycin C was added to produce a final concentration of 80 µg/ml and incubated for 45 minutes at 17°C with occasional shaking. After incubation, the cells were washed three times with TCM and brought to a final concentration of $2x10^7$ cells/ml. Anterior kidney cells treated with 80 µg/ml of mitomycin C were then immediately exposed to ^{60}Co radiation. The cells were exposed to 2000 rads, on ice, in the low flux region of the gamma irradiator. The cells were washed once to remove any toxic free radicals or their products which may have resulted from the radiation (Mishell and Shiigi, 1980) and brought to a final concentration of $2x10^7$ cells/ml. The inactivated cells were added to the wells of the plate which had been treated with fibronectin the night before the assay (Tripp, 1988). Fibronectin

allowed for the binding of adherent cells to the wells. In brief, 1 mg of lyophilized bovine fibronectin (Sigma No. F-4759) was reconstituted with 1 ml of sterile nanopure water. Once reconstituted, the solution was stored in working aliquots (0.20 ml) at -20°C until use. Fifty µl of fibronectin at a concentration of 5 µg/ml in sterile distilled water was added to each well and incubated overnight in a gas box at 17°C. The fibronectin was removed from the wells by flicking the contents of the plate over a sterile surface. Each well was then washed with 100 µl of RPMI-1640. After removal of the RPMI-1640, 50 µl of treated anterior kidney cells, ranging in concentration from $1x10^6$ cells/well to $2x10^5$ cells/well, were added. The cultures were fed on alternate days with 10 µl of the nutritional supplement. The cells were harvested on day 9. Between 30-50 replicate wells were prepared for each responder cell concentration.

Procurement of a sufficient number of anterior kidney cells for use as filler/feeder cells in conventional microculture plates required at least 3 fish per assay. A more efficient method of cell utilization was developed using Terasaki plates instead of the standard 96 well, flat bottom plates. These plates contain wells that hold only one tenth the volume of cells that were used for the conventional 96 well plates. Thus, these plates required a final volume of only 10 µl of cells at $2x10^7$ cells/ml. In these studies, spleen cells were used as the responding cell population and peripheral blood leukocytes (PBL) were used as the feeder and filler cell populations. Ten µl of a 0.0013 µg/µl solution of

fibronectin was added to each well of the Terasaki plates. The plates were incubated overnight at 17°C.

As stated above, PBL were used as the source of filler and feeder cells. Peripheral blood was collected from the caudal vein in a heparinized vacutainer. The whole blood was diluted by 1:4 with RPMI-1640 and centrifuged at 500 x g for 10 min at 17°C. The supernatant was removed and the blood cell pellet was resuspended to the original diluted volume with RPMI-1640. This suspension was placed on an equal volume of leukocyte separation medium, Histopaque-1077 (Sigma, St. Louis, MO). The cells were then centrifuged at 800 x g for 20 min at 17°C. After centrifugation, the white cells (buffy coat) were isolated from the interface between the RPMI-1640 and the Histopaque. The buffy coat was removed with a pasteur pipet and washed once in TCM. The white cell viability was assessed by trypan blue exclusion and the cells diluted to a concentration of $2x10^7$ live cells/ml with TCM. Spleen cells were prepared as previously described in the standard lymphocyte culture and plaque assay section.

Colbalt (^{60}Co) radiation was used to inactivate the PBL. The cells were placed on ice and exposed to 6,000 rads in the low flux region of the Colbalt irradiator. They were washed once to remove any toxic free radicals and their products and then resuspended to a concentration of $2x10^7$ viable cells/ml with TCM.

After the wells of the microculture plate were incubated overnight with the fibronectin solution, the solution was removed just prior to the addition of cells by aspiration with a 26 gauge

needle and syringe. Ten µl of irradiated PBL were added to each well. The plates were incubated in an incubator box under an atmosphere of 9.8% CO_2, 10.2 % O_2 and 80% N_2 at 17°C for one to two hours to allow for cell adherence to the fibronectin. After the incubation, the nonadherent cells were removed by aspiration. Ten µl of RPMI-1640 was added immediately to each well and the wells were aspirated again to remove the RPMI-1640 along with any extra fibronectin and remaining nonadhering cells. The responding cells (spleen) were diluted to the appropriate concentrations with the filler cells (PBL). The cells were exposed to 0.4 µg/ml of the antigen (TNP-LPS). Ten µl of each responding cell concentration was added to replicate cultures (50-60 wells). Three hundred µl of TCM was added around the interior edge of the Terasaki plates to prevent evaporation and the plates were then incubated in an incubator box under an atmosphere of 9.8% CO_2, 10.2% O_2 and 80% N_2 at 17°C. The cells were fed with 2 µl per well of feeding cocktail on days 2 and 5 of culture. Cells from individual wells were harvested for determination of PFC on day 7 of culture as described previously for the microculture plates.

The LDA data were analyzed according to the method of Lefkovits and Waldmann (1979). In order to use the above method, the B cells must be distributed in a Poissonian fashion. The requirements for this distribution were met in that the probability of success (obtaining a positive response) or a specific B cell precursor was rare, relative to the total number of lymphocytes within the population. And secondly, the occurrence

of a positive response does not enhance or diminish the occurrence of another positive response.

The mean number of precursor cells to TNP in a population of lymphocytes can be estimated by the use of the zero term of the Poisson distribution. The zero term is represented by equation 1.

$$F_0 = e^{-u} \qquad 1)$$

F_0 represents the frequency of wells without plaques (products of precursors), e represents the base of the natural logarithum (2.718) and u represents the mean number of precursor cells.

The precursor frequency can be determined graphically. The fraction of nonresponding wells are represented on the log axis (y) of semi-log graph paper and the corresponding number of responder or spleen cells/well is represented on the linear axis (x) of the graph. Linear regression is used for determination of the line of best fit and its corresponding correlation coefficient (R). Interpolation to the number yielding 37% nonresponding wells represents the concentration of cells containing an average of one precursor to the specific antigen (see equation 2 and 3)

$$F_0 = e^{-1} \qquad 2)$$

$$F_0 = 0.368 \text{ or } \sim 37\% \qquad 3)$$

A straight line plot is indicative of single-hit kinetics meaning that only one cell type is limiting, which in this case is necessarily the B cell.

Occasionally data obtained from LDA required correction due to spurious background plaques. These plaques were either due to some residual activity in the feeder/filler cell population or due to spontaneous lysis of portions of the TNP-SRBC lawn. This problem was corrected in either one of two ways: 1) the average number of feeder/filler cells responding to the antigen were subtracted from all of the results when a large number of fillers responded or 2) when only a small number of background plaques occurred (i.e. approx. 1-3) the highest number obtained was subtracted from all of the results. Once corrected for background, the data was plotted as described. Single hit curves not passing through the origin were corrected according to Lefkovits and Waldman (1979). In brief, a parallel straight line was drawn through the origin and the precursor frequency determined using this line.

Clone size is determined by the method described by Lefkovits and Waldmann (1979). In brief, the average clone size is determined by equation 4.

$$c = \frac{\Sigma PFC}{n} \qquad 4)$$

To determine ΣPFC, the plaque forming cells from all wells at a single concentration of the responder cells are summed. n is the number of clones present in the pool and is determined by equation 5.

$$n = \mu * W_t \qquad 5)$$

The mean number of precursors (μ) was described in equation 1. W_t represents the number of replicate wells for that responder cell dilution.

PREPARATION OF BIOTINYLATED ANTI-TROUT IMMUNOGLOBULIN (1-14)

The myeloma which produced the monoclonal antibody 1-14, was injected intraperitoneally, into BALB/c mice. The hybridoma cell line (1-14), which produced a monoclonal antibody to trout immunoglobulin was a generous gift of Dr. G. Warr, Dept. of Biochemistry, Univ. of South Carolina. The isotype for the 1-14 monoclonal is IgG (DeLuca et al. 1983). In our laboratory this monoclonal antibody has been found to be reactive against antibodies in coho (Oncorhynchus kisutch), chinook (Oncorhynchus tshawytscha) as well as to rainbow trout (O. mykiss) antibodies.

After sufficient growth of the tumor, the ascites fluid was aspirated from the peritoneal cavity and precipitated three times

with 50% saturated ammonium sulfate (SAS). This preparation was then extensively dialyzed against PBS and filter sterilized.

Biotinylated 1-14 was prepared using the method described by Kendall et al. (1983). Briefly, 0.30 ml of a 3 mg/ml solution of the SAS precipitated Warr's 1-14 antibody in 0.1 M $NaHCO_3$ was reacted for one hr at room temperature with 17.1 µl of a 0.1 M biotinyl-n-hydroxysuccinimide ester (BNHS) (Cal-Biochem, La Jolla, CA) solution dissolved in dimethyl formamide The mixture was dialyzed extensively against PBS and mixed 1:1 with glycerol and stored at -20°C.

DETERMINATION OF ANTIBODY ACTIVITY

An enzyme-linked immunosorbent assay (ELISA) system has been developed in our laboratory for the quantification of fish anti-TNP antibodies in units of activity per µl of serum (Arkoosh and Kaattari, in press). Wells of a 96-well ELISA plate (Costar, Cambridge, MA) were coated overnight with 0.1 ml of a 0.5 µg/ml solution of trinitrophenylated bovine serum albumin, TNP-BSA, in coating buffer (see Appendix 1) and placed in a moist chamber held at 17°C. After coating, the wells were blocked with 0.1 ml of 1% BSA in tris-buffered saline (TBS) for 1 hr at room temperature. The plates were then washed three times with 1% Tween in TBS (TTBS) followed by 3 washes in TBS. After the plates were washed, various dilutions were made of the serum or plasma samples in TTBS. Fifty µl of each dilution were added to the wells in duplicate.

Each plate contained a standard anti-TNP serum for normalization of the data. Such normalization virtually eliminates the variations that can occur due to daily temperature fluctuations and minor ionic strength changes in the buffers (Tijssen, 1985). The standard serum was also used to assign units of activity per µl of serum. The standard anti-TNP serum may be derived from any salmonid as long as it is used consistently throughout a particular assay. Initially, a coho standard antiserum was used to determine the titer of the samples. However, the samples were retitered for fine specificity analysis and avidity determination with a trout standard antiserum. The dilutions of samples and standard serum were incubated overnight at 17°C in a moist chamber. The plates were washed as above and 0.1 ml of 1/500 dilution of the biotinylated 1-14 monoclonal antibody diluted in TTBS was added. The plates were then incubated for 90 min at room temperature. After incubation, the wells were washed as above and incubated with 0.1 ml of a 1/100 dilution of strepavidin-HRPO (horseradish peroxidase) in TBS for 30 min. The plate was washed and 0.1 ml substrate (see Appendix 1). Optical densities were read at 405 nm on a Biotek EL310 ELISA reader (Burlington, VT) after 25 min of reaction. It was previously determined that at 25 min the reaction rate still demonstrated linear kinetics. Initially, each sample was tested against BSA to ensure that the high sensitivity obtained was specific for the ant-trinitrophenyl-antibodies.

DETERMINATION OF ANTIBODY FINE SPECIFICITY

Fine specificity of the antibodies from the serum/plasma samples was determined by inhibition ELISAs. A quantity, Krel (relative affinity), can be used to define the fine specificity of an antibody population. Krels were determined as originally described by Mäkelä (1976). This determination is accomplished by comparing the binding of an antibody population to the reference hapten with that of a structurally different, or heterologous, hapten (Pressman, 1968). Therefore, Krel determination is useful in describing the effect of structural changes in the hapten on the binding affinity of a population of antibodies. The Krel values of an antibody population are obtained by dividing the 50% inhibition concentration (IC_{50}) of the homologous hapten by the IC_{50} determined for the heterologous hapten. This is presented by equation 4.

$$\text{Krel} = \frac{IC_{50} \text{ for the homologous hapten}}{IC_{50} \text{ for the heterologous hapten}} \qquad 4)$$

As can be seen from this equation, the more heterologous hapten required to inhibit the binding, the lower the Krel value will be for that hapten. The following stock concentrations of haptens were prepared for the fine specificity analysis:

1. ε-Trinitrophenyl-L-lysine monochloride monohydrate, TNP-lys, (Sigma No. 104793) was diluted in TTBS to an initial concentration of 0.001M.
2. Dinitrophenyl-L-Lysine, DNP-lys, (ICN No. 102215) was diluted in 3% BSA-TTBS to an initial concentration of 0.001M.
3. Dinitrophenyl-L-phenylalanine (ICN No. 102617) was diluted in 3% BSA-TTBS to an initial concentration of 0.01M.
4. Dinitrophenol, DNP-OH, (Sigma No. D-7004) was diluted in 3% BSA-TTBS to an initial concentration of 0.01M.
5. Trinitrophenyl-γ-aminobutyric acid (ICN No. 101765) was diluted in 3% BSA-TTBS to an initial concentration of 0.001M.
6. p-Nitro-D, L-phenylalanine (ICN No. 102617) was diluted in 3% BSA-TTBS to an initial concentration of 0.001M.
7. N-2,4-Dinitrophenyl-ε-amino-n-caproic acid, DEAC, (Sigma No. D-7754) was diluted in 3% BSA-TTBS to an initial concentration of 0.001M.
8. N-2,4,-Dinitrophenyl-γ-aminobutyric acid (Sigma No. D-7504) was diluted in 3% BSA-TTBS to an initial concentration of 0.001M.
9. N-2,4-Dinitrophenyl-DL-α-amino-caprylic acid (Sigma No. D-7629) was diluted in 3% BSA-TTBS to an initial concentration of 0.001M.

10. Trinitrophenyl-L-phenylalanine (ICN No. 105178) was diluted in 3% BSA-TTBS to an initial concentration of 0.001M.
11. 2,4-Dinitrophenylacetic acid, DNP-AA, (Aldrich No. 20,956-2), was diluted in TTBS to an initial concentration of 0.01M.

The stock concentrations used represent the highest concentrations in which these haptens remain soluble.

The homologous hapten used was trinitrophenylated lysine (TNP-LYS). In brief, the wells of a 96-well ELISA plate (Costar, Cambridge, MA) were coated either overnight with 0.1 ml of a 0.5 μg/ml solution of trinitrophenylated bovine serum albumin, TNP-BSA, diluted in coating buffer or for 1 hour at 37°C in a moist chamber. Each technique yielded comparable results. After coating, the wells were blocked for 30 min with 0.2 ml of 3% BSA in tris-buffered saline with 1% tween (3% BSA-TTBS) at 37°C. The plate was washed 3 times with TTBS followed by 3 washes in tris-buffered saline (TBS). After the plate was washed, 0.050 ml of the appropriate concentration of inhibitor was added to the ELISA wells, followed by addition of 0.05 ml of the appropriate sample dilution. Both sample and inhibitors were diluted in 3% BSA-TTBS. The plates were then incubated for 90 min at 17°C.

The dilution of plasma or serum yielding 1 unit of antibody activity was added to the various inhibitors. One unit of antibody activity corresponds to the dilution of the sample which produced

50% maximum binding. This amount of activity on the plate guarantees that lack of inhibition by the haptens would not be due to a simple excess of antibody.

After incubation, the plate was washed and 0.1 ml of biotinylated 1-14 monoclonal antibody diluted in 3% BSA-TTBS was added to the wells. After the plate was incubated for 90 min at room temperature, it was washed and incubated for 30 min with a 1/100 dilution of strepavidin conjugated to horseradish peroxidase, (Sigma, St. Louis, MO). The plate was washed and 0.1 ml of substrate was added to each well. Optical densities were read at 405 nm at 0 and 25 min on a Biotek EL310 ELISA reader (Burlington,VT).

The Biotek ELISA reader was linked to a RS232 adaptor port of an IBM computer (PC). In this fashion the plate readings could be transmitted to the computer and stored using Bio-tek Eia application software.

Chi-square analysis was performed to determine the existence of a significant shift in the fine specificity during the primary response relative to the secondary. An important assumption of chi-square analysis is that the expected number of responses in each cell should be at least five. The median Krel value of all the data was chosen in order to fulfill this condition. The four cells used in this assay were Krels (primary) above the median, Krels (primary) below the median, Krels (secondary) above

the median and Krels (secondary) below the median. If this analysis revealed a p value of 0.05 or less, the shift in fine specificity (i.e. from higher to lower Krel values, or vice versa) was considered to be significant.

COMPUTER DETERMINATION OF IC_{50} VALUES

A custom designed computer program was developed by Microsolutions (Corvallis, OR) for determination of IC_{50} values. The program subtracted the stored zero time reading of each ELISA well from its corresponding 25 min reading. In this fashion the staggered introduction of substrate was compensated and thus only 25 min periods were accurately assessed. Once the zero time reading was subtracted from the 25 min read the computer program determined the amount of hapten which inhibited 50% of the antibody reaction (IC_{50}). A logit-log transformation (Banowetz, 1987) of the data was performed by the program. In brief, a plot of the logit of the optical densities versus the log of the inhibitor concentration is generated for each set of data points. The calculations for the logit transformation of the data is performed according to equation 6:

$$\text{logit} = \ln((B/Bo)/1-B/Bo) \qquad 6)$$

Where Bo is the average optical density of the uninhibited sera and B the corresponding average optical density of the test serum

inhibited with a specific concentration of inhibitor. The transformed data was fitted to a regression line and the IC_{50} was determined by interpolation to logit=0. The Krel values and average avidities (Ka) were determined from the IC_{50} as stated previously.

AVIDITY ELISA

The average avidity (Ka) of the antibodies generated during the primary and secondary *in vivo* antibody responses to TNP was determined by the use of an avidity ELISA. The data was normalized from plate to plate and from sample to sample by comparing each sample's IC_{50} value to a standard serum's IC_{50} value. The ELISA was performed with TNP-lysine as the only inhibitor. The Ka value of an antibody population was obtained by dividing the 50% inhibition (IC_{50}) obtained with a standard trout hyperimmune serum by the IC_{50} obtained with the sample. This value was a measurement of the average association constant (Ka). Equation 7 below is used to calculate Ka.

$$Ka = \frac{IC_{50} \text{ for the standard anti-serum}}{IC_{50} \text{ for the serum sample}} \qquad 7)$$

In brief, the wells of a 96-well ELISA plate (Costar, Cambridge, MA) were coated either overnight with 0.1 ml of a 0.5

μg/ml solution of trinitrophenylated bovine serum albumin, TNP-BSA, diluted in coating buffer or for 1 hr at 37°C in a moist chamber. Each technique yielded comparable results. After coating, the wells were blocked for 30 min with 0.2 ml of 3% BSA in tris-buffered saline with 1% tween (3% BSA-TTBS) at 37°C. The plate was washed 3 times with TTBS followed by 3 washes in tris-buffered saline (TBS). After the plate was washed, 0.050 ml of the appropriate TNP-lysine concentration was added to the ELISA wells followed by addition of 0.05 ml of the appropriate serum sample dilution. A dilution of plasma or serum yielding 1 unit of antibody activity was added to the various inhibitors. One unit of antibody activity corresponds to the dilution of the sample which was equivalent to 50% maximum binding of the standard serum. The samples and TNP-lysine were diluted in 3% BSA-TTBS. The plates were incubated for 90 min at 17°C.

After incubation, the plates were washed and 0.1 ml of biotinylated 1-14 monoclonal antibody diluted in 3% BSA-TTBS was added to the wells. After the plate was incubated for 90 min at room temperature, it was washed and incubated for 30 min with a 1/100 dilution of strepavidin conjugated to horseradish peroxidase, (Sigma, St. Louis, MO). The plate was washed and 0.1 ml of substrate added to each well. Optical densities were read at 405 nm at 0 and 25 min on a Biotek EL310 ELISA reader (Burlington, VT).

The Biotek ELISA reader was linked to a RS232 adaptor port of an IBM computer (PC). In this fashion the plate readings could

be sent to the computer and stored using Bio-tek Eia application software. IC_{50} values were determined by a custom design computer program for subsequent determination of Ka.

RESULTS

THE IMMUNE RESPONSE OF RAINBOW TROUT

KINETICS OF THE IN VIVO ANTI-TNP RESPONSE

The kinetics of the primary and secondary in vivo anti-TNP antibody response was monitored through time with a quantitative ELISA (Figure 3). This assay was specifically developed (Arkoosh and Kaattari, in press) for the determination of the quantity of anti-trinitrophenyl (TNP) antibodies in salmonid serum. This is a very sensitive assay which is capable of quantifying the amount of trout serum antibody in units of activity/µl of serum to TNP, even when only very minute volumes (e.g. 1.0 µl) of serum are available. The standard antiserum used in this ELISA was an anti-TNP serum derived from coho salmon (O. kisutch). This serum was determined to contain 250 units of activity/µl of serum. To determine whether enhanced secondary response could be produced in trout, the T-dependent antigen, TNP-keyhole limpet hemocyanin (KLH), was injected into 120 test fish (100 µg of TNP-KLH/fish). Plasma or serum samples were collected at 0, 2, 4, 6, 9, 13 and 17 weeks after the primary injection. The animals were then allowed to rest for 3 weeks after the final bleeding and given a second injection of the antigen. At 2, 4, 6, 9, 13 and 17 weeks after the secondary injection of antigen (20 µg TNP-KLH/fish) the fish were again bled. Also, as a control, a group of non-immunized

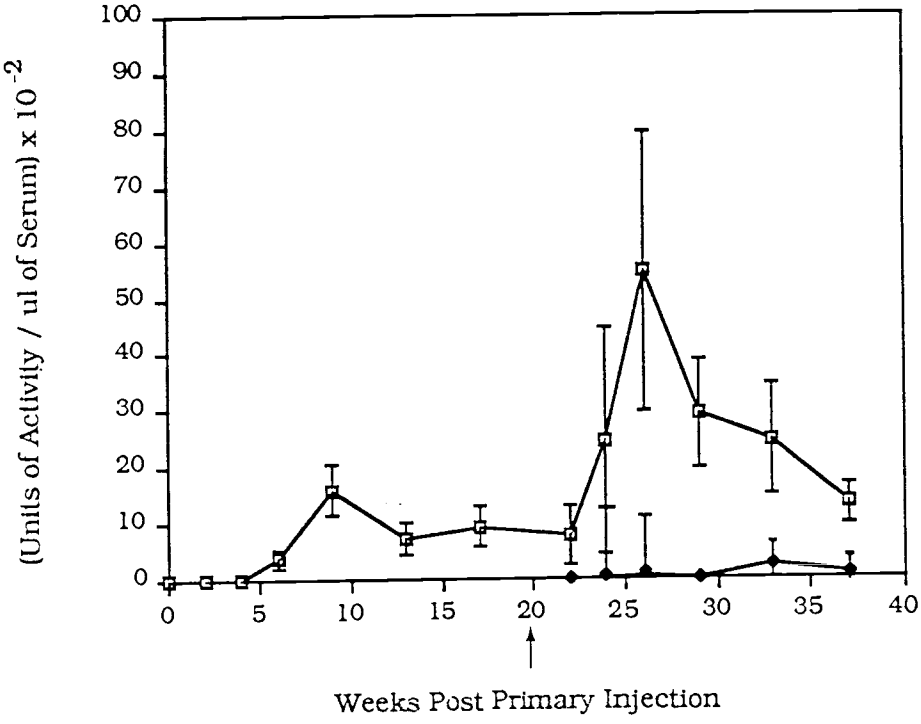


Figure 3. The <u>in vivo</u> antibody response to the trinitrophenyl hapten as determined by an ELISA. The units of activity/µl of serum measured at various time points after injection with TNP-KLH were plotted. The mean value derived from 9 test fish are plotted at each time point. The verticle bars indicate two standard error about the mean. The arrow (↑) represents the time at which the secondary immunization was administered. The unprimed control group (◆), which received the same concentration as administered in the secondary immunization, is also plotted.

fish from the same brood were given a primary injection of the same concentration of antigen (20 μg TNP-KLH/fish) at the same time the primed group of fish received their secondary injection. Sera samples from these fish were collected at the same time periods as the group given the secondary injection.

The secondary in vivo antibody response was characterized by an accelerated rate of antibody generation and a higher concentration of antibody activity/μl at the peak of the response (5300 units of activity/μl) as compared to the peak of the primary response (1500 units of activity/μl). The secondary response required only six weeks to reach its maximum after the injection of antigen while the primary response required nine weeks. The control group of animals which received a primary injection of the same low concentration of TNP-KLH as those simultaneously receiving a secondary of TNP-KLH (week 20 of the experiment) were unable to generate an appreciable antibody response (Figure 3).

ANALYSIS OF THE CONTRIBUTION FREUND'S COMPLETE ADJUVANT TO THE IN VIVO ANTI-TNP SERUM RESPONSE

To determine if the adjuvant nonspecifically contributes to the increased in vivo response seen after the secondary injection of antigen, trout were immunized with antigen and Freund's complete adjuvant (FCA) or with FCA alone. Trout which were given a primary injection of TNP-KLH (100 μg) in FCA produced a primary

antibody response with a mean titer of 164 units of activity/µl, 8 weeks post-immunization (Figure 4). Fish receiving the same amount and volume of FCA alone did not have a mean response over 6.75 units of antibody activity/µl of serum.

After these two groups of fish were given a secondary injection of 20 µg of TNP-KLH in Freund's incomplete adjuvant (20 weeks after the primary injection), their titer was reexamined at 3 weeks post secondary. An excellent secondary response developed in the fish which were given a primary injection with the antigen emulsified in FCA (992 units/µl), while the fish which received FCA only gave a significantly lower mean titer of 19.5 units /µl ($p<0.02$). These results indicate that Freund's complete adjuvant (FCA) does not contribute to the secondary in vivo anti-TNP serum antibody response as determined by the ELISA.

GENERATION OF A PRIMARY AND MEMORY IN VITRO ANTI-TNP ANTIBODY RESPONSE IN SPLEEN CELLS

The ability of rainbow trout lymphocytes to demonstrate a memory response in vitro was accomplished by the use of a modified Mishell-Dutton (1967) culture system and the Cunningham modification of the Jerne hemolytic plaque assay (1968). A plaque, in an antigen coated sheep red blood cell (SRBC) lawn, is the product of a single B cell which is secreting specific antibody against the antigen coated onto the SRBC. Therefore, it was possible to enumerate the B cells responding to TNP in vitro.

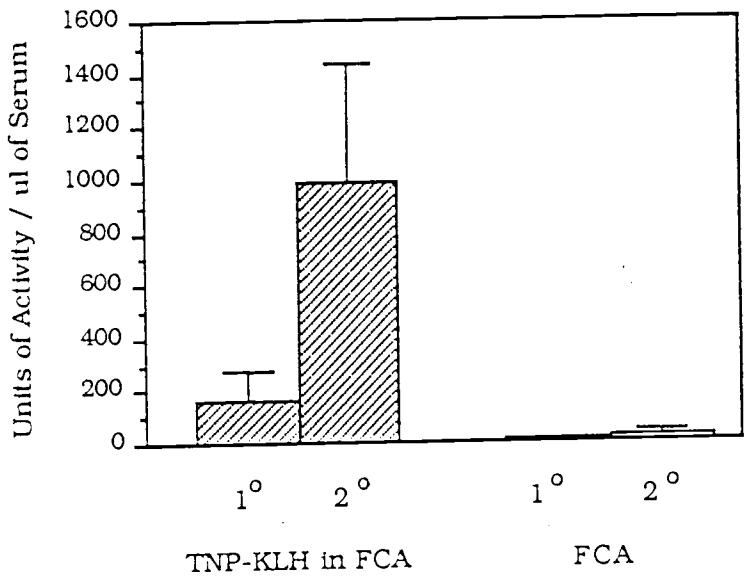


Figure 4. Freund's complete adjuvant contribution to the in vivo secondary antibody response to the trinitrophenyl (TNP) hapten. The units of activity/µl of serum generated during a primary response were plotted. Each histogram represents the mean value of 4 individuals tested for the primary response and 9 individuals tested for the secondary response. The error bars represent one standard error above the mean.

The characteristics of the secondary response that these cells produced to various doses of a T-dependent (TNP-KLH) and a T-independent (TNP-LPS) antigen was studied. A lymphoid organ, the spleen, of the trout were used to determine if a memory response can be generated as determined by an increase in the plaque forming cell (PFC) response over that seen with cells from unprimed fish.

Trout were injected with 100 μg of TNP-KLH emulsified in FCA intraperitoneally (i.p.), or given a sham injection of the same volume of PBS emulsified with FCA. The animals were allowed to rest for a minimum of 21 weeks. The spleens were removed and cultured with various doses of TNP-KLH or TNP-LPS. A plaque assay was performed on day 9 of culture to determine the magnitude of the PFC response.

Spleen cells from trout given a single i.p. injection of TNP-KLH and subsequently exposed to the antigen in vitro were unable to produce a detectable secondary response (Figure 5A). In fact, splenic lymphocytes from nonimmunized fish were able to produce a higher plaque forming cell (PFC) response at two doses of antigen, 200 μg/ml (55 PFC/culture) and 500 μg/ml (51 PFC/culture), than the cells obtained from animals primed in vivo (0 and 3 PFC/culture respectively) ($p<0.01$).

Trout primed with TNP-KLH were able to produce a memory response in vitro against a classical T-independent form of the antigen, TNP-LPS, (Figure 5B). The peak of the secondary PFC response occurred with a dose of 0.04 μg/ml of TNP-LPS (717

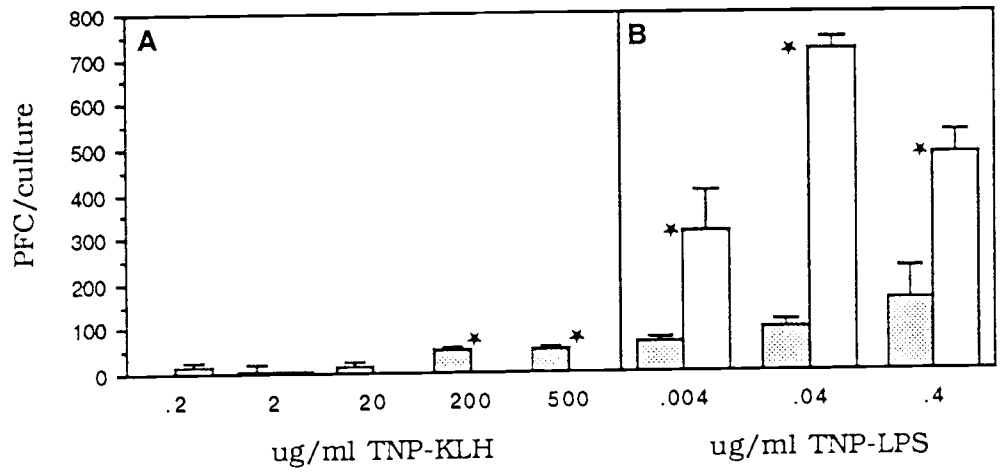


Figure 5. The <u>in vitro</u> antibody response of spleen cells from fish immunized with TNP-KLH in FCA (□) or FCA alone (▣). Plaque forming cells (PFC) to TNP-KLH (A) and TNP-LPS (B) were assayed on day 9 of culture. Each histogram represents the mean of 3 cultures and the error bar represent one standard error above the mean. The (✶) represents a significant difference in the PFC response ($p<0.05$) produced between the two population of cells tested at that particular concentration of antigen.

PFC/culture) while lymphocytes from the FCA injected animals exposed to the same concentration of TNP-LPS in vitro generated only 96 PFC/culture. All doses of TNP-LPS developed secondary PFC responses which were significantly higher than the PFC responses which were generated from the lymphocytes obtained from control animals ($p<0.05$).

In a further attempt to stimulate an in vitro TD memory response, animals were injected twice in vivo with TNP-KLH. After a primary injection of 100 µg of TNP-KLH in FCA, the fish were allowed to rest for 20 weeks and then given a secondary injection of the antigen (20 µg/fish in FIA). The leukocytes were challenged in vitro with various doses of TNP-KLH and TNP-LPS at 17 weeks after the second injection of antigen.

At 17 weeks after the second injection, a significantly higher ($p<0.01$) tertiary response was produced with 20 and 2000 µg/ml of TNP-KLH. A slight difference between primed and unprimed leukocytes occurred at 200 µg/ml ($p<0.20$) (Figure 6). At 26 weeks after the secondary injection this heightened response to TNP-KLH was not induced at any of the concentrations used (data not shown).

At 17 weeks after the secondary injection of antigen the lymphocytes were also stimulated in vitro with various doses of TNP-LPS. All concentrations of antigen, except the lowest (0.0002 µg/ml) were able to produce a significantly ($p<0.02$) higher response than seen with cells from unprimed fish (Figure 7). A slight difference occurred between primed and unprimed leukocytes at 0.0002 µg/ml of TNP-LPS ($p<0.01$).

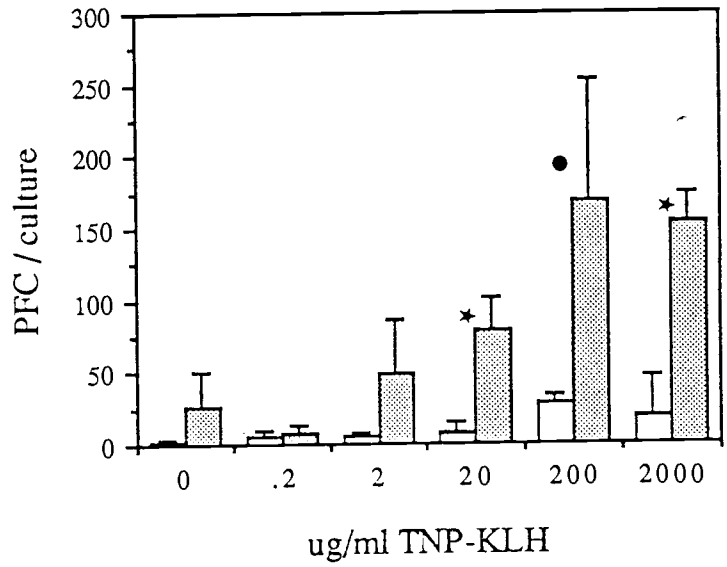


Figure 6. The <u>in vitro</u> antibody response of spleen cells from fish immunized with two injections of TNP-KLH in FCA (▣) or uninjected controls (□). Plaque forming cell (PFC) responses to TNP-KLH were assessed on day 9 of culture. Each histogram represents the mean of 3 cultures and the error bars represent one standard error above the mean. The (★) represents a significant different PFC tertiary response ($p<0.01$) produced between the two population of cells tested at that particular concentration of antigen. The (•) represents a p value > 0.01 but < 0.1 obtained between the primed and unprimed leukocytes.

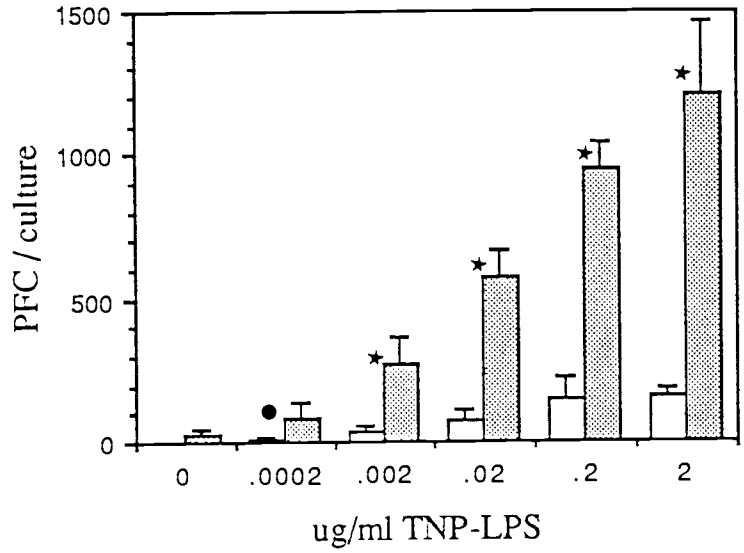


Figure 7. The in vitro antibody response of spleen cells from fish immunized with two injections of TNP-KLH in FCA (▣) or uninjected controls (□). Plaque forming cell (PFC) responses to TNP-LPS were assessed on day 9 of culture. Each histogram represent the mean of 3 cultures and the error bars represent one standard error above the mean. The (✶) represents a significant memory response ($p<0.02$). The (•) represents a p value > 0.02 obtained between primed and unprimed leukocytes but < 0.2. This particular experiment is a representative experiment of approximately 5 other experiments.

ESTIMATION OF PRECURSOR FREQUENCIES AND CLONE SIZES UTILIZING CONVENTIONAL 96 WELL TISSUE CULTURE PLATES

The nature of the increased PFC response to TNP-LPS after priming with TNP-KLH was examined utilizing limiting dilution analysis (LDA). This procedure enables one to quantitatively determine the contribution of initial precursor frequencies versus clonal expansion to the final generation of antibody-producing cells. Trout were, therefore, injected with 100 μg of TNP-KLH in FCA and allowed to rest for at least one year before their lymphocytes were used in this LDA. The analysis was performed as described by Tripp (1988) with a few modifications. At least cells from 3 fish were required in each assay in order to procure enough anterior kidney cells which were used as the filler/feeder cell population.

Priming a cell population in vivo to TNP-KLH resulted in an increase in the number B cell precursors specific to TNP (Figure 8) but not an increase in their clone size. Using spleen lymphocytes from animals which were not primed in vivo produced a precursor frequency to TNP-LPS of 4.44×10^{-5} or 1 antigen-responsive B cell precursor (B precursor)/22,500 leukocytes (Figure 8A). A primed animal produced a precursor frequency of 4.35×10^{-4} or 1 B precursor/2500 leukocytes, which represents a logarithmic increase (Figure 8B) above that seen with unprimed animals. Primed lymphocytes had a mean clone size of 2.4 +/- 0.28 antibody producing cells while the lymphocytes from animals which were not primed had a similar mean clone size of 3.78 +/- 0.35.

Figure 8. Antigen-specific B cell precursor frequencies for splenic lymphocytes stimulated in vitro with TNP-LPS in conventional 96 well plates. Cells were derived from either unprimed trout (A) or from trout receiving one priming injection of TNP-KLH (B). The cells were harvested on day 9 of culture and the fraction of nonresponding wells were plotted against the corresponding number of splenic leukocytes per well. Between 30-50 replicate cultures were analyzed for each spleen cell concentration. The line of best fit (·····) was determined by the method of least squares. The correlation coefficient (R) for A was 0.96 and for B, it was 0.99. The line of best fit was corrected for background (——) as described by Lefkovits (1979) A cell-concentration value (abscissa) corresponding to the 37% negative value (ordinate) was interpolated from the line passing through the origin to determine the precursor frequency. Experiment A and B are representative experiments.

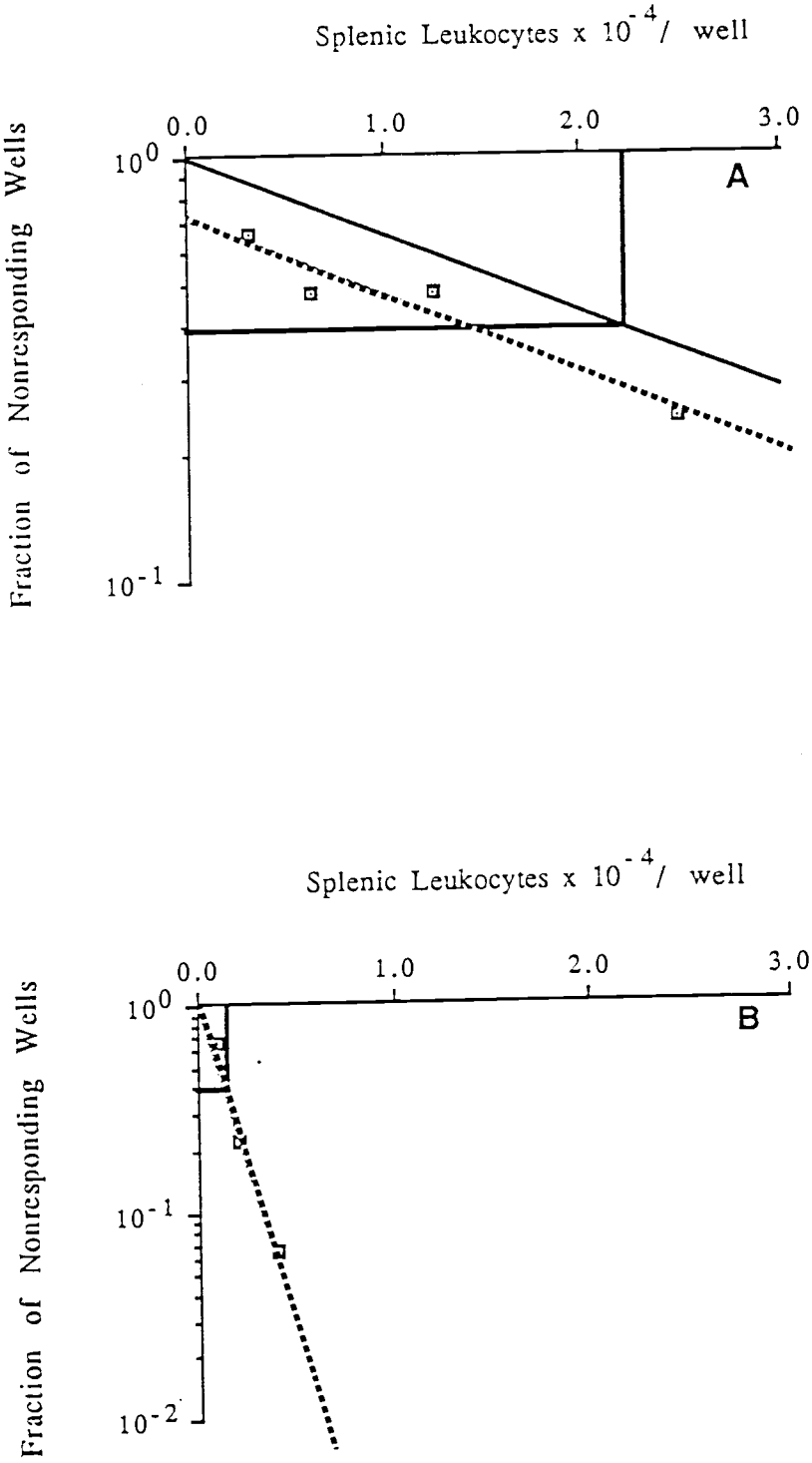

Splenic Leukocytes x 10^{-4}/ well
Fraction of Nonresponding Wells
0.0
1.0
2.0
3.0
10^{0}
10^{-1}
A
Splenic Leukocytes x 10^{-4}/ well
Fraction of Nonresponding Wells
0.0
1.0
2.0
3.0
10^{0}
10^{-1}
10^{-2}
B

Figure 8

B CELL KINETICS IN MICROCULTURE (TERASAKI) PLATES

A microculture system was developed which allowed for the incubation of a small quantity of cells (10^5/well) for the *in vitro* production of PFC. The optimal incubation time for the lymphocytes in microculture was 7 days. (Figure 9B). Leukocytes, from the same fish, incubated in 96 well plates (conventional) required at least 2 more days of incubation for optimal production of plaques. If the number of PFC were normalized to the number of cells used in the conventional plates (10^6), it was possible to determine that the responses generated on the peak day of the microculture system (day 7) was significantly higher than the response generated on that day in the conventional plates (Figure 9A). The optimal TNP-LPS concentration for each plate type was 0.4 μg/ml (data not shown).

ESTIMATION OF PRECURSOR FREQUENCIES AND CLONE SIZE UTILIZING THE MICROCULTURE SYSTEM

To determine the B cell precursor frequency and the clone size of spleen cells responsive to TNP-LPS from individual animals, it was necessary to employ the microculture system for LDA. Trout were injected with 100 μg of TNP-KLH in FCA and allowed to rest at least a year before using them in this analysis. In the microculture system, gamma-irradiated

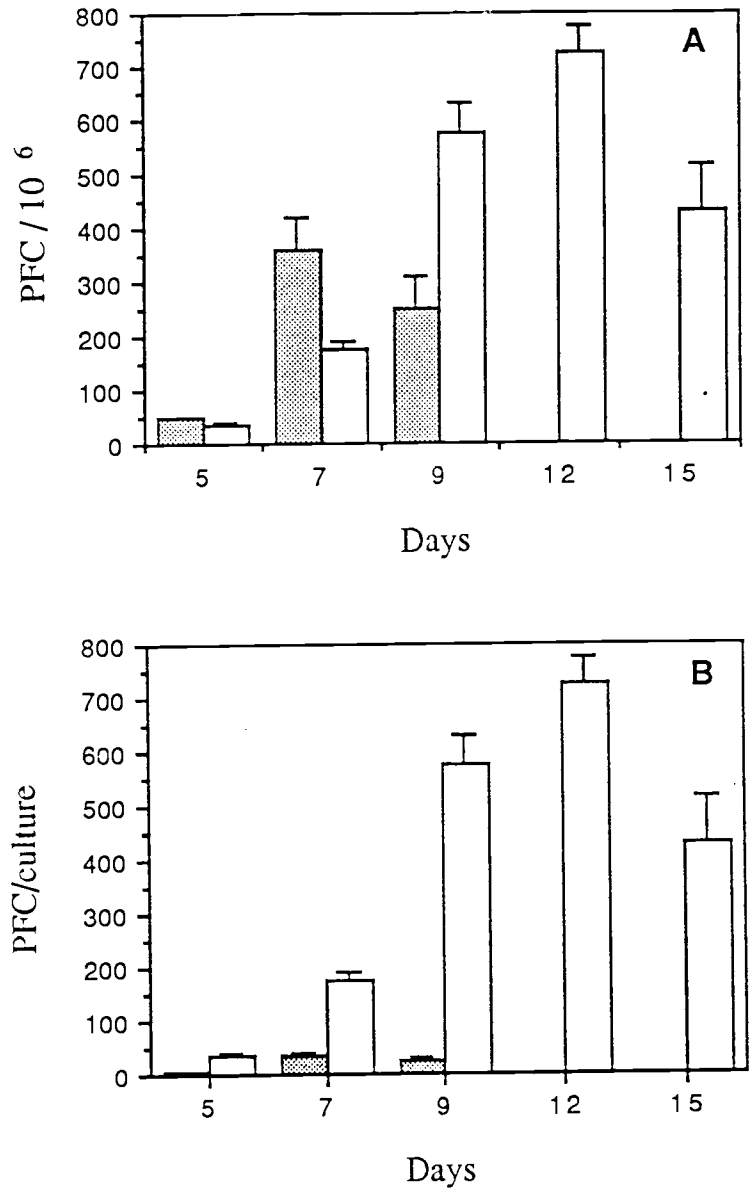


Figure 9. Plaque-forming cells (PFC) kinetics in microculture (Terasaki) plates compared to the kinetics in a conventional 96 well plate. Spleen cells were cultured with 0.4 μg/ml of TNP-LPS in Terasaki (□) or conventional plates (□). The plates were harvested on days 5, 7, 9, 12 and 15 of the culture period. PFC/10^6 (A) and PFC/culture (B) were determined for each set of data. The histogram represents the mean of 6 cultures from the Terasaki plates or the mean of 3 cultures from the conventional plates.

peripheral blood leukocytes were used as the filler/feeder source for the responding spleen cells.

The assays using leukocytes from primed animals demonstrated an increase in the splenic B cell precursor frequency (Figure 11 A, B, C, D) compared to the leukocytes from animals that were not primed (Figure 10 A, B, C). The precursor frequencies obtained from the splenic leukocytes of unprimed animals were; 4.71×10^{-5} or 1 B precursor/21,250 leukocytes (10A); 1.85×10^{-5} or 1 B precursor/45,000 leukocytes (10B); and 8.6×10^{-6} or 1 B precursor/117,500 leukocytes (10C). Precursor frequencies obtained from spleen cells from primed animals primed were; 1.01×10^{-3} or 1 B precursor/989.6 leukocytes (11A); 6.86×10^{-4} or 1B precursor/1458 leukocytes(11B); 9.4×10^{-5} or 1 B precursor/10625 (11C); and 8.57×10^{-5} or 1 B precursor/11,667 (11D). The clone size obtained from primed and normal lymphocytes were equivalent. The mean clone size generated to TNP-LPS from primed animals was 2.66 +/- 0.62 compared to a mean clone size of 2.74 +/- 0.66 obtained from unprimed B cells.

AVERAGE AVIDITY

Determination of the average avidities for the sera and plasma antibodies to TNP was accomplished by the use of the avidity ELISA. Trout were injected with 100 µg of TNP-KLH emulsified in FCA. Sera or plasma samples were collected at 0, 2, 4, 6, 13 and 17 weeks after the primary injection. The animals were

Figure 10. Precursor frequencies for spleen cells stimulated in vitro with 0.4 μg/ml of TNP-LPS in microculture plates. The cells were harvested on day 7 of culture and the fraction of nonresponding wells were plotted against the corresponding number of splenic leukocytes per well in 3 different assays (ABC). Between 50-60 replicate cultures were analyzed for each spleen cell concentration. The line of best fit (·····) was determined by least squares. The correlation coefficient (R) for A, B and C was 0.98, 0.90 and 0.99 respectively. The line of best fit was corrected for background (——) as described by Lefkovits (1979). A cell-concentration value (abscissa) corresponding to the 37% negative value (ordinate) was interpolated from the line through the origin to determine the precursor frequency.

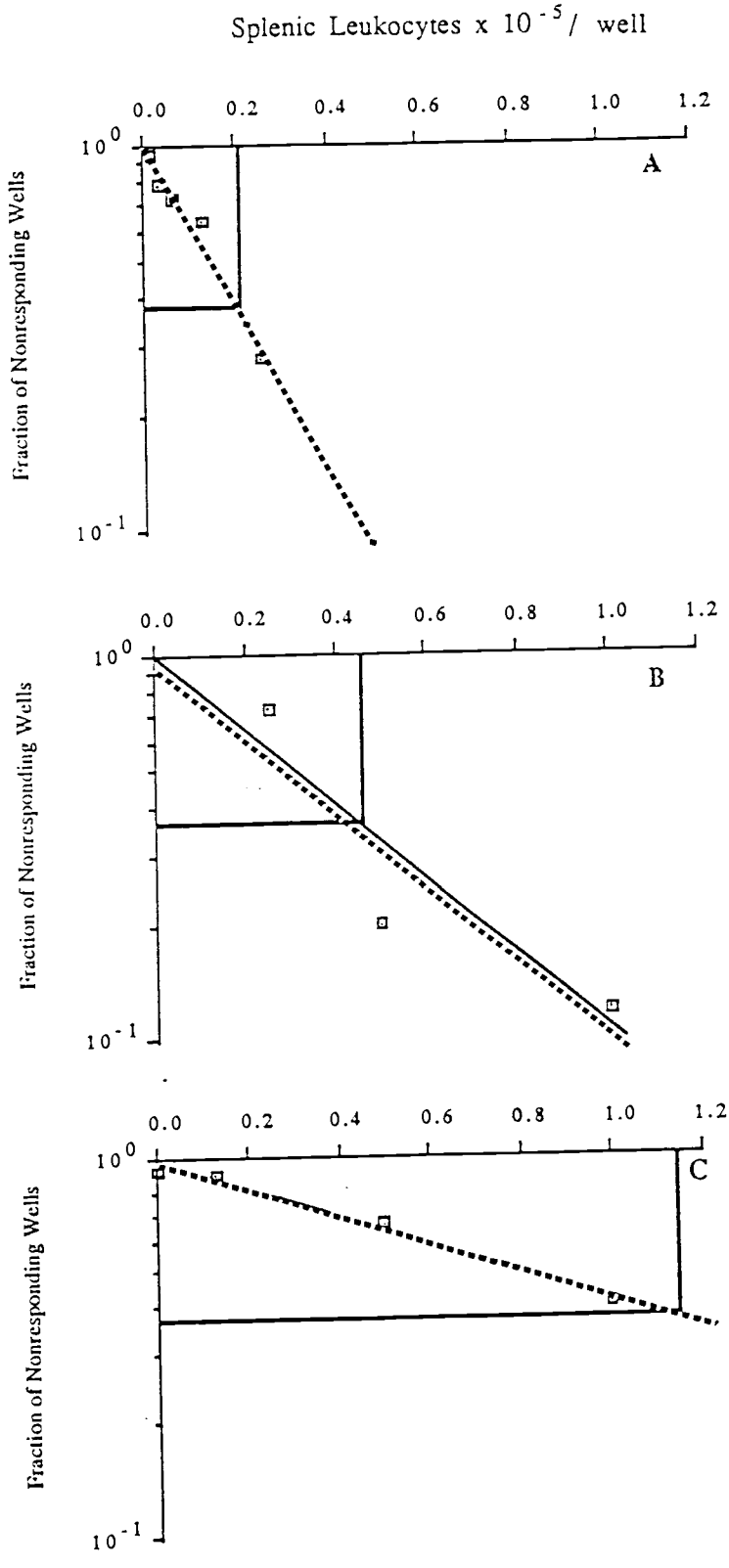

Splenic Leukocytes x 10^{-5} / well
Fraction of Nonresponding Wells
10^{0}
10^{-1}
0.0
0.2
0.4
0.6
0.8
1.0
1.2
A
B
C

Figure 10

Figure 11. Precursor frequencies for spleen cells stimulated in vitro with 0.4 μg/ml of TNP-LPS in microculture plates from fish primed in vivo to TNP-KLH. The cells were harvested on day 7 of culture and the fraction of nonresponding wells were plotted against the corresponding number of splenic leukocytes per well in 4 different assays (A, B, C, D). Between 50-60 replicate cultures were analyzed for each spleen cell concentration on day 9 of culture. The line of best fit (·····) was determined by least squares. The correlation coefficient (R) for A, B, C, and D is 0.994, 0.92, 1.0 and 0.942 respectively. The line of best fit was corrected for background (——) as described by Lefkovits (1979). A cell-concentration value (abscissa) corresponding to the 37% negative value (ordinate) was interpolated from the line passing through the origin to determine the precursor frequency.

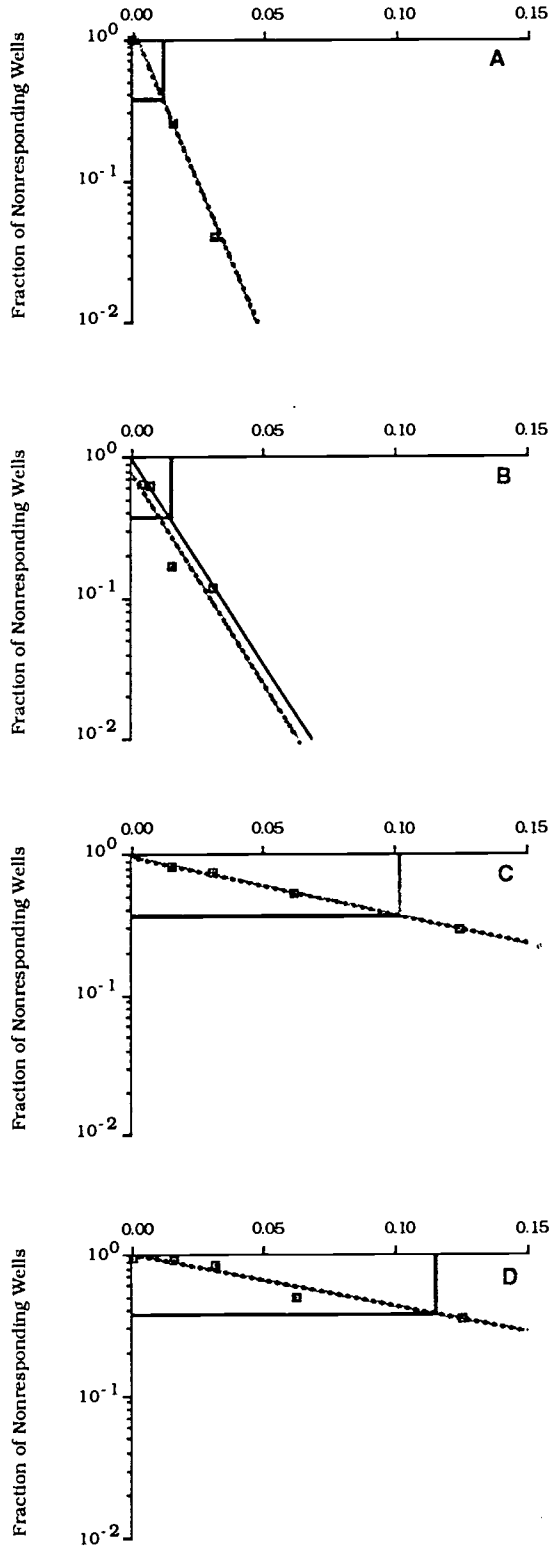

Splenic Leukocytes x 10^{-5} / well
Fraction of Nonresponding Wells
0.00
0.05
0.10
0.15
10^{0}
10^{-1}
10^{-2}
A
B
C
D

Figure 11

then rested for 3 weeks and given a secondary injection of antigen (20 µg/fish) at 20 weeks post primary. Blood was collected from fish again at 2, 4, 6, 9, 13 and 17 weeks after this secondary injection of antigen. These samples were used for determination of the average avidity (Ka) as well as for fine specificity analysis. The trout standard antiserum used for this analysis contained 1824 units of anti-TNP activity/µl of serum. The Ka were determined by testing an average of 6 samples per time period. A significant change ($p<0.05$) in the average avidity of the antibody population to TNP did not occur through time or after a secondary injection of the antigen TNP-KLH (Figure 12) when compared to the Ka from the first time point evaluated.

FINE SPECIFICITY ANALYSIS

The possible occurrence of specificity changes during the generation of a secondary response were determined by the use of a comparative inhibition ELISA using various analogs (Stenzel-Poore et al., 1988) (Figure 13) to the TNP-lysine homolog.

Trout were injected with 100 µg of TNP-KLH emulsified in FCA. Serum and plasma samples were collected at 0, 2, 4, 6, 13 and 17 weeks after the primary injection from each group. The animals were rested for 20 weeks and then given a secondary injection of antigen (20 µg/fish). At 2, 4, 6, 9, 13 and 17 weeks after the secondary injection of antigen the fish were bled again. These

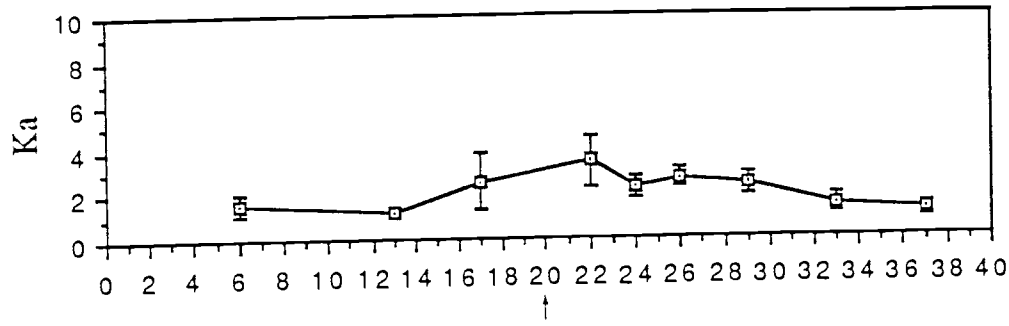
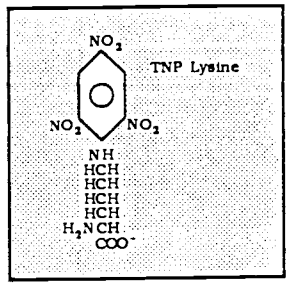
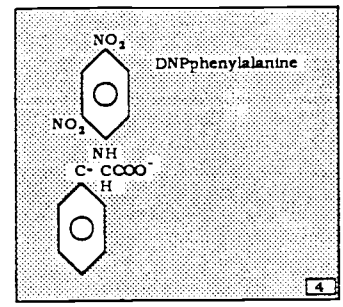
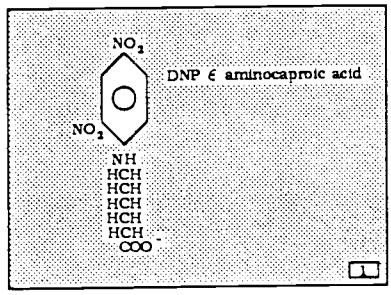


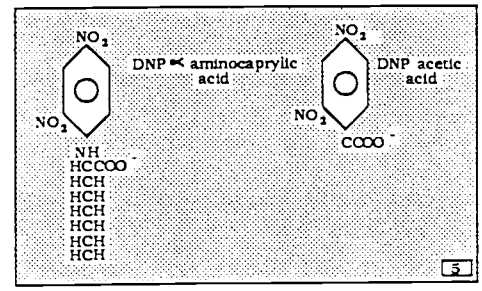
Figure 12. Average avidity (Ka) of antibodies to TNP. Serum samples of fish immunized with TNP-KLH were evaluated through time by the use of an inhibition ELISA. The mean of 6 samples (⊡) were plotted and the error bars represent two standard errors about the mean. The arrow (↑) represents the time at which the primed trout received a second injection of antigen.

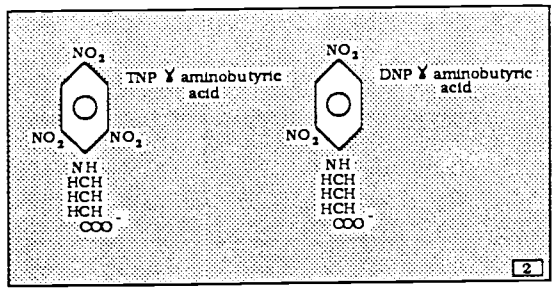
Figure 13. Structure of TNP-lysine and its analogues. The analogues were ranked (1-7) according to how well they were able to inhibit an anti-TNP antibody response in an inhibition ELISA. The assignment of 1 implies that the analog was the best inhibitor while those assigned 7 possess the least affinity for anti-TNP antibody.

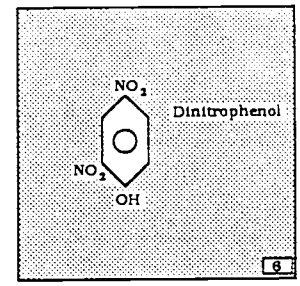

TNP Lysine

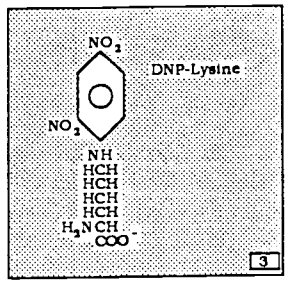

DNPphenylalanine
4


DNP ε aminocaproic acid
1


DNP α aminocaprylic acid
DNP acetic acid
5


TNP γ aminobutyric acid
DNP γ aminobutyric acid
2


Dinitrophenol
6


DNP-Lysine
3

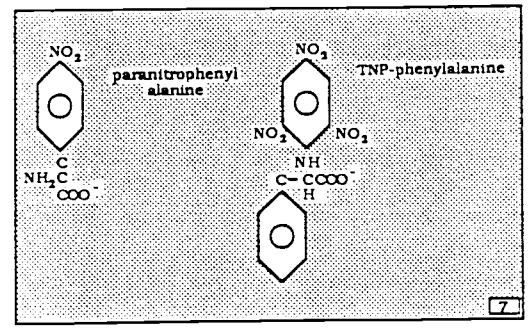

paranitrophenyl alanine
TNP-phenylalanine
7

Figure 13

samples were used for fine specificity analysis and the determination of average avidity.

The relative affinity was determined by comparing the amount of the homologous hapten (TNP-lysine) required for 50% inhibition of the antibody activity to the amount of heterologous hapten (analog) required for equivalent inhibition. The sera were all titered so that a standard amount of antibody would be tested for the determination of all Krel values. Changes in the fine specificity generated during the secondary response relative to the primary response were analyzed by the chi-square statistic to determine significance. The data's median was determined from the individual Krel values for each analog (Figures 14-23) in these studies. The analogues which demonstrated a significant decrease in Krel values, were dinitrophenol (Figure 16), dinitrophenyl-ε-aminocaproic acid (DEAC) (Figure 19) and dinitrophenyl-γ-aminobutyric acid (Figure 20). The statistical analyses of this data are summarized in Table 2.

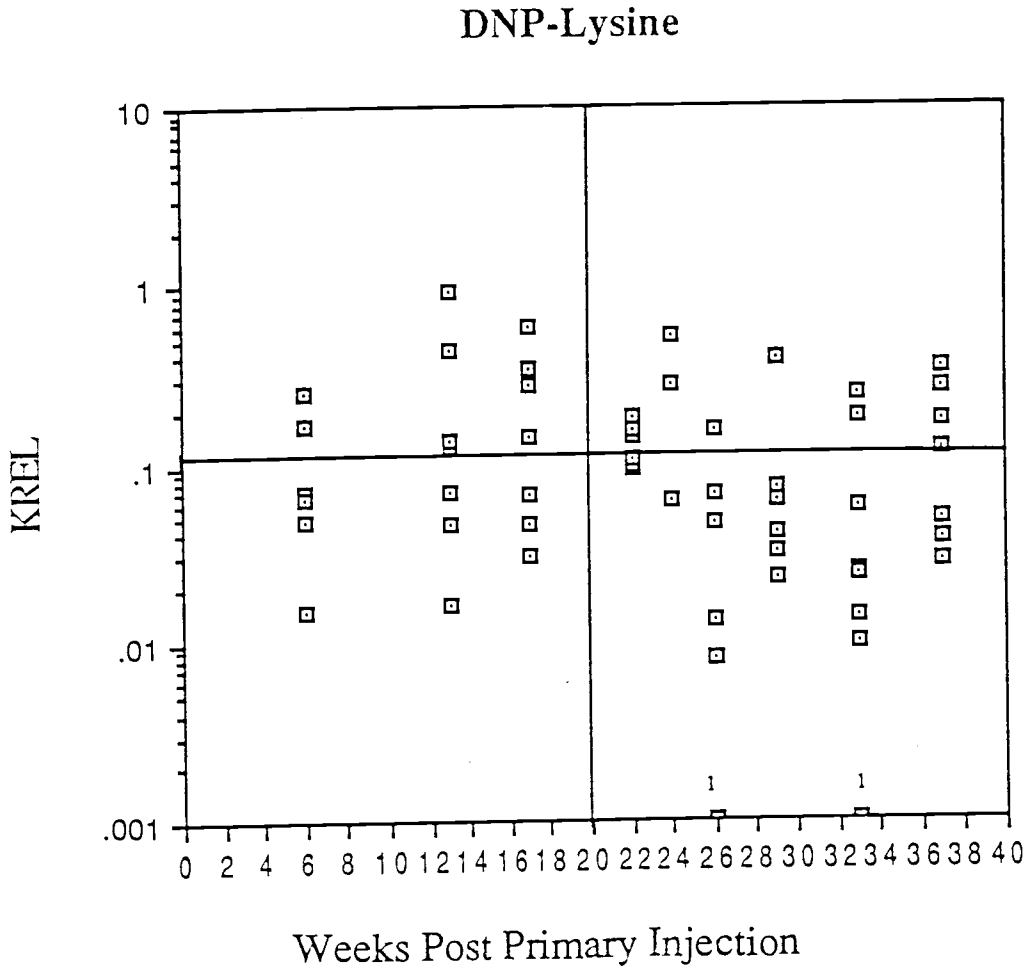


Figure 14. Krel values of the anti-TNP antibody samples determined for the analog, DNP-lysine. The line drawn horizontally represents the median of all data points. The line drawn vertically represents the point in time when the fish were given a secondary injection of TNP-KLH. Chi-square analysis was performed using the resulting four squares as cells to determine if the Krel values obtained in the primary response were significantly different from those obtained in the secondary response. The numbers immediately above the abscissa represent the number of samples tested at that time point which were not inhibited.

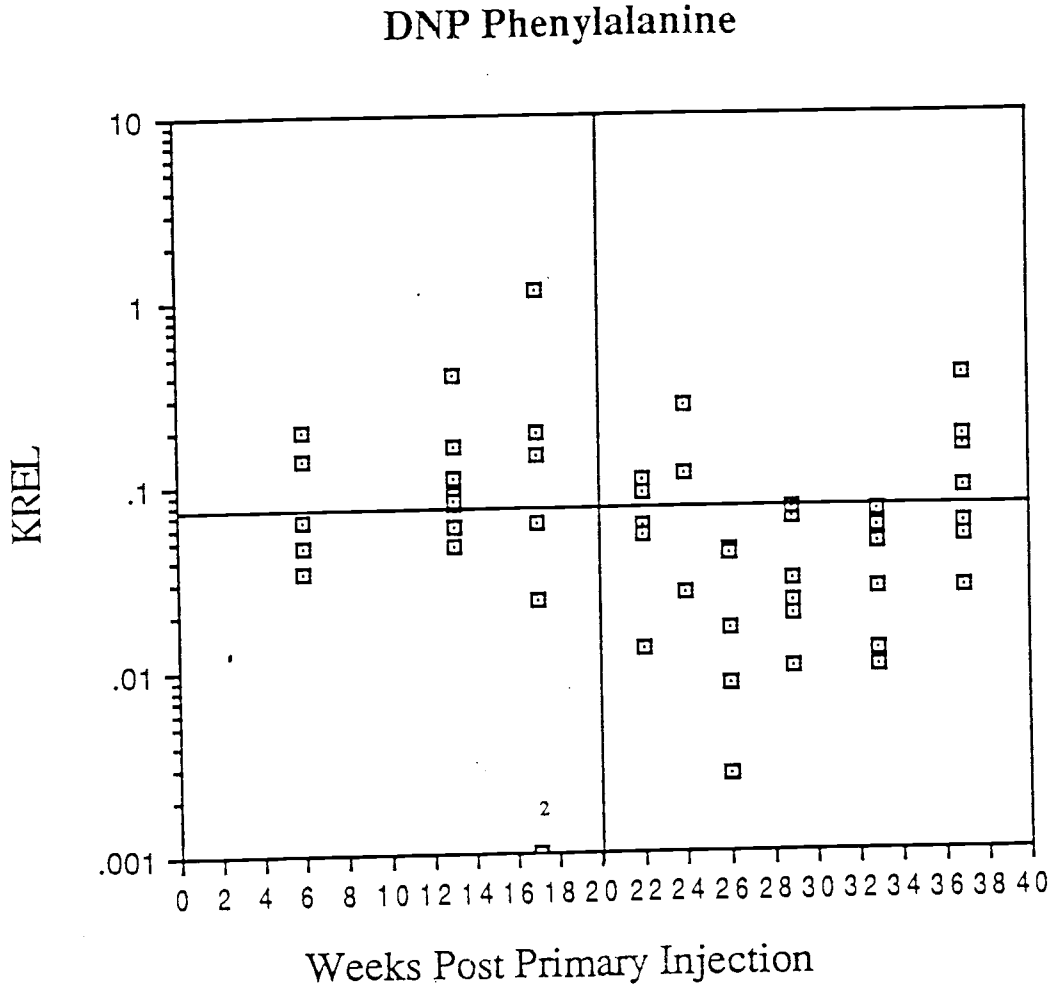


Figure 15. Krel values of the anti-TNP antibody samples determined for the analog, DNP-phenylalanine. The line drawn horizontally represents the median of all data points. The line drawn vertically represents the point in time when the fish were given a secondary injection of TNP-KLH. Chi-square analysis was performed using the resulting four squares as cells to determine if the Krel values obtained in the primary response were significantly different from those obtained in the secondary response. The numbers immediately above the abscissa respresent the number of samples tested at that time point which were not inhibited.

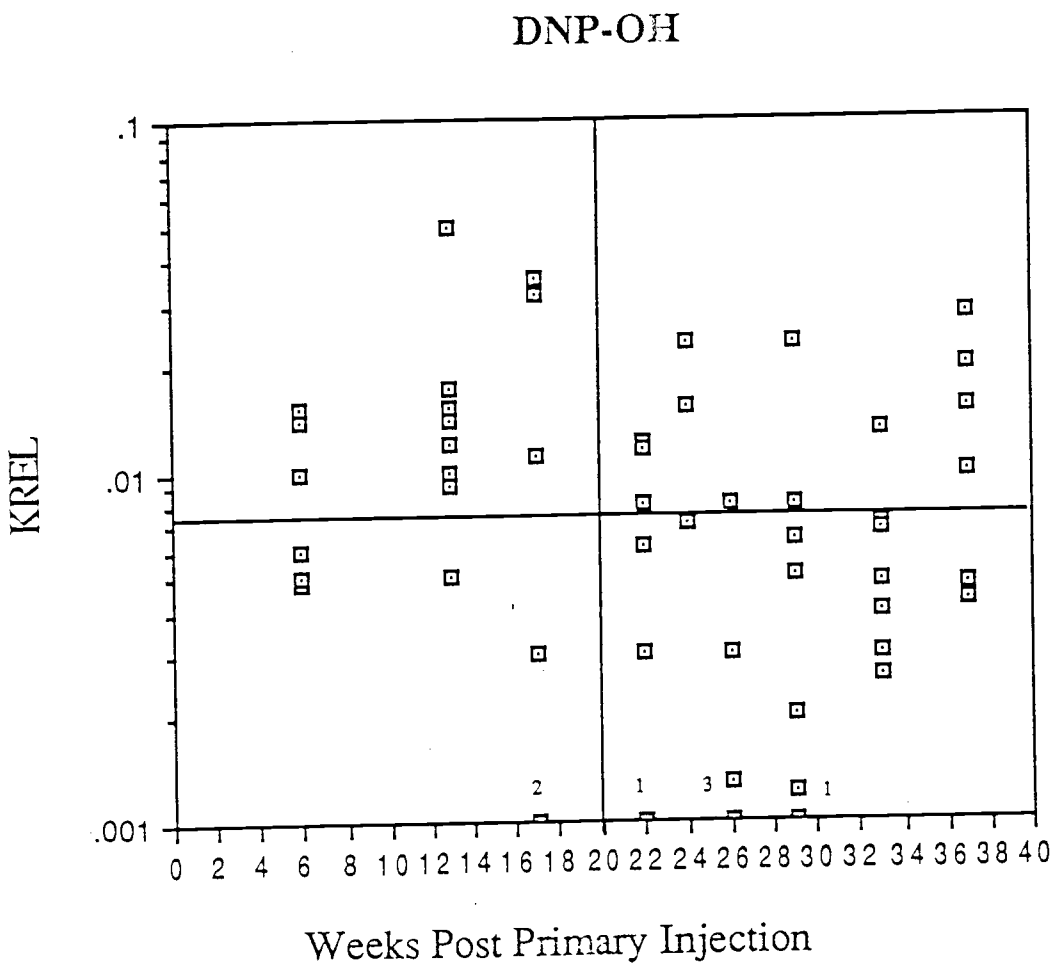


Figure 16. Krel values of the anti-TNP antibody samples determined for the analog, DNP-OH. The line drawn horizontally represents the median of all data points. The line drawn vertically represents the point in time when the fish were given a secondary injection of TNP-KLH. Chi-square analysis was performed using the resulting four squares as cells to determine if the Krel values obtained in the primary response were significantly different from those obtained in the secondary response. The numbers immediately above the abscissa respresent the number of samples tested at that time point which were not inhibited.

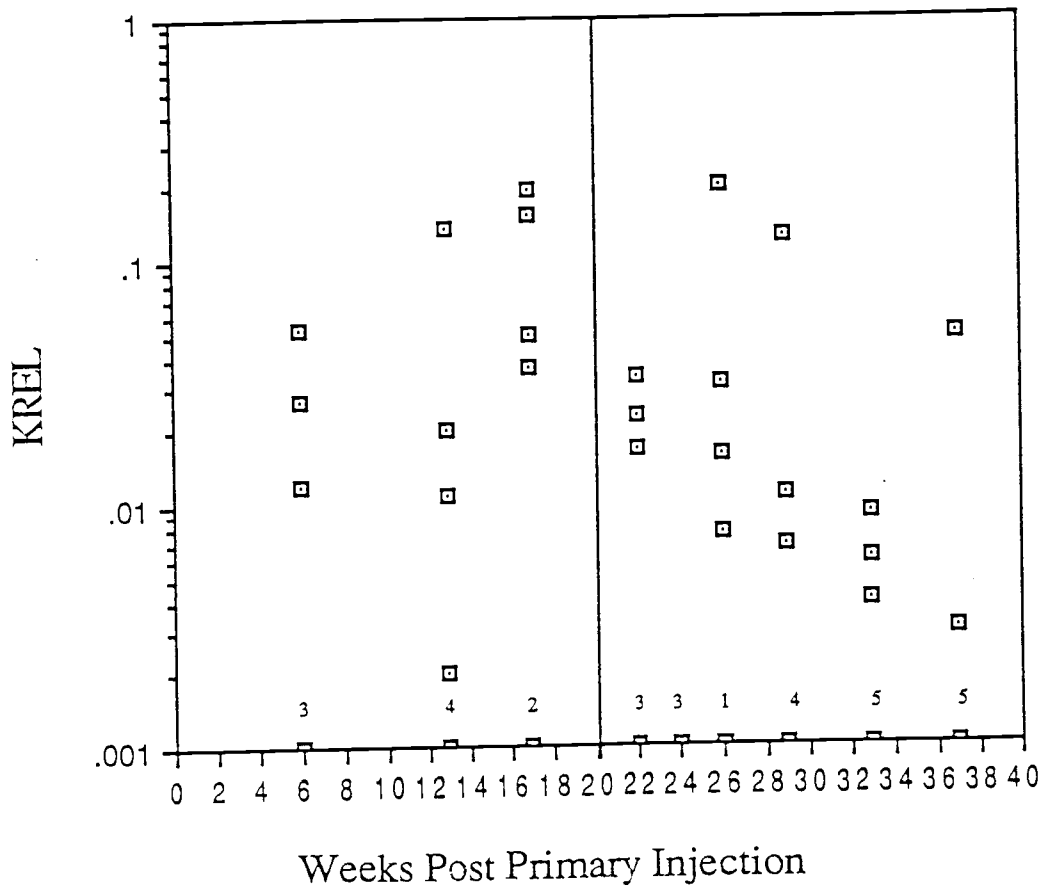


Figure 17. Krel values of the anti-TNP antibody samples determined for the analog, paranitrophenylalanine. For this inhibitor, there were as many samples uninhibited as inhibited. Therefore, the median would be at the 0.001 Krel value. The line drawn vertically represents the point in time when the fish were given a secondary injection of TNP-KLH. Chi-square analysis was performed using the resulting four squares as cells to determine if the Krel values obtained in the primary response were significantly different from those obtained in the secondary response. The numbers immediately above the abscissa respresent the number of samples tested at that time point which were not inhibited.

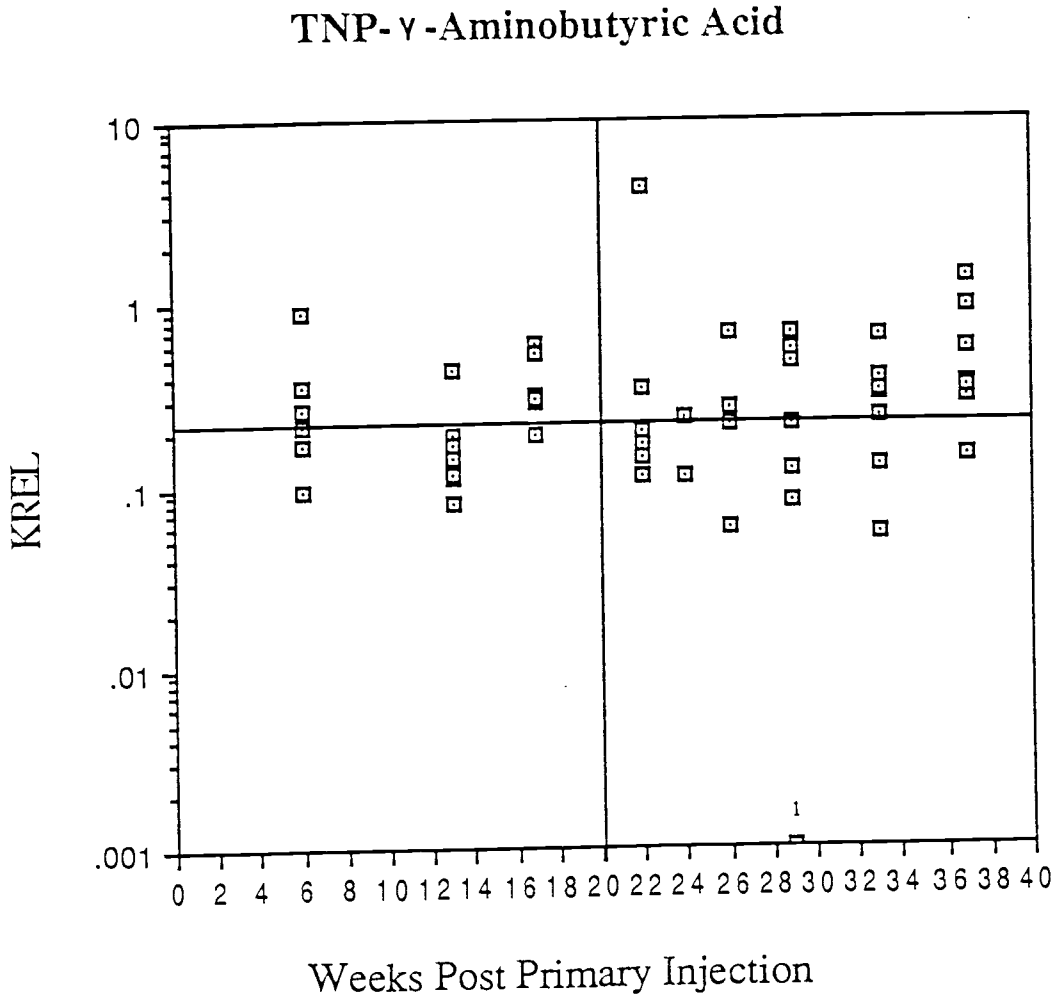


Figure 18. Krel values of the anti-TNP antibody samples determined for the analog, TNP-γ-aminobutyric acid. The line drawn horizontally represents the median of all data points. The line drawn vertically represents the point in time when the fish were given a secondary injection of TNP-KLH. Chi-square analysis was performed using the resulting four squares as cells to determine if the Krel values obtained in the primary response were significantly different from those obtained in the secondary response. The numbers immediately above the abscissa respresent the number of samples tested at that time point which were not inhibited.

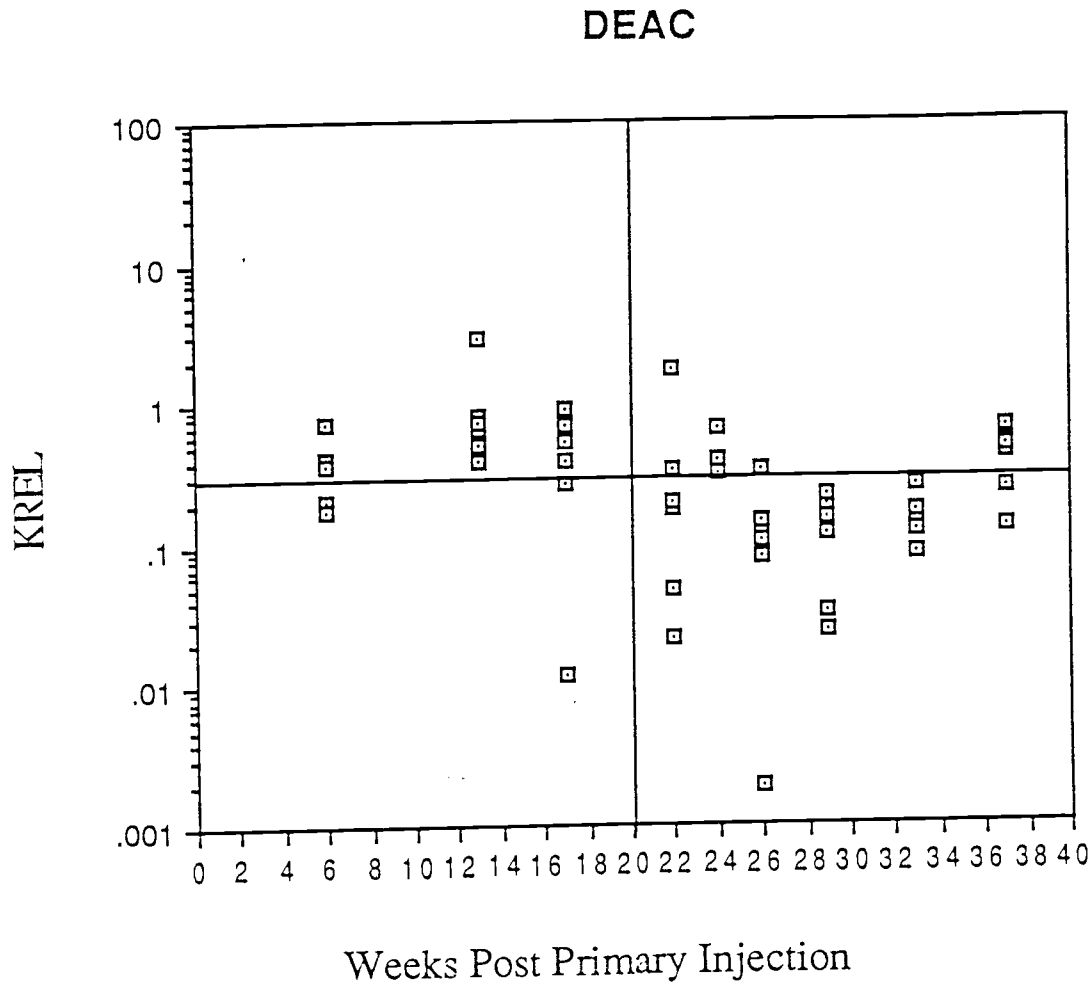


Figure 19. Krel values of the anti-TNP antibody samples determined for the analog, DEAC. The line drawn horizontally represents the median of all data points. The line drawn vertically represents the point in time when the fish were given a secondary injection of TNP-KLH. Chi-square analysis was performed using the resulting four squares as cells to determine if the Krel values obtained in the primary response were significantly different from those obtained in the secondary response.

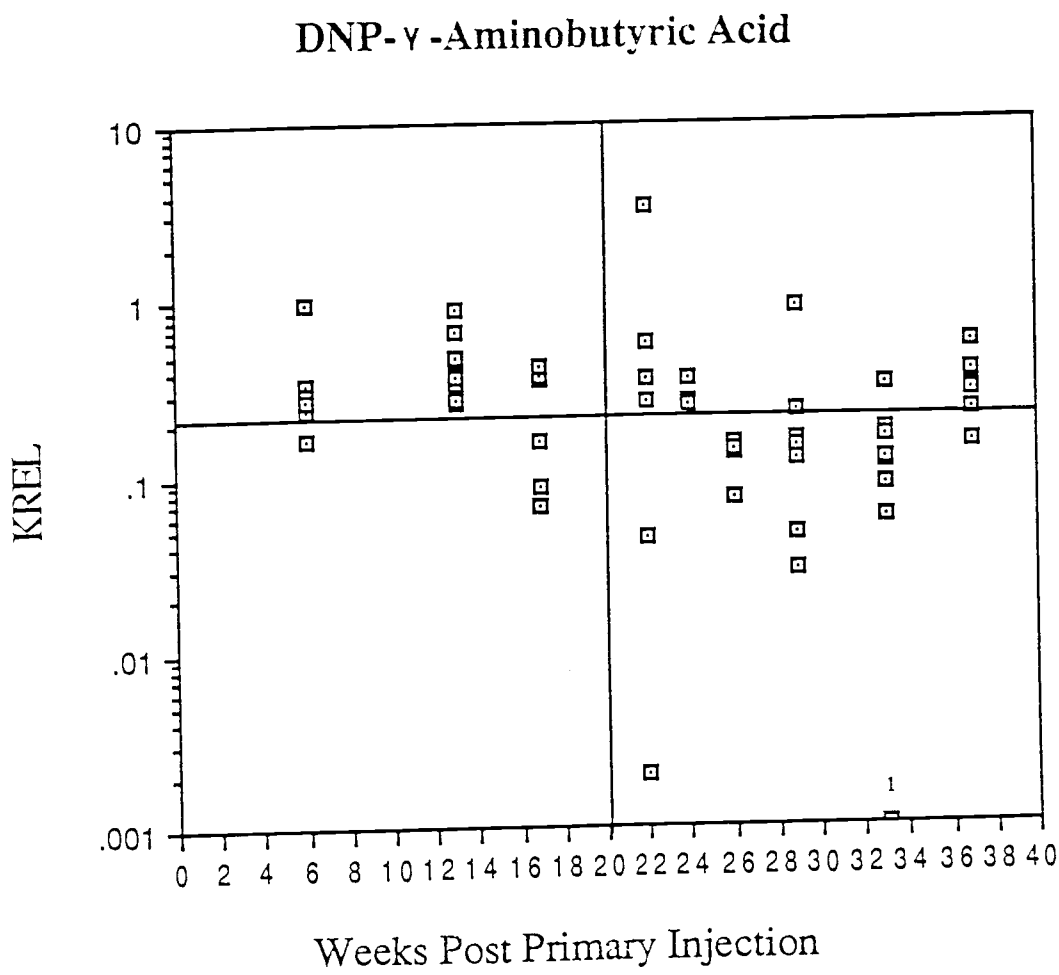


Figure 20. Krel values of the anti-TNP antibody samples determined for the analog, DNP-γ-aminobutyric acid. The line drawn horizontally represents the median of all data points. The line drawn vertically represents the point in time when the fish were given a secondary injection of TNP-KLH. Chi-square analysis was performed using the resulting four squares as cells to determine if the Krel values obtained in the primary response were significantly different from those obtained in the secondary response. The number immediately above the abscissa respresent the number of samples tested at that time point which were not inhibited.

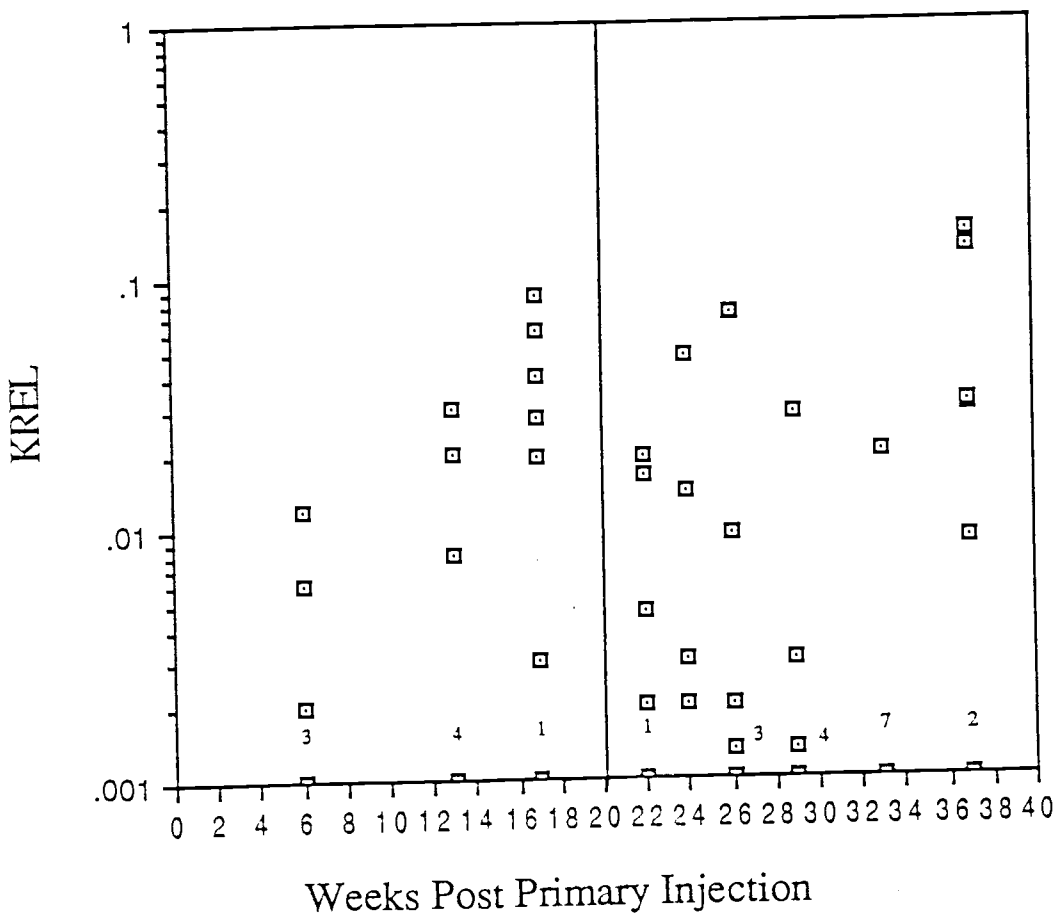


Figure 21. Krel values of the anti-TNP antibody samples determined for the analog, TNP-phenylalanine. For this inhibitor, there were as many samples uninhibited as inhibited. Therefore, the median would be at the 0.001 Krel value. The line drawn vertically represents the point in time when the fish were given a secondary injection of TNP-KLH. Chi-square analysis was performed using the resulting four squares as cells to determine if the Krel values obtained in the primary response were significantly different from those obtained in the secondary response. The numbers immediately above the abscissa respresent the number of samples tested at that time point which were not inhibited.

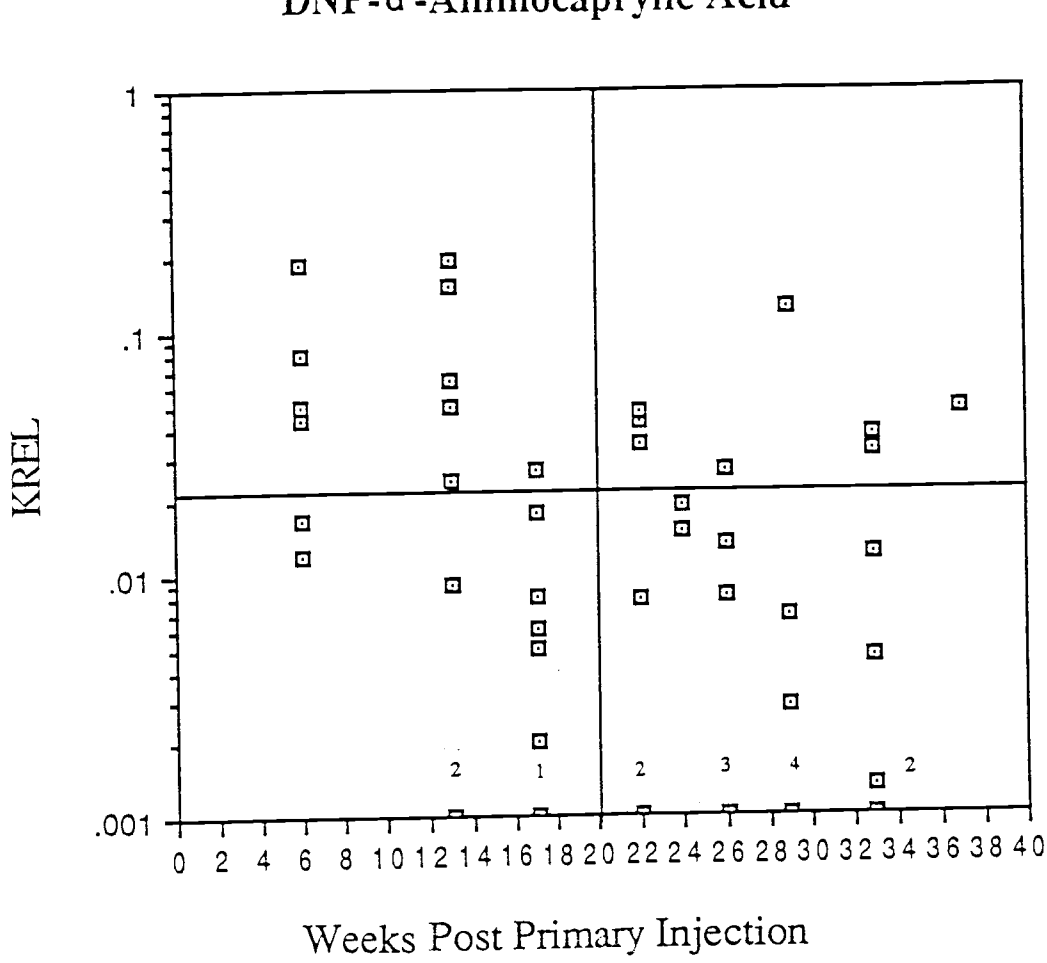


Figure 22. Krel values of the anti-TNP antibody samples determined for the analog, DNP-α-aminocaprylic acid The line drawn horizontally represents the median of all data points. The line drawn vertically represents the point in time when the fish were given a secondary injection of TNP-KLH. Chi-square analysis was performed using the resulting four squares as cells to determine if the Krel values obtained in the primary response were significantly different from those obtained in the secondary response. The numbers immediately above the abscissa respresent the number of samples tested at that time point which were not inhibited.

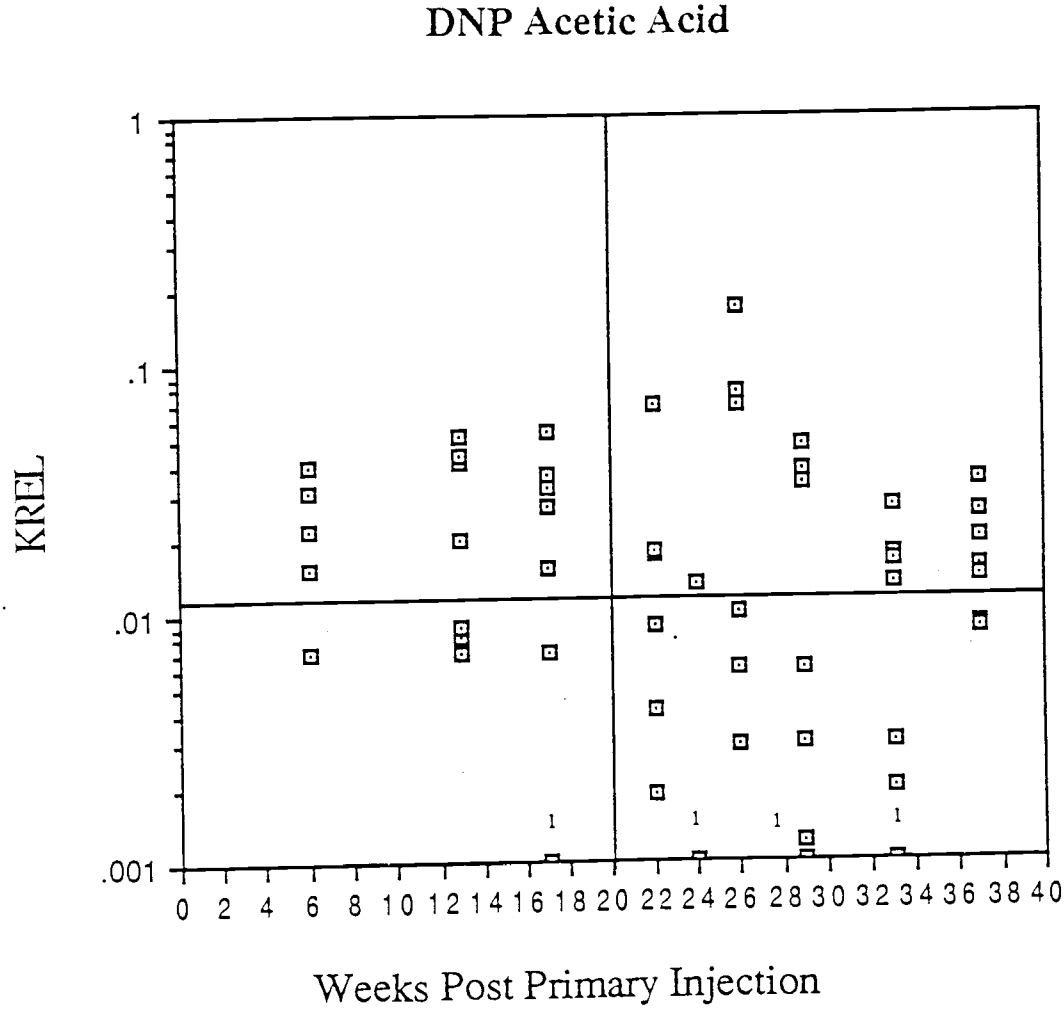


Figure 23. Krel values of the anti-TNP antibody samples determined for the analog, DNP-acetic acid. The line drawn horizontally represents the median of all data points. The line drawn vertically represents the point in time when the fish were given a secondary injection of TNP-KLH. Chi-square analysis was performed using the resulting four squares as cells to determine if the Krel values obtained in the primary response were significantly different from those obtained in the secondary response. The numbers immediately above the abscissa respresent the number of samples tested at that time point which were not inhibited.

Table 2. Summary of Chi-square analysis of the fine specificity data obtained from normal fish.

Inhibitors	Median[1]	Krel shift[2]
DNP-Lysine	.1	N.S.[3]
DNP Phenylalanine	.06	N.S
DNP-OH	.007	↓(P<.05)[4]
Paranitrophenylalanine	<.001	N.S.
TNP-γ-aminobutyric acid	.2	N.S.
DNP-ε-aminocaproic acid (DEAC)	.3	↓(P<.05)
DNP-γ-aminobutyric acid	.2	↓(P<.025)
TNP-phenylalanine	<.001	N.S.
DNP-α-aminocaprylic acid	.02	N.S.
DNP acetic acid	.01	N.S.

[1] Median Krel value for all individual samples tested.

[2] ↓ indicates a decrease in the Krel values during the secondary response.

[3] No significant changes (P>0.05) between primary and secondary responses.

[4] P values calculated by use of Chi-square analysis.

THE EFFECT OF AFLATOXIN ON THE IMMUNE RESPONSE OF RAINBOW TROUT

KINETICS OF THE IN VIVO ANTI-TNP RESPONSE IN AFLATOXIN EXPOSED TROUT

In parallel with the previous study, using normal fish (Figure 3), a population of aflatoxin-exposed animals were also studied. The primary in vivo antibody responses of aflatoxin exposed and normal trout were equivalent with respect to both the amount of antibody activity/µl of the samples tested and in the kinetics of the response (Figure 24). However, a dramatic difference between aflatoxin exposed and normal trout was observed at the peak of the secondary response. Sera from trout not exposed to aflatoxin produced a peak secondary response which was approximately 5300 units of antibody/µl of sample. This was at least twice the concentration of antibody that was produced by the aflatoxin exposed animals. These animals had a peak mean antibody titer of 2500 units/µl. Also, the peak of the secondary antibody response in aflatoxin-exposed animals, did not occur until 3 to 7 weeks after the peak secondary antibody response in normal animals. Therefore, the peak period of the secondary antibody response in aflatoxin-exposed animals was virtually indistinguishable from that seen in the primary response (9 weeks).

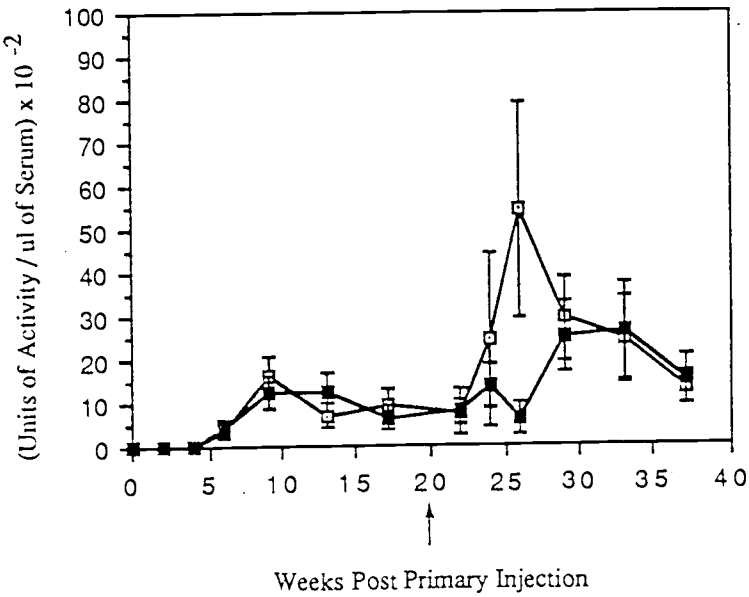


Figure 24. The in vivo antibody response to the TNP hapten as determined by an ELISA. The units of activity/µl of serum measured at various time points after injection with antigen were plotted. A mean of 9 fish were tested at each time point. The verticle bars indicates two standard error about the mean. The arrow (↑) represents the time at which the secondary immunization was administered to normal (⊡) and aflatoxin-exposed fish (■).

EFFECT OF AFLATOXIN ON THE PRODUCTION OF AN IN VITRO ANTI-TNP RESPONSE

The primary response generated in the splenic leukocytes of aflatoxin-exposed fish to a T-independent antigen, TNP-LPS, and to a T dependent antigen, TNP-KLH, was equivalent to the response produced by normal lymphocytes (Figure 25) at 17 weeks post primary. These lymphocytes were, also, unable to produce a heightened in vitro secondary response to either TNP-KLH (Figure 25A) or a TNP-LPS (Figure 25C), whereas spleen cells from normal trout demonstrated an enhanced in vitro memory response to TNP-KLH (Figure 25B) and to TNP-LPS (Figure 25D). However, the spleens from aflatoxin exposed animals were unable to generate a memory response to either form of the antigen (Figure 25A, C). Thus, this in vitro correlate of immunity parallels what is seen in vivo.

The in vitro response to TNP-LPS was tested again at 26 weeks after the second injection of antigen. (The characteristic heightened secondary response in the spleen of normal trout to TNP-KLH did not persist to this point in time). The B cells from aflatoxin-exposed animals were again able to make an equivalent primary response to that seen with normal animals (Figure 26A) but were unable to demonstrate an increase in PFC during the secondary response (Figure 26B) unlike the spleen cells derived from primed normal animals. The response in primed cells from normal animals was 140-fold greater than in aflatoxin-exposed fish

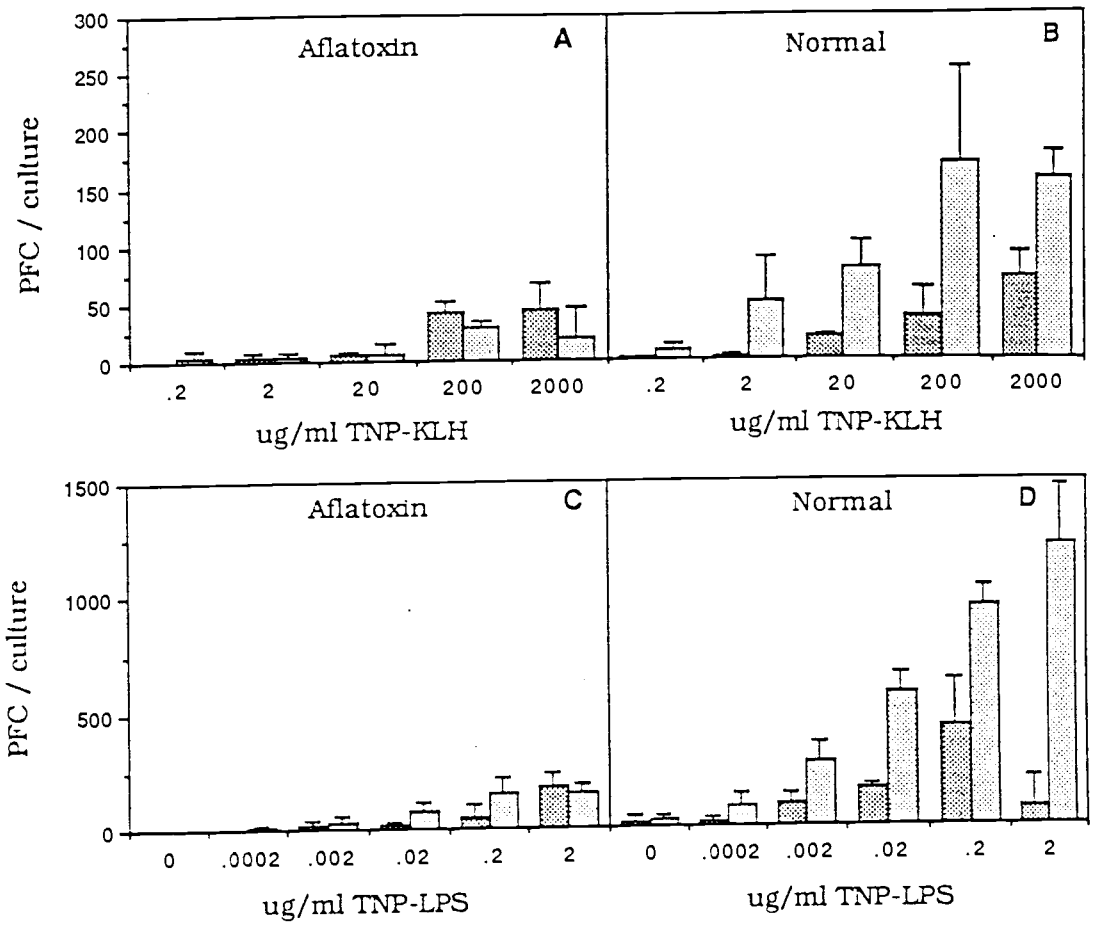


Figure 25. The effect of aflatoxin on the generation of a splenic memory response <u>in vitro</u> to TNP-KLH and TNP-LPS. Seventeen weeks after a second injection of antigen, spleen cells (▢) from aflatoxin exposed animals and normal controls were cultured with various concentrations of TNP-KLH (A, B) and TNP-LPS (C, D). Also at this time unprimed spleen cells (▨) from aflatoxin exposed animals and normal controls were cultured <u>in vitro</u> with various concentrations of TNP-KLH (A, B) and TNP-LPS (C, D). The histograms, representing the mean of 3 cultures, were plotted. The error bars represent one standard error above the mean. The data from these normal animals were also shown in figure 6 and 7.

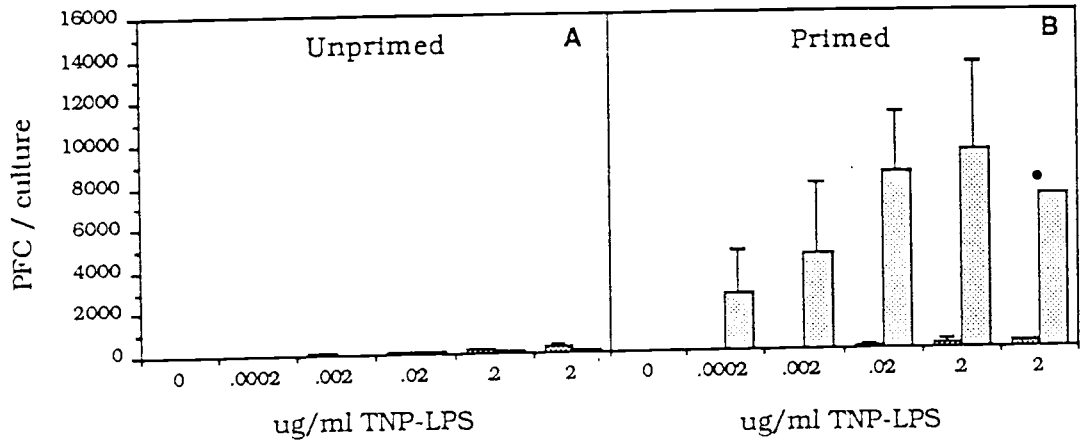


Figure 26. The effect of aflatoxin on the generation of splenic primary and memory responses <u>in vitro</u> to TNP-LPS. Twenty-six weeks after a second injection of antigen, spleen cells from unprimed aflatoxin-exposed animals (▨) and normal controls (□) were cultured with various concentrations of TNP-LPS (A). At the same time primed spleen cells from aflatoxin exposed animals (▨) and normal controls (□) were also cultured with various concentrations of TNP-LPS (B). The histograms, representing the mean of 3 cultures, were plotted. However, for one concentration (2.0 μg/ml) only one culture well was tested (•). The error bars represent one standard error above the mean.

at the antigen concentration which produced the peak mean of the PFC response (0.2 μg/ml).

During the course of this experiment, the weights of normal and aflatoxin-exposed fish were monitored to ensure that all the animals matured at the same rate. It was found that the growth rate was identical for both groups of fish (Table 3). Therefore size was not a variable that could affect the immunocompentency of the fish.

AVERAGE AVIDITY

To determine if the average avidity (Ka) of an anti-TNP antibody population changes due to short term aflatoxin-exposure the avidity ELISA was employed. For determination of avidity, TNP-lysine, (Figure 13) was used as the inhibitor. The Ka was determined by comparing the amount of TNP-lysine that produces 50% inhibition of the sample population with the amount of TNP-lysine which inhibits 50% of the trout standard. An average of six fish were assayed at each time point.

The Ka was monitored during the primary and secondary response in normal (Figure 12) and aflatoxin-exposed animals. At each time point monitored, the Ka did not differ significantly ($p>0.05$) between normal and aflatoxin exposed animals (data not shown). The Ka of serum samples from aflatoxin-exposed fish were also monitored to determine if the Ka of aflatoxin-exposed animals changed through time in comparison to the first time point assayed.

Table 3. Mean weights of normal and aflatoxin exposed fish throughout the assay period.

Weeks[1]	Normal[2]	Aflatoxin[3]
6	5.83	5.12
9	7.33	6.75
13	10.00	8.50
17	14.00	12.20
22	25.37 ± 6.30	18.24 ± 4.60
24	24.80 ± 8.30	26.70 ± 7.60
26	44.80 ± 16.20	39.30 ± 18.95
29	40.98 ± 11.70	39.50 ± 12.80
33	60.14 ± 10.99	61.86 ± 8.50
37	61.30 ± 20.10	80.80 ± 19.30

1. Weeks weighed after the primary injection.

2 Average body weight of normal fish in grams. An average of 9 fish were weighed for each time point. Fish were initially weighed as one large group because of their small size. However, at 22 weeks post primary injection, fish could be weighed as individual and standard errors were subsequently determined.

3 Average body weight of aflatoxin exposed fish in grams. An average of 9 fish were weighed for each time point. Fish were initially weighed as one large group because of their small size. However, at 22 weeks post primary injection, fish could be weighed as individual and standard errors were subsequently determined.

Figure 27. Krel values of the anti-TNP antibody samples determined for the analog, DNP-lysine, in normal (A) or aflatoxin-exposed (B) fish. The line drawn horizontally represents the median of all data points. The line drawn vertically represents the point in time when the fish were given a secondary injection of TNP-KLH. Chi-square analysis was performed using the resulting four squares as cells to determine if the Krel values obtained in the primary response were significantly different from those obtained in the secondary response. The numbers immediately above the abscissa respresent the number of samples tested at that time point which were not inhibited.

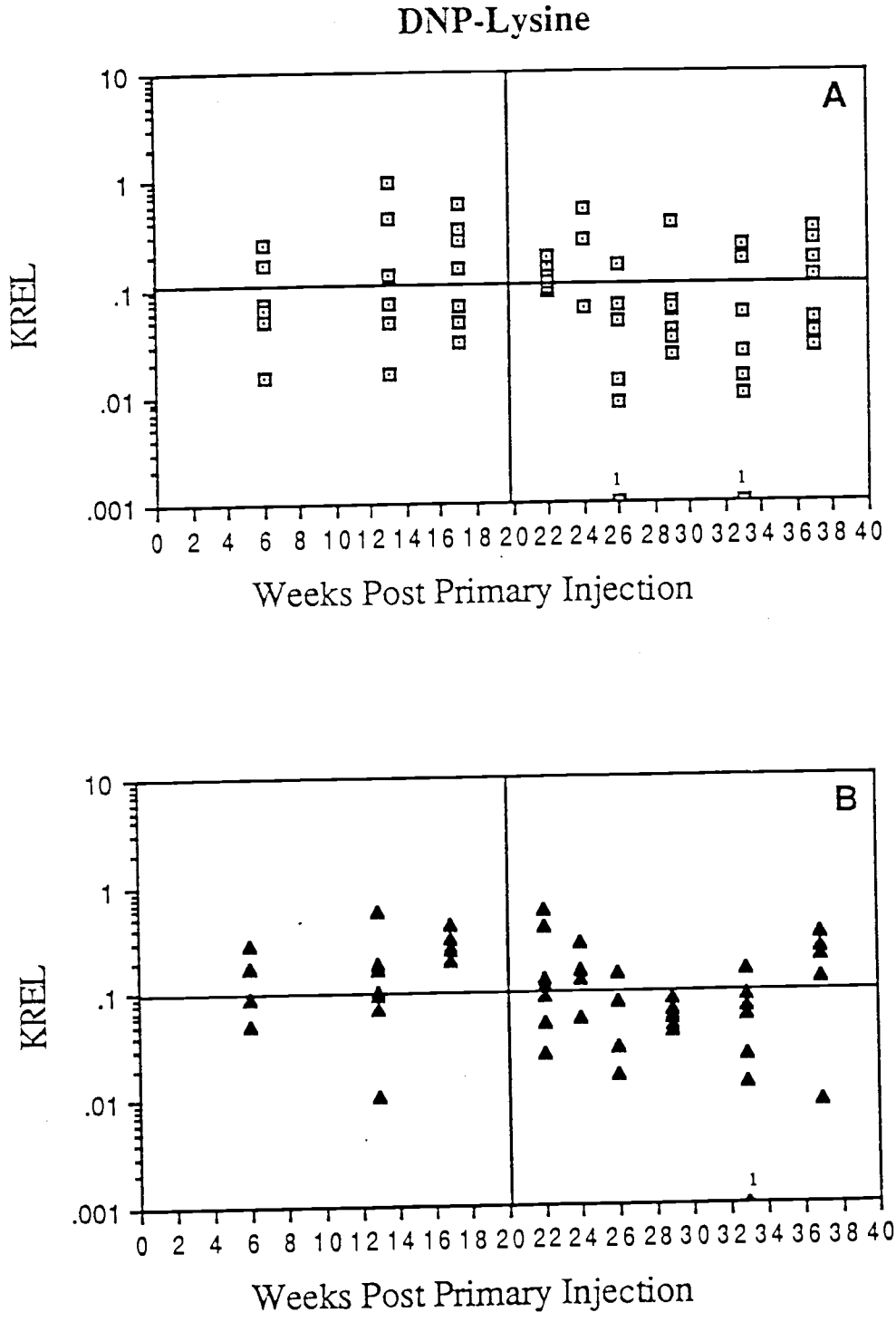

DNP-Lysine
A
KREL
10
1
.1
.01
.001
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Weeks Post Primary Injection
B
KREL
10
1
.1
.01
.001
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Weeks Post Primary Injection

Figure 27

Figure 28. Krel values of the anti-TNP antibody samples determined for the analog, DNP-phenylalanine, in normal (A) or aflatoxin-exposed (B) fish. The line drawn horizontally represents the median of all data points. The line drawn vertically represents the point in time when the fish were given a secondary injection of TNP-KLH. Chi-square analysis was performed using the resulting four squares as cells to determine if the Krel values obtained in the primary response were significantly different from those obtained in the secondary response. The numbers immediately above the abscissa respresent the number of samples tested at that time point which were not inhibited.

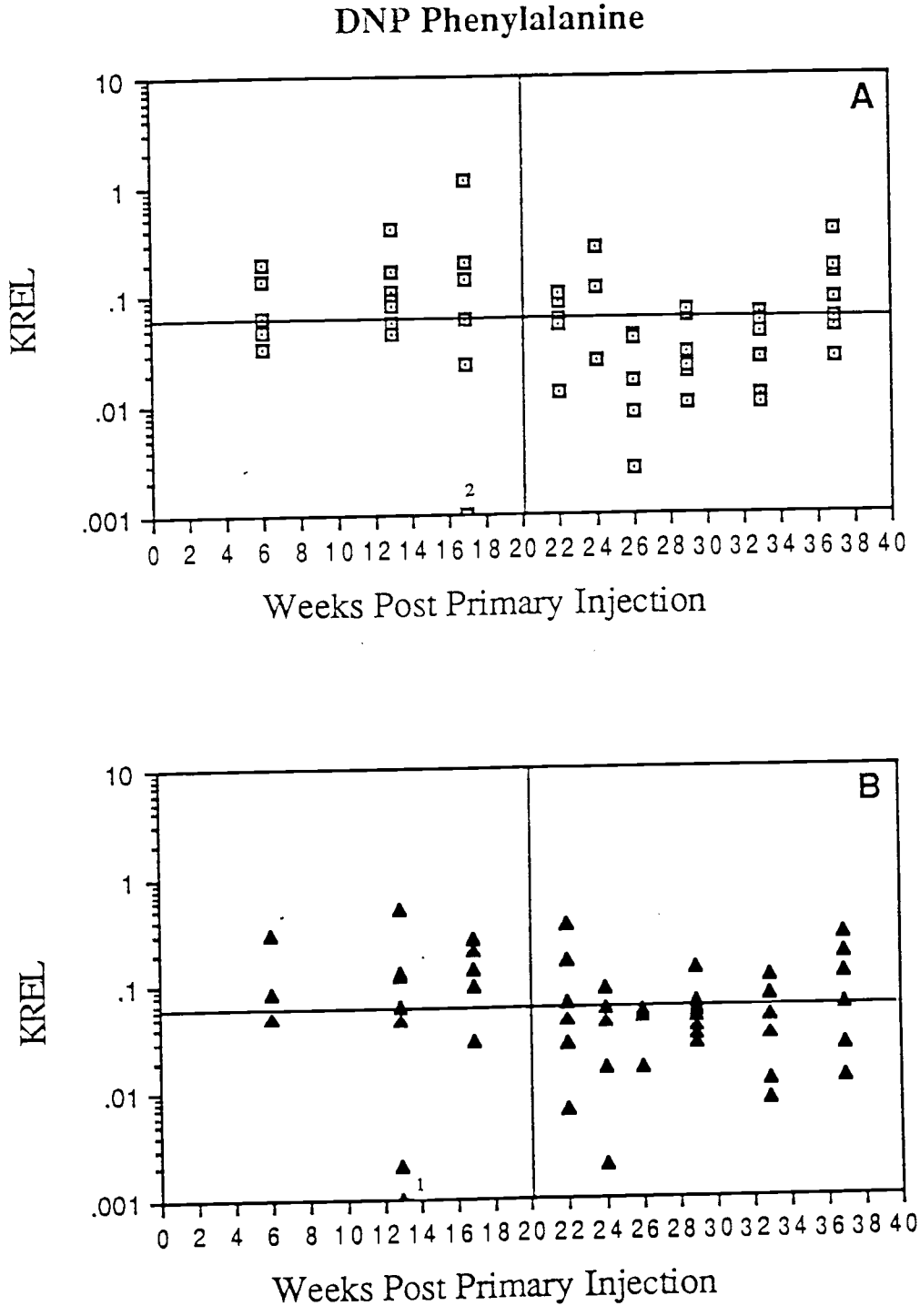
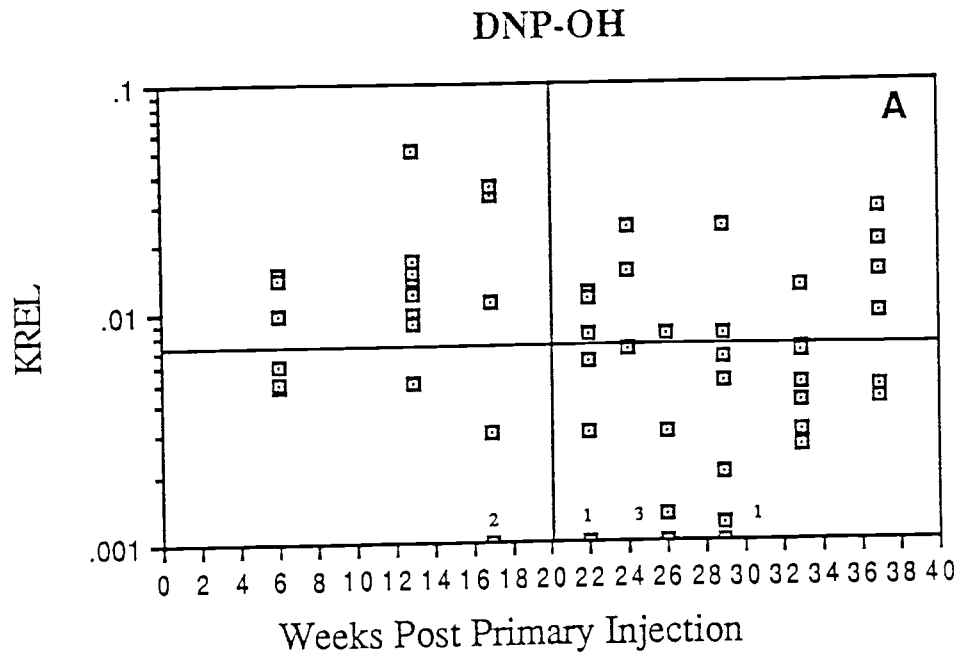

DNP Phenylalanine
A
KREL
10
1
.1
.01
.001
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Weeks Post Primary Injection
B
KREL
10
1
.1
.01
.001
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Weeks Post Primary Injection

Figure 28

Figure 29. Krel values of the anti-TNP antibody samples determined for the analog, DNP-OH, in normal (A) or aflatoxin-exposed (B) fish. The line drawn horizontally represents the median of all data points. The line drawn vertically represents the point in time when the fish were given a secondary injection of TNP-KLH. Chi-square analysis was performed using the resulting four squares as cells to determine if the Krel values obtained in the primary response were significantly different from those obtained in the secondary response. The numbers immediately above the abscissa respresent the number of samples tested at that time point which were not inhibited.


DNP-OH
A
KREL
.1
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0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Weeks Post Primary Injection

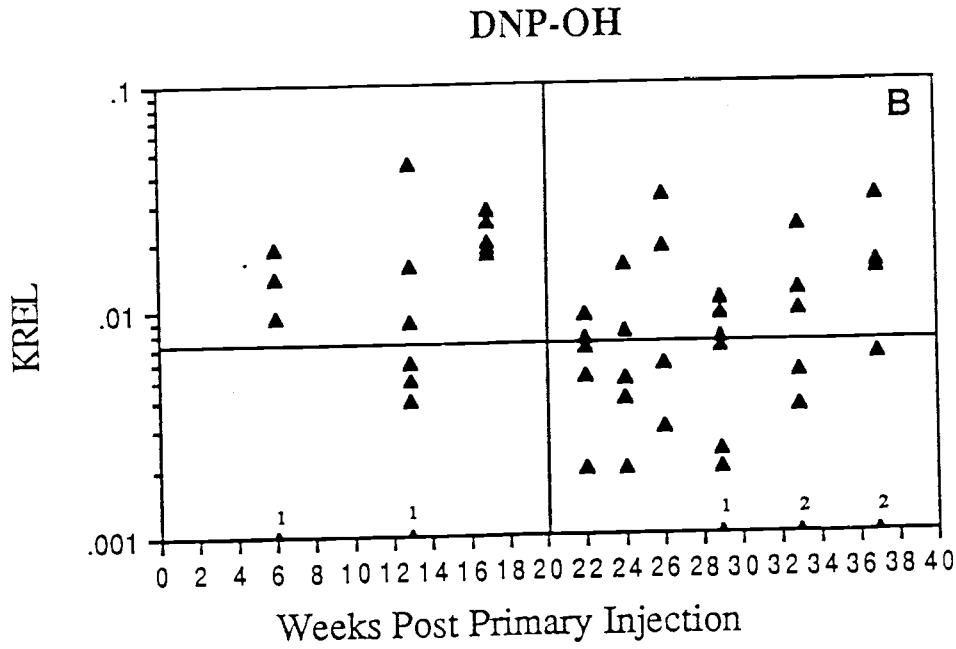
DNP-OH
B
KREL
.1
.01
.001
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Weeks Post Primary Injection

Figure 29

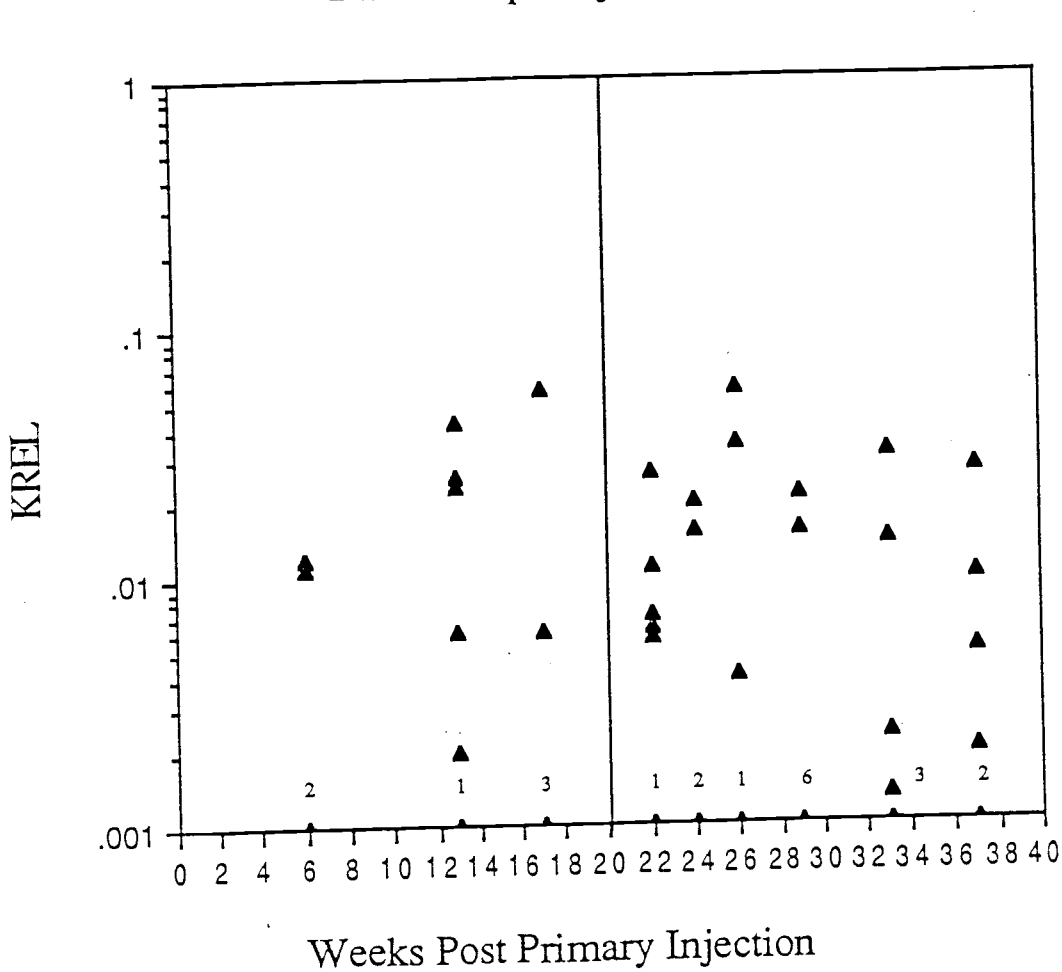

Figure 30. Krel values of the anti-TNP antibody samples determined for the analog, paranitrophenylalanine. For this inhibitor, there were as many samples uninhibited as inhibited. Therefore, the median would be at the 0.001 Krel value. The line drawn vertically represents the point in time when the fish were given a secondary injection of TNP-KLH. Chi-square analysis was performed using the resulting four squares as cells to determine if the Krel values obtained in the primary response were significantly different from those obtained in the secondary response. The numbers immediately above the abscissa respresent the number of samples tested at that time point which were not inhibited.

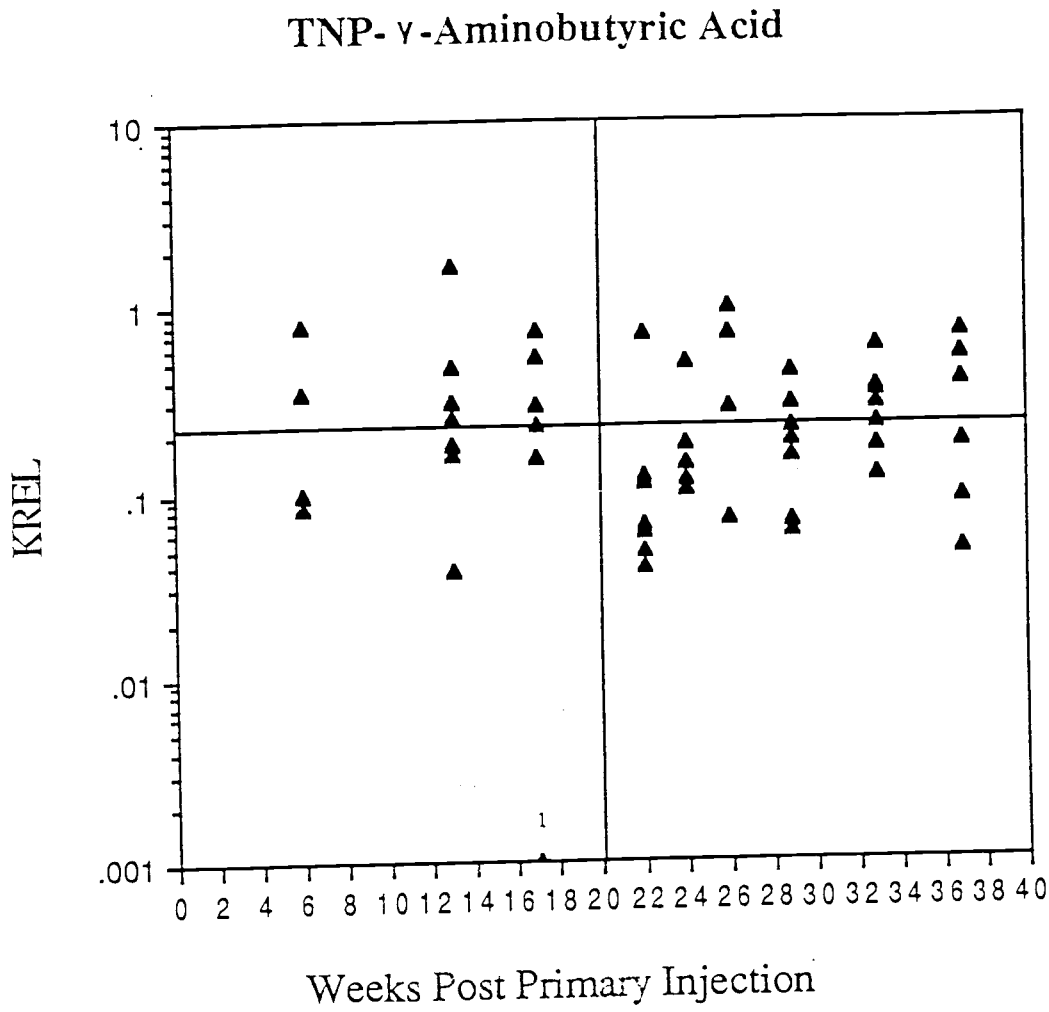

Figure 31. Krel values of the anti-TNP antibody samples determined for the analog, TNP-γ-aminobutyric acid. The line drawn horizontally represents the median of all data points. The line drawn vertically represents the point in time when the fish were given a secondary injection of TNP-KLH. Chi-square analysis was performed using the resulting four squares as cells to determine if the Krel values obtained in the primary response were significantly different from those obtained in the secondary response. The numbers immediately above the abscissa respresent the number of samples tested at that time point which were not inhibited.

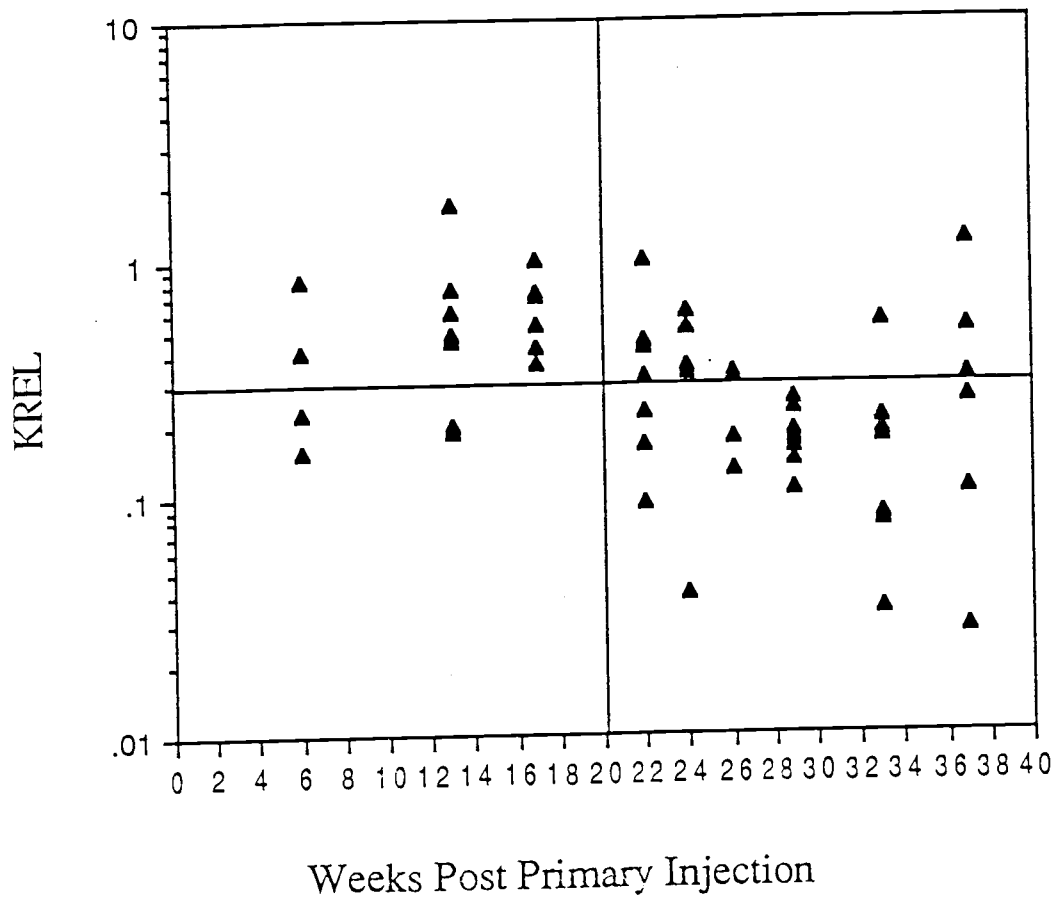

Figure 32. Krel values of the anti-TNP antibody samples determined for the analog, DEAC. The line drawn horizontally represents the median of all data points. The line drawn vertically represents the point in time when the fish were given a secondary injection of TNP-KLH. Chi-square analysis was performed using the resulting four squares as cells to determine if the Krel values obtained in the primary response were significantly different from those obtained in the secondary response.

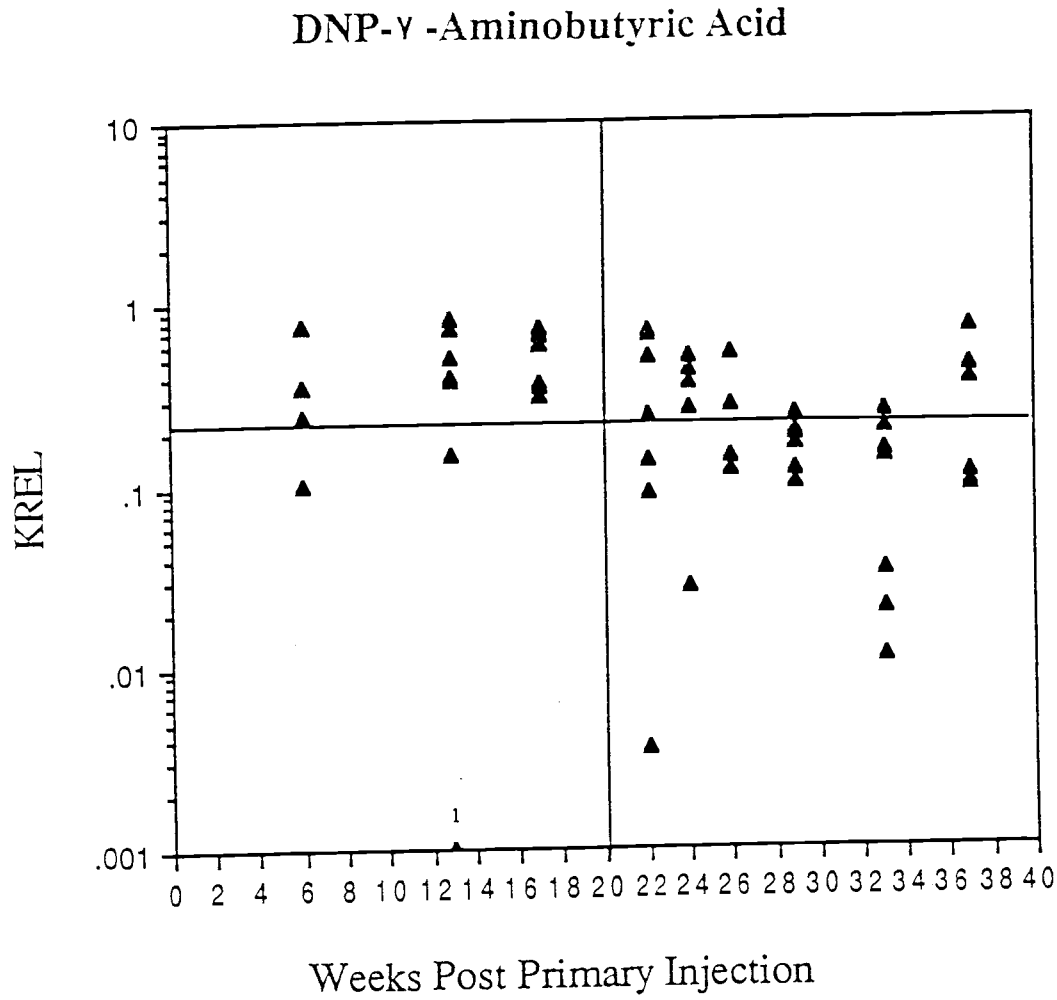

Figure 33 Krel values of the anti-TNP antibody samples determined for the analog, DNP-γ-aminobutyric acid. The line drawn horizontally represents the median of all data points. The line drawn vertically represents the point in time when the fish were given a secondary injection of TNP-KLH. Chi-square analysis was performed using the resulting four squares as cells to determine if the Krel values obtained in the primary response were significantly different from those obtained in the secondary response. The numbers immediately above the abscissa respresent the number of samples tested at that time point which were not inhibited.

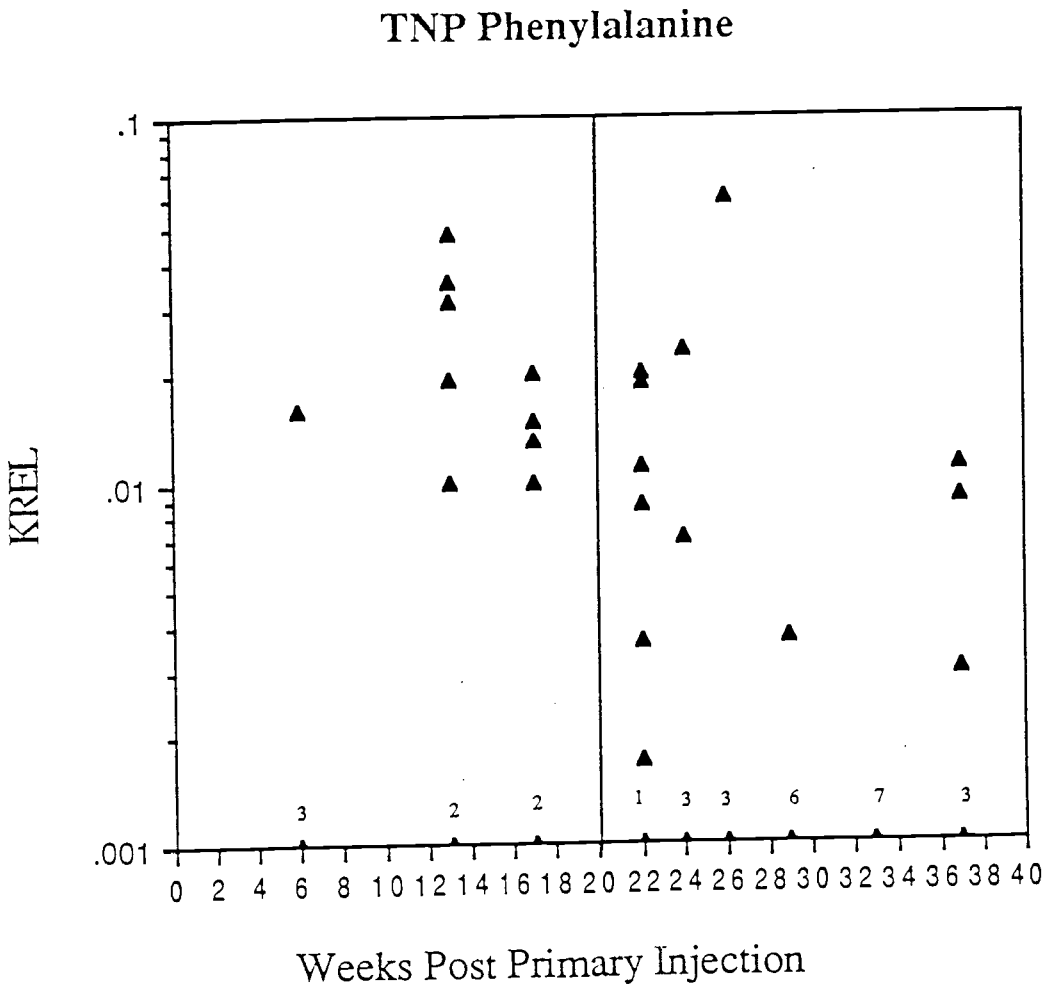

Figure 34. Krel values of the anti-TNP antibody samples determined for the analog, TNP-phenylalanine. For this inhibitor, there were as many samples uninhibited as inhibited. Therefore, the median would be at the 0.001 Krel value. The line drawn vertically represents the point in time when the fish were given a secondary injection of TNP-KLH. Chi-square analysis was performed using the resulting four squares as cells to determine if the Krel values obtained in the primary response were significantly different from those obtained in the secondary response. The numbers immediately above the abscissa respresent the number of samples tested at that time point which were not inhibited.

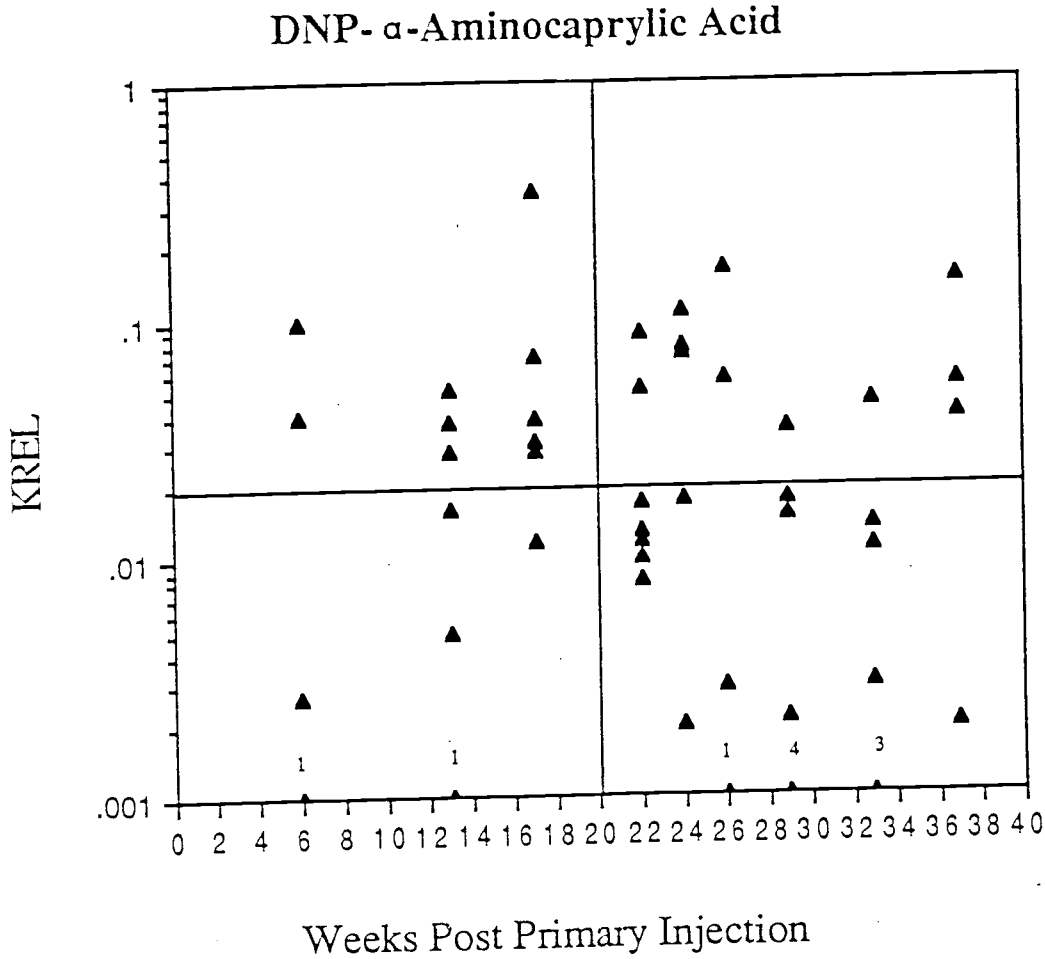

Figure 35. Krel values of the anti-TNP antibody samples determined for the analog, DNP-α-aminocaprylic. The line drawn horizontally represents the median of all data points. The line drawn vertically represents the point in time when the fish were given a secondary injection of TNP-KLH. Chi-square analysis was performed using the resulting four squares as cells to determine if the Krel values obtained in the primary response were significantly different from those obtained in the secondary response. The numbers immediately above the abscissa respresent the number of samples tested at that time point which were not inhibited.

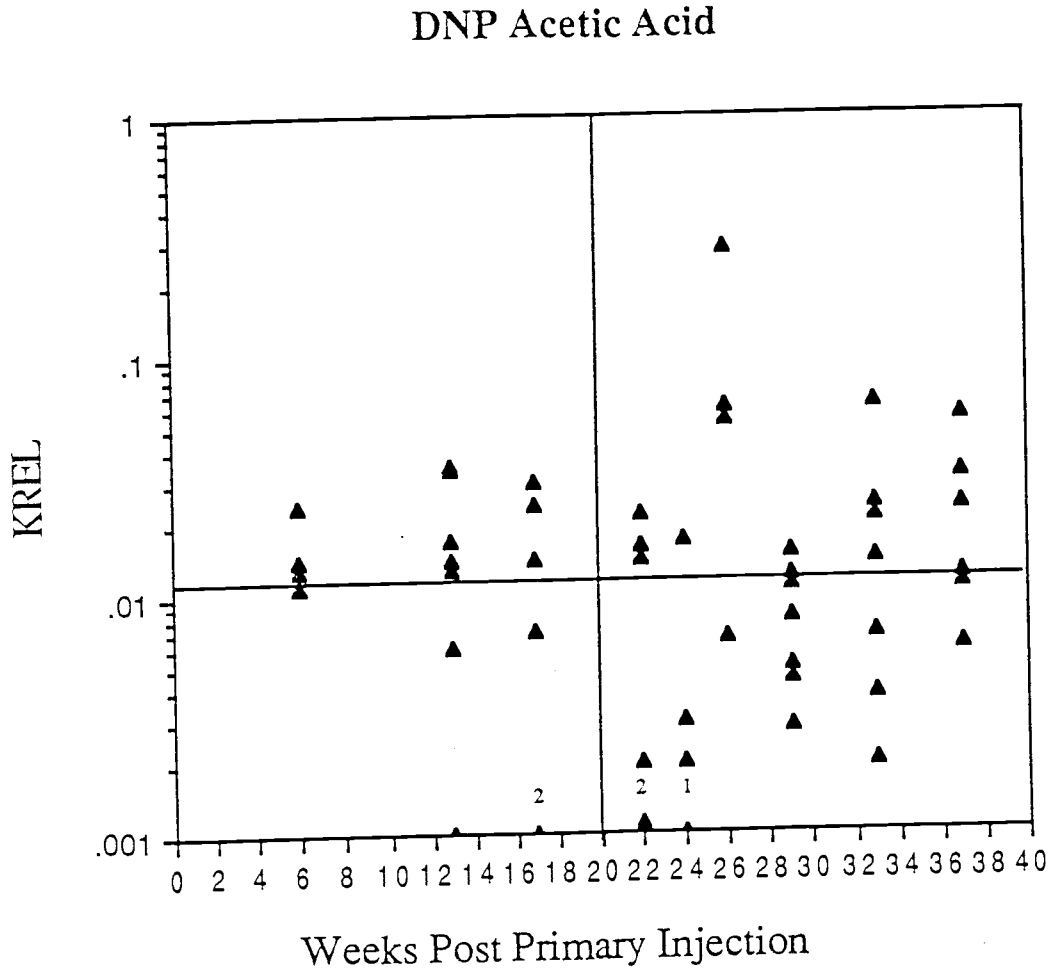

Figure 36. Krel values of the anti-TNP antibody samples determined for the analog, DNP acetic acid. The line drawn horizontally represents the median of all data points. The line drawn vertically represents the point in time when the fish were given a secondary injection of TNP-KLH. Chi-square analysis was performed using the resulting four squares as cells to determine if the Krel values obtained in the primary response were significantly different from those obtained in the secondary response. The numbers immediately above the abscissa respresent the number of samples tested at that time point which were not inhibited.

No significant differences between the initial plasma samples collected and the rest of the time periods were found except for two weeks post secondary ($p<0.05$).

FINE SPECIFICITY ANALYSIS

Determination of the possible occurrence of specificity changes during the generation of a secondary response was determined by the use of a comparative inhibition ELISA using various analogs (Stenzel-Poore et al., 1988) (Figure 13) to the TNP-lysine homolog.

Changes seen during the primary and secondary response in the sera of aflatoxin-exposed and normal animals were analyzed for significance by the chi-square statistic. The median Krel value was determined from the individual Krel values generated for all serum sample each analogue for the generation of the chi-square statistic (Figures 27-36). The analogues which demonstrated a significant difference in the fine specificity of the induced antibody in both aflatoxin and normal fish were, dinitrophenyl-ε-aminocaproic acid (Figure 32) and dinitrophenyl-γ-aminobutyric acid (Figure 33). The statistical analyses of this data are summarized in Table 4. However, changes in the antibody specificity occurring only in aflatoxin-exposed fish were demonstrated by 2 inhibitors, dinitrophenyl lysine (Figure 27) and dinitrophenyl phenylalanine (Figure 28). A change in antibody specificity, occurring only in

Table 4 Summary of Chi-square analysis of the fine specificity data obtained from normal and aflatoxin-exposed fish.

Inhibitors	Median[1]	P values[2] Not exposed	Aflatoxin exposed
DNP-Lysine	.1	N.S.[3]	↓(P<.025)
DNP Phenylalanine	.06	N.S	↓(P<.025)
DNP-OH	.007	↓(P<.05)[4]	N.S.
Paranitrophenylalanine	<.001	N.S.	N.S.
TNP-γ-aminobutyric acid	.2	N.S.	N.S.
DNP-ε-aminocaproic acid (DEAC)	.3	↓(P<.05)	↓(P<.010)
DNP-γ-aminobutyric acid	.2	↓(P<.025)	↓(P<.010)
TNP-phenylalanine	<.001	N.S.	N.S.
DNP-α-aminocaprylic acid	.02	N.S.	N.S.
DNP acetic acid	.01	N.S.	N.S.

1 Median Krel value for all individual samples tested.

2 ↓ indicates a decrease in the Krel values during the secondary response.

3 No significant changes (P>0.05) between primary and secondary responses.

4 P values calculated by use of Chi-square analysis for normal and aflatoxin-exposed fish.

normal fish, was demonstrated by the inhibitor dinitrophenol (Figure 29).

DISCUSSION

Immunological memory in mammals can be functionally defined by the following parameters: 1. The generation of an enhanced antibody titer after a second exposure to antigen (Eisen, 1980); 2. A immunogenic or non-immunogenic concentration of antigen [which is lower than the dose needed to initiate a primary response (Tittle, 1978)] can be used to induce the enhanced secondary or memory response; 3. A shorter lag period [time from antigen exposure to the first appearance of antibodies (Eisen, 1980)] relative to the primary response.; 4. The occurrence of isotype switching events (Baker and Stashak, 1969; Barthold et al., 1974; Braley-Mullen, 1974; Braley-Mullen, 1975). Therefore, to determine if rainbow trout (O. mykiss) are capable of producing an anamnestic or memory response, the effect of a secondary exposure to a non-immunogenic dose of the antigen on the serum antibody titer was first determined. Trout were given a secondary injection (20 µg/fish) of TNP keyhole-limpet hemocyanin (TNP-KLH) twenty weeks after a primary injection of the same antigen (100 µg/fish). Serum or plasma samples were collected at various time points and the units of antibody activity/µl were determined. The in vivo primary anti-TNP antibody response peaked at nine weeks while the secondary antibody peak response occurred six weeks after the secondary injection of antigen (Figure 3). Also, the peak of the secondary response was almost four times greater in magnitude than the peak of the primary response. Control animals

receiving a primary injection at this time of 20 µg/fish, demonstrated that this dose of antigen was non-immunogenic for unprimed fish.

At present, monoclonal antibody reagents (Lobb et al., 1984) and molecular genetic techniques (Ghaffari and Lobb, 1989) are being developed which can eventually be used to determine if isotype switching events occur in fish. Thus, this parameter of memory can not be addressed at this time.

Based on the above findings it can be concluded that, immunological memory in trout parallels, at least in part, the classical definition for mammalian immunological memory. Specifically, there is an increase antibody titer above that observed in a primary response (parameter # 1), a shorter lag period in the secondary response (parameter #2), and a non-immunogenic dose of antigen is capable of stimulating a memory response (parameter # 3).

The increase of the in vivo antibody response after a secondary injection of antigen has been demonstrated in other fish with a variety of antigens (Clem and Sigel, 1965; Avtalion, 1969; Trump and Hildemann, 1970; Ambrosius and Frenzel, 1972; Desvaux and Charlemagne, 1981; Tatner, 1986). Those instances where investigators were unable to demonstrate an increase were those where either a T-independent (T-I) antigen was used to prime the animals, or when a method with much less sensitivity than the ELISA was employed. Tatner (1986) and Dunier (1985) were unable to produce an enhanced secondary response because

they were using a T-I antigen, Aeromonas salmonicida or dinitrophenyl-lipopolysaccharide (DNP-LPS) respectively, for priming. In the mouse complete memory does not develop to T-I antigens (Umetsu et al., 1979). Generally, in mammals, a secondary exposure does not elicit a memory response if the primary exposure was to a T-I antigen (Eisen, 1980). Dunier (1985) was, also, unable to demonstrate a memory response with a T-dependent (T-D) antigen, DNP-KLH. This may have been due to the hemagglutinin technique which she used. This technique is less sensitive then the ELISA for the determination of antibody titer.

A shorter lag period during the secondary response versus the primary response has also been demonstrated to occur in other fish species. The goldfish, for example, exhibits a shorter lag period upon secondary exposure to red blood cells or to bovine serum albumin (BSA) (Trump and Hildemann, 1970; Desvaux and Charlemagne, 1981). A decrease in the lag period has also been demonstrated in the carp, using the antigens dinitrophenyl-bovine gammaglobulin (DNP-BγG) and BSA.

The effect of Freund's complete adjuvant (FCA) on the generation of the trout antibody responses was deemed essential due to concerns over natural antibodies which have been reported in teleosts (Gonzalez et al., 1988). These antibodies have been postulated to increase spontaneously upon exposure to non-crossreactive antigens (P. De Kinkelin, Laboratoire b'Ichthyopathologie, Thieerval-Grignom, France, personal communication). Thus, it was important to determine if the

increase in serum titer to TNP (Figure 3) could be due to the FCA alone. The possible induction of these antibodies was assessed by comparing titers from trout injected with PBS emulsified in FCA or with TNP-KLH emulsified in FCA. All of the trout received a secondary injection of TNP-KLH in Freund's incomplete adjuvant (oil without Mycobacterium) twenty weeks later. Three weeks later, the serum was collected and the units of antibody activity/µl was determined. Trout receiving only FCA for a primary injection, were unable to produce an enhanced secondary in vivo response to TNP. Therefore, it is felt that the response generated to TNP is specific for the immunizing antigen and not due to the adjuvant.

The concept of immunological memory has grown more sophisticated with advances in cellular techniques. With the advent of culture techniques such as the in vitro immunization of leukocytes (Mishell and Dutton, 1967), and the enumeration of specific antibody or "plaque" forming cells (PFC) (Jerne, 1963) it became possible to study memory development at the cellular level. Using such systems, Tittle and Rittenberg (1978) were able to elicit an in vitro memory responses from murine lymphocytes. Mice previously injected with the T-D antigen, TNP-KLH, were shown to produce an enhanced IgG secondary or memory response upon reexposure to the same antigen in vitro. However, Tittle and Rittenberg (1978) were also able to elicit an enhanced secondary response with these cells using a T-I antigen, [E. coli phage (T4) conjugated to TNP (TNP-T4)]. They further determined that this phenomenon was due to the existence of two populations of

memory cells: B1γ memory cells, which respond to a T-I (type 2), and B2γ memory cells, which respond to a T-D antigen (Tittle and Rittenberg 1978). T-I antigens are characteristically large molecules with repeating antigenic determinants (i.e. polysaccharides) (Feldmann and Basten, 1977). In mammals T-I antigens have been divided further into type 1 and type 2 antigens. Type 1 (lipopolysaccharides) antigens trigger an immature population of B cells whereas type 2 (polysaccharides, such as ficoll and dextran) antigens activate a more mature population of B cells (Mosier et al., 1978). Tittle and Rittenberg (1980) also demonstrated that type 1 T-I antigens (TNP-LPS and TNP-BA) are able to stimulate both B1γ and B2γ subpopulations. Therefore, their studies revealed that either T-D or T-I forms of antigen can elicit a memory response from lymphocytes primed in vivo with the T-D form of antigen (Figure 37).

This phenomenon has now been documented in trout, however, it is of particular interest that there is a dichotomy in the responsiveness of primed trout lymphocytes to the T-I and T-D forms of antigen. The in vivo secondary exposure produces a significant increase in the anti-TNP titer (Figure 3), however, a secondary in vitro exposure to TNP-KLH does not induce an enhanced secondary anti-TNP response (Figure 5) while exposure to TNP-LPS did produced a substantial response (Figure 5). An in vitro memory response, however, can be elicited to TNP-KLH if the trout are given two in vivo exposures to this antigen (Figure 6).

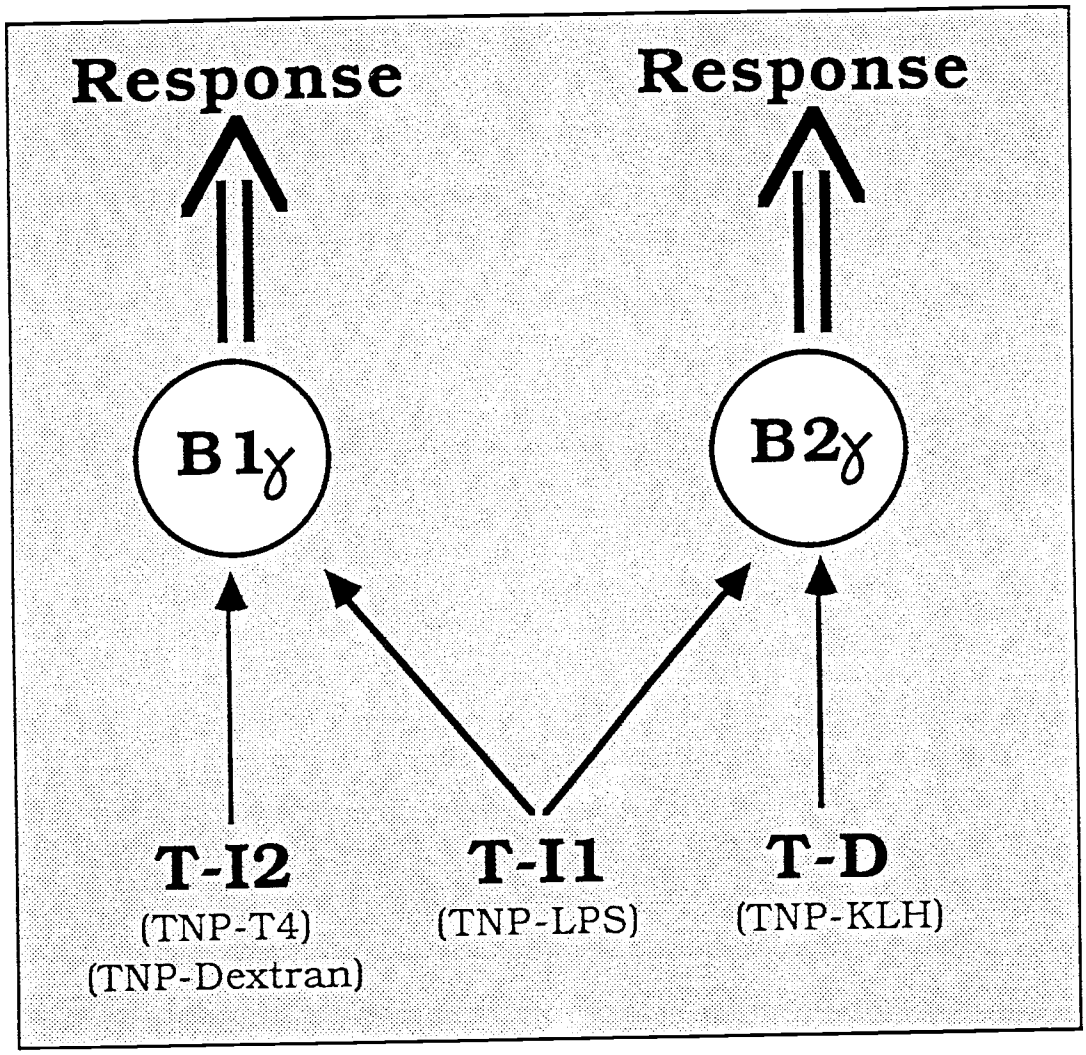

Figure 37. Activation of memory B cell subpopulations in mice by different molecular forms of TNP.

This dichotomy indicates that a secondary in vitro anti-TNP response can not be induced by TNP-KLH, although these same spleen cells are capable of producing an anti-TNP response if given a different form of antigen.

This in vitro system has revealed a dichotomy which, when resolved, should lead to a greater understanding of B cell function in the trout. Specifically, three basic hypotheses can be postulated which would explain this dichotomous behavior: 1. An originally, non-uniform distribution of B cell subsets or other required cells (i.e. T-Helper cells), which become more uniform in the spleen by trafficking. 2.) A difference in the antigen-induced triggering requirements of a single, representative B cell subpopulation. 3. The existence of two functionally distinct B cell subpopulations, with differing capabilities, or sensitivities to regulatory phenomena.

If the difference observed in the secondary in vivo and in vitro antibody response is due to dispersal of cellular populations via cellular trafficking in vivo, it would appear that one subpopulation of cells responsive to TNP-LPS resides in the spleen prior to injection of antigen or homes to the spleen after the primary injection of antigen. The TNP-KLH responsive cells reside elsewhere until the secondary in vivo exposure to antigen. At this point, the T-D responding cells would migrate to the spleen. This would suggest the existence of distinct subpopulations which respond differently to the two forms of antigen. A non-B, but possibly requisite cell for the response to the T-D antigen (i.e. T-

cell), could also be distributed and dispersed in a similar manner as described above.

Alternatively, if there are no differences in the organ distribution of leukocytes required for a T-D response, it may be that in vitro culturing more readily distinguishes between different triggering requirements of a single population (Figure 38) of B cells. The simplest model would be one where only a single population of responding B cells exists which necessarily must be more responsive to the T-I form of antigen than the T-D form in vitro. A single injection of TNP-KLH would, therefore, generate these cells which would be highly responsive to the T-I form of the antigen in vitro, and only marginally responsive to the T-D form in vitro. However, after two injections with a T-D antigen, enough stimulus has been provided to expand this population of cells or enhance their sensitivity to T-D antigens which would then enable them to produce a detectable response to the T-D antigen in vitro (Figure 38).

A third possibility may be that two B cell subpopulations exists in the spleen, in which the priming injection stimulates the clonal development of T-D and T-I responsive B cell subpopulations. The B cell which will ultimately be stimulated in vitro with TNP-LPS may have a much higher precursor frequency than the B cell subpopulation which would be stimulated in vitro with TNP-KLH (Figure 39). The existence of two or more functionally distinct subpopulations, (as suggested by this or by

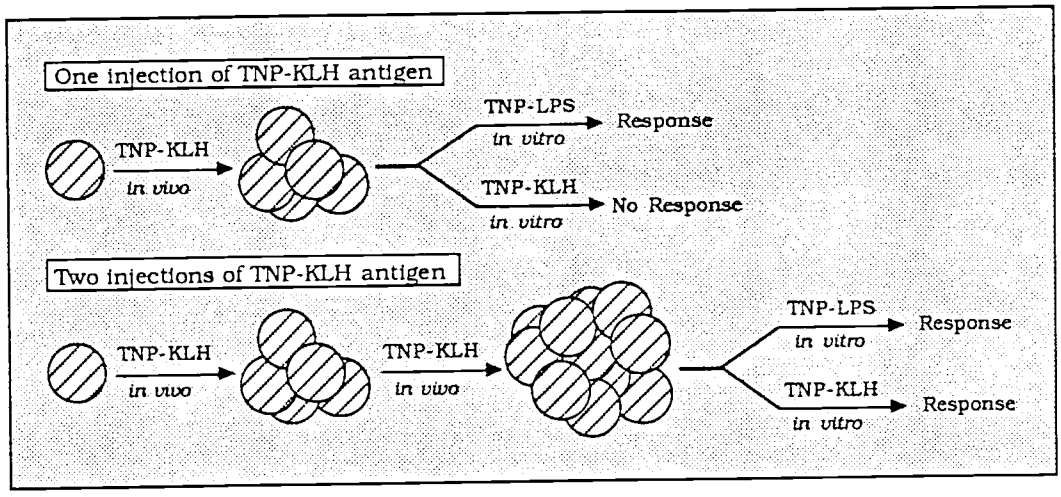

Figure 38. A postulated model for the differential triggering requirements of a memory B cell (⊘) population to TNP-KLH *in vitro* or *in vivo*.

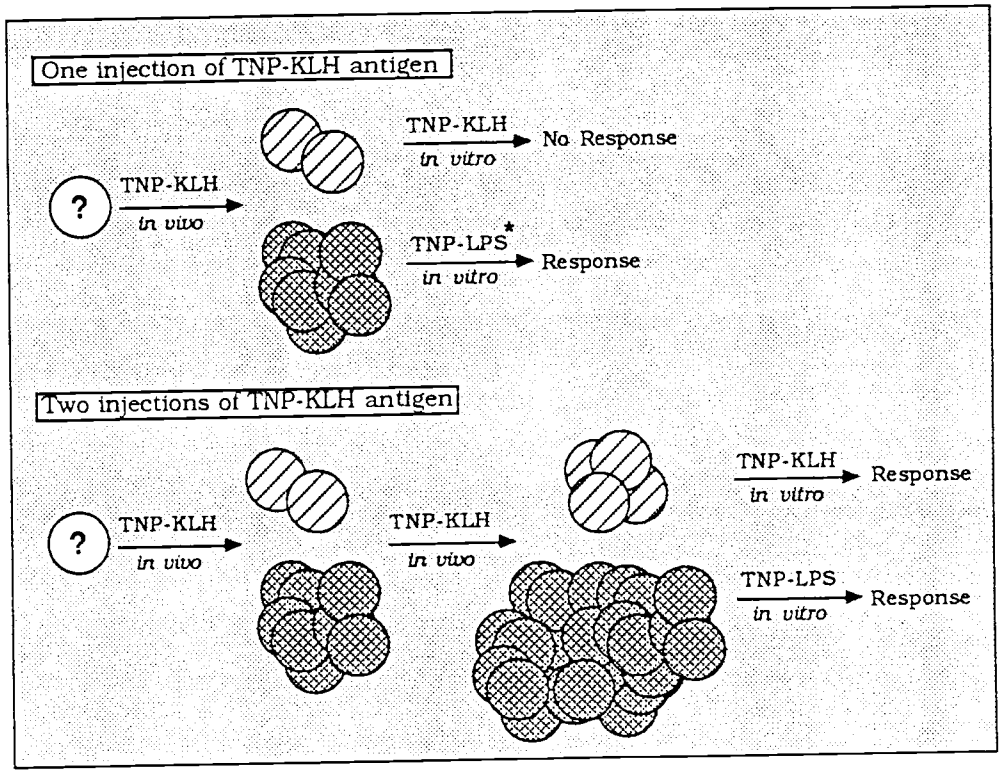

Figure 39. Memory B cell subpopulations (⊘, ⊗) to TNP-KLH or TNP-LPS with different precursor frequencies. The possibility that these memory cells arose from a single or distinct lineage(s) of primary B cells is indicated by the question mark.

* Although not represented in the schematic, it may be possible that TNP-LPS, a T-I1 antigen, stimulates both subpopulations of trout memory cells as is the case in mammals (Tittle, 1978).

differential trafficking of subpopulations) which are responsive to different forms of antigen can only be addressed by the use of antigen addition experiments (Tittle and Rittenberg, 1978) or selective suicide experiments where antigen-sensitive populations are selectively eliminated by antiproliferative drugs (Tittle and Rittenberg, 1978).

The enhanced secondary response (anamnestic) to the T-I antigen* must either involve an increase in the number of B cell precursors and/or an increase in clonal proliferation. Both of these changes have been found to occur in the rat (Brooks and Feldbush, 1981). If the enhanced secondary response is achieved by a simple increase in the number of primary B cells this would suggest that the memory B cells are not functionally different from the primary cell. However, if the clone size increases due to priming, this would suggest that the memory B cell is a functionally distinct cell because, upon antigenic stimulation, it was capable of proliferating to a greater extent than the primary cell. This question was resolved by the use of a LDA.

In order to obtain sufficient cells for the standard LDA, a minimum of three fish were initially required. The precursor frequency obtained from unprimed animals to TNP-LPS in this type of assay was 1 precursor cell per 22,500 cells or 4.44×10^{-5} (Figure 8A). However, the precursor frequency obtained from the

*Further in vitro studies focused solely on the TNP-LPS response because of its relative ease of elicitation and due to the fact that further studies on T-D responses would require the development of T cell separation and purification techniques. These techniques are not currently available for rainbow trout.

primed animal was 1 precursor frequency per 2,500 cells or 4.35×10^{-4} (Figure 8B). This represents a ten-fold increase in the precursor frequency over that seen in unprimed fish. The mean clone size obtained from unprimed and primed fish were approximately the same, 2.40+/-0.28 and 3.78 +/-0.35 respectively. In order to perform this analysis on individual fish it was necessary to utilize a technique originally developed for mice (Lefkovits, 1972). This technique involved the culturing of cells from a single animal in microculture or Terasaki plates. Cells cultured from the same animal in Terasaki plates with TNP-LPS show a distinctly different form of kinetics than did the same cells cultured in conventional 96 well, flat bottom plates (Figure 9A,B) with the optimal response occurring on day 7 rather than on day 9. LDA performed in Terasaki plates were, thus, harvested on day seven of culture. The optimal TNP-LPS concentration was the same for both plate types (data not shown).

The advantages of using Terasaki plates over conventional plates are numerous. Cells from various outbred individuals no longer must be pooled to secure sufficient cells to complete a single assay. This becomes especially important when the number of animals is very limited or the organ under study consists of limited numbers of cells. This procedure also decreases the volume of medium and antigen required for the generation of an in vitro response. Assays performed in microculture plates are, thus, performed for one tenth the cost of those performed in conventional 96 well plates.

The results obtained by the use of conventional plates were reconfirmed by the use of Terasaki plates. Lymphocytes from primed fish possess larger precursor frequencies to TNP-LPS than lymphocytes from unprimed fish. Precursor frequencies obtained with the microculture system using a primed cell source ranged from 1 B cell precursor per 989.6 leukocytes to 1 B cell precursor per 11,667 leukocytes. While the precursor frequency to TNP-LPS in unprimed animals ranged from 1 B cell precursor per 21,250 leukocytes to 1 B cell precursor per 117,500 leukocytes. Again, the clone sizes in primed (2.66+/-0.62) and unprimed (2.74+/-0.66) spleen cells in trout were the same. The limiting dilution data indicate that memory development may be due simply to an increase in B cell precursors capable of responding to TNP. The absence of any change in clone size also suggests that this memory population is not functionally different from the primary population with respect to its proliferative potential.

These LDA studies also suggest that species-specific or organ dependent differences and similarities may exist among salmonids. Although Tripp (1988), did not examine the secondary response in coho salmon, he did determine that the primary precursor frequency and mean clone size of anterior kidney B cells responding to TNP. Using conventional 96 well plates, he found that the anterior kidney cells from unprimed coho salmon have a precursor frequency to TNP-LPS of 1 B cell precursor per 41,250 leukocytes. This is within the range determined for spleen cells in the trout. However, he also found that the mean clone size in the

anterior kidney of the coho was 17. This is almost eight fold larger than the clone size observed in the spleen of trout. Whether this is due to species-dependent differences or organ-dependent differences is not, at this time, known. Unfortunately, anterior kidney cells from the trout were not amenable to use in these studies due to technical difficulties related to the use of these cells in the plaque assay (including sporadic episodes of cell clumping, and non-PFC related red blood cell lawn destruction).

In mammals, there is a paucity of LDA literature describing the generation of immunological memory. This lack of information impairs any critical comparative analysis of the generation of memory precursor frequencies and clone sizes between fish and mammals. One study, demonstrated that rat popliteal lymph node cells have an increased precursor frequency and clone size in primed cells compared to unprimed cells when responding to the T-D antigen, DNP-ovalbumin (Brooks and Feldbush, 1981). However, there is substantial literature on the precursor frequencies and clone sizes generated during a primary response in mice. Kettman et al. (1985) determined that the precursor frequency in the spleen cells of mice (BDF_1) to a T-I antigen TNP-BA was 1 B cell precursor per 5,000 leukocytes. This is higher than the range obtained for the primary B cell precursor in trout. Kettman et al. (1985) also determined the precursor frequency for the T-D antigen, SRBC, in the spleen of mice to be 1 precursor per 10^4 leukocytes. Another investigator, Lefkovits (1972), also determined the precursor frequency to SRBC in the spleens of mice

from a different strain (NMRI/HAN) to have a range between 1.5-10.5 precursor B cells per 10^6 leukocytes.

Affinity maturation (Eisen and Siskind, 1964) of the antibody response is another important aspect of not only in the development of memory response, but also of the qualitative changes in the serum antibody generated during a primary response (Berek et al., 1985 and Griffiths et al., 1984). Specifically, the theory of affinity maturation describes a mechanism by which an increase in the affinity of antibody can occur over time. This increase in affinity can occur during the late primary, secondary or tertiary responses in mammals (Eisen and Siskind, 1964; Berek et al., 1985; Kimball, 1972). However, affinity maturation does not appear to occur during a primary or secondary response in rainbow trout (Figure 12). Although this study represents, necessarily a populational study, the results are statistically significant. It would, however, be of interest to monitor the response of single fish over time to confirm these findings. O'Leary (1981) also studied the affinity of anti-TNP antibodies generated in rainbow trout over time and was unable to demonstrate a change in affinity. However, he looked only at serum antibodies generated at time points subsequent to hyperimmunization (multiple injections). This examination of later time points may not be sufficient for such an analysis, since large changes in affinity may primarily occur early in the response. Other fish species such as the channel catfish (Lobb, 1985), horned shark (Litman et al., 1982; Mäkelä and Litman, 1980) gray snapper (Clem and Sigel, 1970),

and the giant grouper (Clem and Small, 1970) all fail to show an increase in affinity of their antibodies over time to a variety of antigens.

This lack of affinity maturation, the presence of which has become a hallmark of mammalian antibody responses, indicates either major differences in the salmonid antibody repertoire or in the regulation of antibody expression. A limited or relatively restricted repertoire of antibody could account for the lack of affinity maturation, because there would essentially be little antigen-driven selection of high affinity clones. This lack of clonotypes has been postulated to be true for rainbow trout (Coissarini-Dunier et al., 1986) due to the lack of spectrotype diversity as found by isoelectric focusing. Thus, if few clonotypes exist and these clonotypes display the same relative affinity there should be little competition for antigen between the different clones and, thus, there would not be an eventual dominance of any selected clones.

Another possibility that exists which would explain the lack of affinity maturation would be the lack of somatic mutation. Somatic mutation in mammals is credited for the generation of high affinity clones (Kochs and Rajewsky, 1989) If there is a lack of mutation, the variable region of the immunoglobulin genes would not evolve new species which may then be of higher affinity. The genetic organization of one fish species, the shark, has been found to possess a distinctly different organization of its heavy chain genes than does the mouse (Figure 2). Thus, if the possibility of

the occurrence of somatic mutation is related to the organization of this gene complex, a dramatic difference in organization may have a significant effect upon the generation of somatic mutation, and thus the ability to generate a broad repertoire from which high affinity antibody producing cells may be selected over time.

A third possibility may be that trout actually possess a comparable number of clonotypes to that found in mammals, however, the high affinity clonotypes are more heavily regulated or suppressed by T suppressor cells. Takemori and Tada (1974) have demonstrated in the mouse that KLH primed murine thymocytes and spleen cells can produce a significant decrease in the average avidity of anti-DNP antibodies produced during a primary and adoptive secondary response. The avidity distribution of the antibodies indicated that the high avidity antibody or plaque forming cells were suppressed more than the low affinity population. They suggest that clones with high affinity for antigen are affected by T suppressor cells which bind the carrier portion of the antigen.

The fine specificity analysis of an antibody population allows one to examine the general features of the architecture of the antigen-binding site and also to determine if these features change during an immune response. A change in fine specificity of an antibody population indicates that either different clones are being preferentially stimulated or that new specifities are being generated and, further, are coming into prominence.

The fine specificity of an antibody can be determined by its relative affinity (Krel) to various analogues of the immunizing hapten, in this case TNP. A Krel value is, therefore, determined by comparing the average affinity of an antibody population to the homologous hapten (TNP-lysine) to the antibody's affinity for a structurally different or heterologous hapten (Pressman, 1968). If a population of antibody molecules binds the homologous hapten with a higher affinity than it binds to a heterologous hapten, it is concluded that the antibody population is more specific for the homologous hapten than for the heterologous hapten by a factor of

$$\frac{\text{Ka (homologous hapten)}}{\text{Ka (heterologous hapten)}}$$

Ka represents the association constant of the antibody population for the hapten. Heterologues are used which possess various differences in their substituent groups. Examination of the Krel values for a particular antibody population can, thus, reveal which antigenic structures are essential for antibody binding.

TNP-lysine was used as the homologous hapten in these studies because conjugation of TNP to protein involves a reaction which preferentially haptenates lysyl side chains via their epsilon amino groups. Thus TNP-lysine is identical to the conjugated regions of the immunizing antigen, TNP-KLH. TNP-KLH was not used as the coating antigen in the ELISA because antibodies directed towards the KLH carrier would bind, making it impossible to distinguish the interactions of the antibodies with the hapten.

An extensive library of heterologues were employed since no a priori information exists which would allow for predictions of the antigenic structural requirements of trout antibodies.

Different binding sites are present within a population of serum antibodies. Thus, a Krel value actually represents a distribution of binding constants (Ka) which describes a general binding characteristic of a population of antibodies to a hapten (Pressman and Grossberg, 1968).

If Krel values change between the primary and secondary responses, an overall structural of fine specificity change in the antibody populations would be indicated. The chi-square statistic was used to determine a significant shifts ($P<0.05$) in the magnitude of the Krels occurred during these responses. Three inhibitors demonstrated such a downward shift in relative affinities between the primary and secondary responses. These inhibitors were DNP-OH, DEAC and DNP-γ-aminobutyric acid. Only these three out of the ten inhibitors were able to demonstrate such a change, thus the use of a relatively large battery of inhibitors for such analyses was, retrospectively, deemed essential.

This observed change in the binding site specificities of antibodies from trout signify that a large proportion of the antibodies are changing between the primary and secondary response. These subtle changes in the antibody binding sites would normally be expected if affinity maturation was occurring it is, therefore, particularly intriguing since affinity maturation to TNP does not occur in this system (Figure 12). At least three

hypotheses can be postulated as to how these different specificities may come into existence without the simultaneous expression of affinity maturation. It may be possible that clones of a particular specificity undergo proliferation and differentiation at relatively different rates upon a secondary stimulation than do other TNP-specific B cell clones. In this fashion the relative proportion of the responding B cell clones would be changed in the secondary response versus the primary response. This could lead to the changes in fine specificity exhibited during the second antibody response relative to the primary. Another hypothesis is that new clonotypes arise during the secondary response which possess novel specificities. This mechanism could only be possible if somatic mutations are occurring which change the fine specificity but not the average affinity. Since it has been demonstrated that the precursor frequency increases approximately ten-fold during the generation of a memory response (Figures 8,11), if selected clones contribute preferentially to this precursor increase then either two of these hypothesis are possible. The third hypothesis incorporates the idea that particular clones may become more restricted with respect to their ability to expand as the animal ages. In mice, Ly-1 B cells give rise to immunoglobulin-negative progenitors only during fetal and neonatal life. After that time, they give rise only to immunoglobulin-positive progenitors, unlike the conventional B cells which are replenished with immunoglobulin-negative progenitors throughout the life of the individual. After 6-8 weeks of age there are no new Ly-1 B cell

specificities produced (Herzenberg et al., 1986). It has been further shown, that the Ly-1 B cell clones becomes increasingly restricted with age (Tarlinton et al., 1988). Such a restriction may account for the change in specificity as seen through time in trout. The change in specificity may not be due to the generation of memory but simply due to an age-related change in clonality of a population of cells through time.

Another use of fine specificity, analysis not related to characterization of immunological memory is the determination of the structural requirements for TNP specific binding of trout antibodies. This topic is covered in appendix 2.

The hepatocarcinogen aflatoxin B_1, an environmental mycotoxin, is known to affect a variety of immunological parameters in a number of different species (Panangala et al., 1986; Pier et al., 1977; Harvey et al., 1988; Mohapatra and Roberts, 1985; Yang et al., 1985). However, this is the first study which examined the effects of a short term embryonic exposure to this carcinogen on the primary and secondary antibody response of trout. In parallel with the quantitative ELISA study which determined the existence of a memory antibody response to TNP in the serum or plasma of normal fish (Figure 3), a population of similarly immunized aflatoxin-exposed fish were also examined. The primary in vivo antibody response of aflatoxin-exposed and normal trout were equivalent both with respect to the amount of antibody induced to TNP, and the kinetics of the response (Figure 22). However, upon receiving a secondary injection of antigen, a

dramatic difference between the aflatoxin-exposed and normal trout was observed. Exposed trout were only able to produce approximately half the antibody titer that was produced in normal fish at the peak of their respective responses. The peak of the secondary response in normal trout occurred at six weeks while the antibody peak in aflatoxin-exposed trout occurred between 9-13 weeks. This is reminiscent of the peak of the primary response which occurred 9 weeks after injection.

This impaired secondary response in aflatoxin-exposed animals was also observed in vitro (Figure 25 and 26). While spleen cells from TNP-KLH primed (injected twice), non-exposed trout produced an enhanced in vitro secondary response to both TNP-LPS and TNP-KLH, over that seen with cells from unprimed fish; cells from primed, aflatoxin-treated trout could not produce the enhanced secondary response with either form of the antigen. Both the in vivo and in vitro studies demonstrate that exposure of trout embryos to aflatoxin B_1 markedly suppresses the generation of a secondary antibody response, while leaving the primary response virtually intact. These initial studies suggest that the primary immunological effect of aflatoxin exposure is not the impairment of B cell function per se, but rather an interference with the process of memory B cell development. These findings are of particular interest since previous studies in the mouse have demonstrated that: 1. Memory B cell development is a T cell-dependent process (Lafreuz and Feldbush, 1981) and 2. Studies indicate that aflatoxin exposure acts to selectively suppress cell-

mediated or T cell related functions (Richard et al., 1983; Giambron et al., 1985; Richard et al., 1983). Thus, aflatoxin may be preventing the induction of B cell memory via suppressive or lymphotoxic effects on trout T cells. However, there exists the possibility that there may have been an irreversible defect induced in stem cells which develop into B cells. This defect may not allow for significant clonal expansion upon a secondary antigenic exposure. It is also possible that such a defect, if genotoxic, would allow for the development of memory B cells with different antibody fine specificities than those produced in a normal population of fish.

The above model, however, requires an irreversible, long term alteration of T or B cell function to occur following only a short embryonic exposure. An alternative explanation for this suppression of memory may be related to the production of liver tumors by aflatoxin exposure. Although the majority of fish in this study did not demonstrate any readily observable tumors at the time of these assays, it may be possible that minute tumors or preneoplastic foci may be capable of producing a general anergic state in these fish. Tumors, including hepatocellular carcinomas, have been reported to produce general states of immunosuppression as well as specific suppression of T cell functions (Rowland et al., 1973). Also, hepatocellular carcinomas also are known to produce large quantities of alpha-fetoprotein (McIntire et al., 1972). This molecule is known to bind specifically to T cells (Dattwyler et al., 1975), and exhibits a preferential

inhibition of T cell function (Murgita and Tomasi, 1975). Although production of alpha-fetoprotein by trout with aflatoxin-induced hepatocellular carcinomas has not been demonstrated, trout do possess receptors for alpha-fetoprotein (Dannevig and Berg, 1985) and, thus, may also be capable of producing this substance.

Examination of the effect of aflatoxin on the avidity of anti-TNP antibodies produced during a primary and secondary response to TNP revealed, as in the normal animals (Figure 12), no differences (Figure 24). Samples collected at one time point (two weeks post secondary) may have shown an increase in avidity. However, this would seem not to be indicative of affinity maturation since the effect did not persist. It is apparent from this study that aflatoxin exposure does not significantly affect the avidity of antibodies through time.

Fine specificity analysis of anti-TNP antibodies from aflatoxin exposed animals were ran in parallel with samples from normal fish. Two out of the three analogues to TNP, which demonstrated a difference in the response of normal fish, also demonstrated a significant ($P<0.05$) difference in the fine specificity of the secondary response relative to the primary response in aflatoxin exposed fish (Table 4). These analogues were DEAC and DNP-γ-aminobutyric acid. Two additional analogues which demonstrated a significant change in fine specificity in the secondary response relative to the primary response were DNP-lysine and DNP-phenylalanine. An analogue which did not demonstrate a significant difference in the fine specificity due to aflatoxin was

DNP-OH. This data, taken together, may suggest that different sorts of specificities are becoming dominant due to aflatoxin exposure.

A first step in the elucidation of the effects of aflatoxin on the production of altered fine specificity profiles of the antibodies would be to determine the precursor frequencies and clone sizes generated from the spleen cells of primed and unprimed aflatoxin-exposed animals. It would stand to reason, from the in vivo and in vitro studies presented, that the precursor frequency during the secondary response would not change relative to the primary response. In other words one might not expect the tremendous increase in the TNP-specific precursor pool as seen during a memory response generated in the spleen of normal trout. This would then suggest the possibility that some of the clones selected in normal animals may not be expanded, due to aflatoxin-exposure. This elimination of clones may be either be a functional or physical deletion of various clonotypes. It is possible that the elimination of these clones could occur at the cellular level, or due to possible genotoxic events which may not allow for the development of certain clonotypes in aflatoxin altered stem cells during lymphoid development. Chromosomal effects or damage (Al-Sabti, 1985), due to, for example DNA adduct formation (Bailey et al., 1982) occurring in the stem cells of what is considered to be the lymphopoietic center in salmonids (Irwin, 1987: Zapata, 1979) may irreversibly alter the repertoire of antibody genes. Thus, this may give rise to a functional deletion of specific clonotypes. In either

case, any such deletion would account for the different fine specificities generated during the secondary response.

Although not examined for in this study, differences in fine specificity may be due to different specificity preferences in aflatoxin-exposed fish in the primary response relative to the primary response in the normal individuals. This would indicate that aflatoxin's effect is not strictly selective for the memory response.

SUMMARY AND CONCLUSIONS

The Shasta strain of Rainbow trout (O. mykiss) were capable of generating an immunological memory response. Memory generated in trout parallels that observed in mammals to some extent, however, trout do display certain unique features. The in vivo antibody response produced to a T-dependent antigen in trout was similar to what is observed for mammals. Trout were capable of producing an increase in antibody titer after a second exposure to a non-immunogenic concentration of antigen, and a shorter lag period in the generation of this secondary response. Trout were also able to produce an in vitro memory response with T-dependent and T-independent antigens after in vivo priming with the T-dependent form. Limiting dilution analysis revealed that spleen cells from primed animals have an increased B cell precursor frequency relative to the spleen cells from unprimed animals. In contrast to mammals, the average B cell clone size from primed and unprimed animals remain the same and affinity (avidity) maturation of the antibody response did not occur during any stage of the response. However, antibodies generated during the secondary response do change functionally in their specificities as determined by the relative affinities of the antibodies to three analogues of TNP-lysine. Fine specificity analysis also allowed for the determination of a hierarchy of analogue binding, which in turn revealed the primary structural requisites for binding to TNP.

Aflatoxin B_1, an environmental toxicant, affects the ability of trout to produce a memory response. Neither in vivo nor in vitro primary responses were affected by the toxicant, but the ability to produce a memory response was eliminated. Aflatoxin did not change the avidity of the population of antibodies but it did change the fine specificity of the secondary response relative to the primary response. This suggests that different antibody specificities were becoming dominant due to aflatoxin-exposure.

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APPENDICES

APPENDIX 1

BUFFERS

I. Cacodylate buffer (0.28 M)

1. 44.8 gm Cacodylate acid (Sigma, St. Louis, MO)

2. 9.0 pellets NaOH (sodium hydroxide)

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

II. Phosphate buffer saline (PBS) (0.70 M)

1. 1.0 gm KH_2PO_4 (potassium phosphate-monobasic) (Sigma, St. Louis, MO)

2. 9.25 gm Na_2HPO_4 (sodium phosphate-dibasic) (Sigma, St. Louis, MO)

3. 8.5 gm NaCl (sodium chloride) (Sigma, St. Louis, MO)

Dissolve in one liter of distilled water. Mix at room temperature. Reduce the pH with 1.0 N HCl to a pH 7.4.

III. Borate buffer

1. 6.18 gm Boric acid (Sigma, St. Louis, MO)
2. 9.54 gm $Na_2B_4O_7$ $10{\cdot}H_2O$ (sodium tetraborate)

Dissolve in one liter of distilled water. Do not adjust pH. Prepared as described by Garvey et al. (1977)

IV. Borate Buffer Saline

1. 6.18 gm Boric acid (Sigma, St. Louis, MO)
2. 9.54 gm $Na_2B_4O_7$ $10{\cdot}H_2O$ (sodium tetraborate)
3. 8.5 gm NaCl (sodium chloride) (Sigma, St. Louis, MO)

Dissolve in one liter of distilled water. Do not adjust pH. Prepared as described by Garvey et al. (1977).

V. Coating buffer

1. 1.53 gm Na_2CO_3 (sodium carbonate) (Sigma, St. Louis, MO)
2. 2.93 gm $NaHCO_3$ (sodium bicarbonate) (Sigma, St. Louis, MO)
3. 0.20 gm NaN_3 (sodium azide) (Sigma, St. Louis, MO)

Dissolve in one liter of distilled water. Adjust pH to 9.6 and store at 4° no longer than two weeks.

VI. Tris-buffered saline (TBS)

1. 6.07 gm Trizma base (Sigma, St. Louis, MO)
2. 0.37 gm EDTA (ethylenediaminetetracetic acid) (Sigma, St. Louis, MO)

3. 8.7 gm NaCl (sodium chloride) (Sigma, St. Louis, MO)

Dissolve in one liter of distilled water. Adjust pH to 8.0.

VII. 1% Tween-Tris buffered saline (TTBS)

Add 10 ml Polyoxyethylenesorbitan monolaurate (Tween-20) (Sigma, St. Louis, MO) to 1 liter TBS (VI).

VIII. RPMI-1640

1. 1 packet of RPMI-1640 with L-glutamine, without sodium bicarbonate (Gibco, Grand Island, NY)
2. 2.0 gm $NaHCO_3$ (sodium bicardonate) (Sigma, St. Louis, MO)

Dissolve in one liter of nanopure water. Adjust pH to 7.2 and filter sterilize. Store at 4°C.

IX. Modified barbital buffer (MBB), 5X

1. 1 vial barbital buffer (0.05 moles sodium barbital, 0.01 moles barbital) (Sigma, St. Louis, MO)
2. 0.083 gm $CaCl_2$ (calcium chloride-anhydrous) (Sigma, St. Louis, MO)
3. 0.508 gm $MgCl_2 \cdot 6H_2O$ (magnesium chloride) (Mallinckratt, Paris, Kentucky)
4. 42.5 gm NaCl (sodium chloride) (Sigma, St. Louis, MO)

Dissolve in one liter of distilled water over low heat on a thermolyne hot plate until ingredients are dissolved. Adjust pH to 7.5.

To prepare the standard MBB solution (1X), dilute a 100 ml of the 5X solution in 400 ml distilled water.

X. Benzocaine

1. 10 gm ethyl p-aminobenzoate (Sigma, St. Louis MO)
2. 90 ml ethanol

Mix thoroughly

XI. Substrate solution

1. 75.0 µl ABTS (2,2'-azino-bis(ethylbenzthiaolinesulfonic acid) 1 µg/ml in distilled water)
2. 5.0 µl H_2O_2
3. Citric buffer (0.2 gm citric acid in 100 ml distilled water, adjust pH to 4.0)

Mix these reagents together immediately prior to use.

APPENDIX 2

FINE SPECIFICITIES OF SERUM ANTIBODIES GENERATED TO TNP

The median of the Krel values for each inhibitor and all time points (Figures 14-23) was used to determine a hierarchy of analog binding. As stated previously, by using the structurally related analogs of TNP-lysine it was possible to gain an estimate of the structural requirement necessary for a population of antibody combining sites to bind the trinitrophenyl hapten.

The requirement for a long carbon chain which positions a negatively charged carboxyl group away from the phenyl ring appeared to be a requirement for strong antibody binding. DNP-ε-aminocaproic acid (DEAC), (median Krel = 0.3) which its has the carboxyl group positioned two more carbon groups distal from the phenyl ring than DNP-γ-aminobutyric acid (median Krel = 0.2). As a result, DEAC was a slightly better inhibitor. The reduced binding of serum antibodies to DNP-α-aminocaprylic acid (Krel = 0.02) and DNP acetic acid (median Krel = 0.01) also demonstrated the requirement for placing the carboxyl group more distal from the phenyl ring for stronger binding with the antibodies. These two analogs have the negatively charged carboxyl group immediately adjacent to the phenyl ring which results in poor inhibition of the anti-TNP reaction.

The third nitro group can either increase, decrease or have no effect on antibody binding, depending upon which substituents are

present on the phenyl ring. For example, DNP-lysine (median Krel = 0.1) is unable to bind the antibody population as well as the homologue, TNP-lysine (Krel = 1.0) due to the loss of the third nitro group at position 6. However, this third nitro group on TNP-phenylalanine virtually eliminates its ability to bind TNP antibodies (median Krel < 0.001), whereas DNP-phenylalanine (Krel = 0.06), which differs by the loss of this nitro group is a much better inhibitor. This likely is due to the effects of steric hindrance. The antibody combining site can not accommodate a nitro group and an additional phenyl ring structure near position six. However, antibody binding was not affected by this third nitro group at position 6 in TNP-γ-aminobutyric acid when compared to DNP-γ-aminobutyric acid. These analogues both generated a median Krel of 0.2. Thus it appears that the proximity of electronegative groups to the phenyl ring and bulkiness associated with the phenylalanine can prevent efficient binding.

Results of fine specificity analysis performed on serum antibodies parallels, in some respects, what has been observed for antibodies generated in vitro by the spleen and anterior kidney of coho salmon (Oncorhynchus kisutch) (Irwin, 1987). Irwin (1987), demonstrated that the hapten must possess at least two nitro groups on its phenyl ring (positions 2 and 4). A third nitro group (position 6) however does not alter the binding efficiency of the antibodies. Irwin (1987) concluded by this observation that DNP-lysine was a comparable inhibitor to TNP-lysine. The results from the present data clearly shows the need for a large battery of

inhibitors to demonstrate that a third nitro group at position six could hinder as well as aid the binding of an antibody population. Lastly, he demonstrated that electronegative groups must be distal from the phenyl ring, which was confirmed by these studies.