CHARACTERIZATION OF IDIOTYPE INTERACTIONS DURING THE IMMUNE RESPONSE TO FERREDOXIN. IDIOTYPE AND EPITOPE SPECIFIC INTERACTIONS DETERMINE THE OUTCOME OF CHALLENGE WITH ANTIGEN

by

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ABSTRACT

Anti-idiotype antisera were raised in rabbits to two monoclonal antibodies, Fd-1 and Fd-2, with specificity for each of the two antigenic epitopes found on the ferredoxin (Fd) molecule. The anti-idiotype antisera (anti-Fd-1 and anti-Fd-2) were used to demonstrate that one of the idiotypes (Fd-1) was expressed at significant levels in most anti-Fd antisera raised in B10.BR mice while the second idiotype (Fd-2) was infrequently expressed. Examination of anti-Fd sera raised in other mouse strains demonstrated that expression of the Fd-1 idiotype mapped to the IgH gene complex and was found in the antisera of all mouse strains examined with the $Ig\text{-}1^b$ allotype. When splenocytes from Fd-immune B10.BR mice were treated with anti-Fd-1 and transferred to irradiated syngeneic recipients, the adoptive secondary response was significantly higher in animals receiving treated cells as opposed to control animals which received normal rabbit serum treated cells. This response produced a net increase in antibody to both epitopes and the relative amount of Fd-1 idiotope was not significantly altered. Further studies with separated cell populations showed that the overall increase of anti-Fd antibody produced was attributable to the effects of the anti-idiotypic serum on a population(s) of T cells. Treatment of mice with the Fd-1 monoclonal antibody (which should react with anti-idiotypic cells) had an analogous effect to that of the anti-idiotype. Treated mice produced heightened levels of antibodies directed to both epitopes of Fd. Treatment of mice with second anti-idiotype, anti-Fd-2, was found to enhance the anti-Fd response of B10.BR mice and abrogate the non-responder status of DBA/2 mice. Additional evidence indicates that the Fd-2 idiotype could be expressed on a suppressor cell population which may be a predominant regulatory element in both B10.BR and DBA/2 mice.

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CHAPTER 1: INTRODUCTION

While the physical universe of Immunology occupies a relatively finite space, the theoretical boundaries which have been crossed in an attempt to understand the workings of the immune system are quite remarkable. Many of its frontiers have been shared with allied studies in genetics and molecular biology. The growth of Immunology represents developments on many different fronts so much so that even research, for instance on the expression of immunoglobulin genes, might be viewed as much a biochemical problem as an immunological one. In the present era, the search for new models inevitably seems to draw the attention of theorists to the problems of Immunology. Much speculation has focused on mechanisms which are involved in the generation of diversity and regulation of immune responses. To date, no contemporary theory has gone as far towards unifying these two apparently incompatible issues as has the concept of network theory which was first proposed in an address to the Pasteur Institute by Niels Jerne in 1974 (1).

Historical outline

From the initial observations of Landsteiner, interactions between antibody and antigen molecules have been defined according to the principle of complementarity in which an immunoglobulin molecule or ligand combined with a particular epitope by virtue of its immunological specificity (2). It has become apparent that this degree of specificity necessitates the existence of an extensive repertoire of antibody specificities. This original concept was reaffirmed in 1957 when Burnet published the Clonal Selection theory which placed diversity in the context of population genetics by defining a clonal distribution of antibody specificities amongst unique populations of antigen reactive cells (3). Burnet's theory therefore defined cell interaction models on the

basis of the ability of cells to bind the same antigen, or to associate by virtue of shared specificity. All forms of immunological interaction were necessarily antigen driven events controlled by contact with the external environment. It became clear, however, that assumptions made concerning the role of self-tolerance in this model could not be made in view of a number of experimental findings which demonstrated the existence of autoantibodies which were directed towards host components, including reports that the combining sites of antibody molecules could be immunogenic in the host immune system (4,5).

In 1974 Niels Jerne proposed a theory whereby cells could communicate through the sharing of variable region receptors by lifting the restrictions on self tolerance and permitting cellular interactions to occur through the production of complementary receptors (6). This concept can easily be viewed as a reflection of clonal selection theory back on itself, transcending the isolated immune system of Burnet into an elaborate network displaying the novel ability to communicate with itself via the capacity for self recognition and to interact with its environment through contact with external antigen. Quoting from Jerne's simple description: "The immune system is a network of antibody molecules and lymphocytes that recognize and are recognized by other antibody molecules and lymphocytes." (6).

Definitions of idiotype

Interactions within this form of network are based upon a concept of idiotype which has undergone several changes in definition since its inception. According to Oudin, an idiotype was defined as the set of unique determinants presented by antibodies induced by a given antigen (7). This definition takes into account the existence of individual antigenic specificities of immunoglobulin molecules which appeared to be associated with the antigen binding activity of the antibody molecule. The idiotypic property of an antibody was

therefore related to its antigen specificity and thereby became correlated with the phenomenon of antibody diversity. In subsequent years, it was made evident that antibody molecules were more highly diversified than the degree envisaged by Oudin, whose definition was later shown to include a sizeable degree of molecular heterogeneity. More recent studies utilizing monoclonal antibodies of either myeloma or hybridoma origin, have resulted in a further refinement of the term. The idiotype of a given monoclonal antibody molecule is, in actuality, a composite property composed of a number of individual antigenic determinants or epitopes. Jerne used a more specific term -idiotope, which was taken to represent an individual idiotypic epitope. An important distinction is therefore made in Jerne's theory between the notion of idiotope and combining site per se. The only criterion for this definition is that they be confined to the variable portion of the molecule. Since each of these specificities are operationally defined by the existence of a complementary anti-idiotype antibody, idiotypic relatedness between antibody molecules can vary in terms of the number of shared specificities and hence, number of anti-idiotopes with which they interact. It is also useful to note that the quantum unit in all network interactions is an idiotope-anti-idiotope pair. In other words, an idiotope is indirectly defined by the specificity of the antibody which recognizes it. Because idiotypic complementarity is the central principle of network theory it is this concept which is alluded to in all subsequent uses of the term idiotype throughout this thesis.

Structural correlates of idiotype markers

Efforts to define the nature of idiotypic determinants more precisely have relied on two avenues of investigation: serological and biochemical analysis of antibody molecules of defined idiotypy. A number of serological approaches have been used in attempting to define idiotype expression in vivo. As these

have often involved raising of anti-idiotype under differing conditions, it is important to consider several qualifications which are implicit in Jerne's theory yet have sometimes been overlooked. In the following paragraphs several experimental definitions of idiotypy are described which reveal that the terms of each are not necessarily equivalent and would hold different consequences within a Jernian network.

Jerne states that regulation in the network occurs through a process of self-recognition. As mentioned previously, exceptions to the rule that self-reactive lymphocytes do not exist have been described in the literature. For example, in a number of diseases which appear to have an autoimmune basis antibodies are present which bind to endogenous IgG and DNA (8,9). Experimentally, it has been found that host idiotypes are not particularly immunogenic in the sense that large quantities of anti-idiotype cannot readily be obtained. Only through prolonged immunization usually with the idiotypic antibody conjugated to a foreign carrier protein has it been possible to drive the network in the direction of producing significant amounts of anti-idiotype (10). In contrast, allogeneic idiotypes do appear to be readily immunogenic, that is, antibodies which block combining sites of allogeneic antibody can be easily produced. Functionally, these allo- or xenoantisera act analogously to auto-anti-idiotype. The actual idiotypic determinant recognized by these two reagents could be unrelated, while being indistinguishable on a serological or functional basis. A problem arises when an attempt is made to determine idiotypic relatedness between antibody molecules using these two forms of anti-idiotype. It is possible that molecules which are idiotypically related in an autologous network may not be recognized as such by a foreign immune system. Jerne's definition inherently depends upon the concept of idiotypic complementarity, an abstract relationship for which it is difficult to define a serological

equivalent. It must therefore be acknowledged in the absence of concrete structural evidence, that present definitions of idiotypy based on dissimilar genetic systems only constitute formal approximations of the relationship proposed by Jerne.

Immunization across species barriers has been preferentially utilized in serological studies in favour of both the speed with which this mode of immunization can yield anti-idiotypic specificity as well as the quantities of reagent that can be isolated. Some of the earliest studies showed that idiotypes were not shared by different animals when immunized with the same antigen (11). Some evidence that cross-reactions might be observed between different individuals of the same species was discovered although the incidence was found to be very low (12). Interest in using idiotypes as genetic markers developed only after reports of idiotypic cross-reactions were reported with defined antigen systems in mice. For example, the response of Balb/c mice to the phosphorylcholine hapten is almost entirely dominated by antibodies bearing the T15 idiotype (13). In another hapten system, 4-hydroxy-3-nitro-phenyl-1-acetyl (NP), 85% of primary anti-NP antibodies express a major idiotype (14). In the arsonate system, it was found that 20-70% of anti-arsonate antibodies bear a common idiotype (15) in A/J mice. Between 30 and 50% of antibodies to group A streptococcal carbohydrate in A/J mice share a common idiotype which is designated A5A (16). In the sense that pooled serum antibodies are composed of the combined products of many different lymphocyte clones, their anti-idiotypes are in reality oligoclonal in specificity and define idiotypy which is biased in favour of the more predominant idiotypes in the existing antibody population.

It has only become possible to probe the idiotypic heterogenity of oligoclonal systems with the advent of cell fusion technology (17). It is now possible to perform idiotypic studies using monoclonal antibodies obtained by cell

fusion in place of pooled serum antibodies. Using a large set of monoclonal antibodies with specificity for the arsonate moiety, the idiotype system designated as CRI has been resolved into a large number of public and a small number of private idiotypic specificities (18). Parallel findings were observed in the NP hapten system using a panel of monoclonal anti-idiotype antibodies raised to a single NP-binding hybridoma protein in mice (19). These cases demonstrate similarities which have also been observed in a number of other systems (20). The predominance of individual hybridoma idiotypes was found to be low, suggesting that the total repertoire of serum antibodies may be very diverse. These examples aptly illustrate the serological distinction between Oudin's criterion and Jerne's concept of idiotype.

Accurate biochemical data has only recently been obtained by exploiting the precision afforded by the existence of monoclonal probes and the precise markers which they define. Comparative amino acid sequence analysis of antibody V-regions has shown that for heavy chains there exist four regions of extreme variability or hypervariable regions which map to residues 31-37, 51-68, 84-91 and 101-110 (21). It was subsequently shown that the residues in three hypervariable regions constituted antigen-contacting residues in the actual antigen binding site, hypervariable regions have also been referred to as complementarity determining regions (22). This role has been strongly supported by affinity labelling and X-ray crystallographic data which showed that residues within hypervariable regions were distributed over the frontal surface of the immunoglobulin molecule in positions which were directly exposed to the solvent and for reasons of conformation could conceivably fill the role of antigen combining site (23).

In taking into account the sequence data of framework domains of V-regions, several groups have shown that the actual alpha-carbon backbone of immunoglobulin

V-regions is almost identical between different molecules (24). It would appear that even between distantly related species a very high degree of conformational homology has been retained. This structural similarity has been used as a basis for model building using an established framework prototype as a foundation for predicting combining site structures based on information from X-ray diffraction analysis and primary sequence data (25).

Sequence analysis of antibody molecules which comprise an idiotypic family of myeloma and hybridoma proteins with dextran binding activity has revealed several important facts concerning the contribution of heavy and light chains towards the actual idiotope (26). Light chains of dextran antibodies are all of the lambda type and appear to be almost identical according to their isoelectric focusing patterns. Preliminary amino acid sequence data on two myeloma proteins M104E and J558 revealed that their V_L regions are identical. Complete amino acid sequences of ten individual heavy chain variable regions have provided primary structural correlates of several V-region idiotype markers. A correlation between an individual idiotype (IdI) on protein J558 (defined by a monoclonal anti-idiotype) and the presence of a specific sequence -Arg-Tyr- at positions 100/101 of the J558 heavy chain was found by comparative sequencing (27). This represents the first demonstration of an idiotypic marker with the D segment of V_H. Support for this assignment was demonstrated in a modification study which showed direct evidence for the participation of the tyrosyl residue at position 101 in the interaction between J558 and its anti-idiotype. Indirectly, the presence of a second category of idiotypic determinant IdX has been correlated to amino acid residues 54 and 55 of J558 confirming the independence of these markers on the basis of differential modification.

Two dextran-binding myelomas J558 and Hdex24 which possess the same individual idiotype (IdI) were diazotized to low levels. Both proteins lost the

IdI marker under these conditions. When diazotization was performed in the presence of hapten (1-O-methyl-alpha-D-glucopyranoside) J558 retained the IdI marker whereas Hdex24 did not. It would appear that the idiotypic determinant modified in the case of J558 is in the hapten binding site whereas in the case of Hdex24 it was not. This finding implies that residues from different locations in the tertiary structure may be juxtaposed to form the same idiotypic determinant. In the case of J558, this determinant is located within the D segment and its expression may reflect the phenomenon of junctional diversity proposed by Early et al or could represent an example of a germline D segment minigene (28,29).

Genetic mapping of idiotypic determinants therefore remains a complex issue. Despite the best efforts of investigators to obtain a molecular correlate, the ultimate definition may escape detection. For example, for an amino acid correlate ascribed to the D-segment, various mechanisms could account for its presence. Where it can be shown that the same marker can be correlated with different sites on different molecules, it might also be predicted that a given idiotype may be dependant on conformation and require particular sequences in other portions of the V regions for its expression. A given minigene may thereby produce an IdI in one context but fail to do so in another. This uncertainty is reflected in the following comment: "Indeed, one can never be certain what is being mapped- a V segment, a D segment, a J segment, the ability to produce certain somatic variants, or some combinations of these." (27).

Experimental demonstrations of idiotypic interactions

Although idiotypes were originally detected on antibody molecules as early as 1953, the existence of such individual antigenic specificities on immunoglobulins remained a serological curiosity until the phenomenon could be placed into the frame of reference of antibody diversity (1). The potential utility of

this property became apparent as information linking idiotypy to antigen specificity became known and was subsequently exploited to probe idiotypic cross-reactivity between genetically related individuals. Expression of idiotype markers has since been linked to the Ig-1 locus and is a subject which has been reviewed (30). In addition, it has been widely demonstrated that anti-idiotype antisera can be raised between individuals of the same inbred strain (isoimmunization) as well as in the same individual from which the immunizing idiotype was originally obtained (autoimmunization) (31). Evidence demonstrating the existence of auto-anti-idiotypic immunity during the course of the normal immune response in vivo fulfills Jerne's postulate on the existence of this form of interactions within the host network (1). Experimental confirmation of other network postulates has been found in a number of systems. Support for the role of idiotypic interactions in regulation of immune responses and the predominance of T lymphocytes in this mode of regulation was shown by several investigators (32-34). These examples of T-cell manipulations using antisera raised to antibody variable regions also confirm the existence of shared idiotypic repertoires between T and B cells. In a number of instances this finding has been extended to idiotypes on T-cell derived regulatory factors (35). Administration of minute quantities of anti-idiotype in vivo can therefore result in profound and long-lasting consequences in the immune system. Stable alterations in the balance of regulatory T lymphocytes have been obtained as a result of idiotypic perturbations during the course of immune responses. This suggests that idiotypes may be one level at which immune responses can be effectively manipulated.

While the concept of idiotypic interaction is now widely accepted and continues to provide concise experimental data on isolated phenomena, it has been used mainly to demonstrate temporal interactions, for example, generation of

T_H or T_S cells as members of an idiotypic series. Within a functional network, however, alterations in the activity of one component must have a compensatory effect on other elements with which they are in equilibrium. It is essential in order to probe the more comprehensive issues in network theory to design experiments which allow one to measure not only the behaviour of a single network parameter, but to follow the changing relationship between linked idiotypic events simultaneously. The lack of clear experimentation along these lines has so far kept network theory from gaining wide acceptance into the field of cellular immunology. This is largely due to the fact that idiotypes have been investigated in macromolecular systems in which the measurable idiotype specific portion constitutes a very small portion of the overall immune response. Antigen-linked (hapten-carrier) interactions form the basis for Ir gene controlled responses but under these conditions interactions between idiotypically distinct compartments cannot be measured. This lack of correlation with the known models of Ir gene control has led various authors to attempt a theoretical integration of network concept with existing data on Ir gene controlled cell interactions (36).

Need for a simple experimental model

The existence of idiotypy among pathways regulating the immune response has been shown in a number of independent systems. A common approach of such studies has been in the method of preparation of anti-idiotype reagent. In general, affinity purified antibody has been used to raise heterologous anti-idiotype which has been used to define major strain specific idiotypes (13-15). Subsequent analysis of monoclonal antibodies with specificity for haptens, amino acid copolymers, and dextrans have indicated that the previously defined major intrastrain idiotypes can be further resolved into families of closely related but not necessarily identical molecules (18-20). The degree of

heterogeneity amongst antibodies expressing a collective idiotype is, consequently, uncertain. Cross-reactivity of an anti-idiotype which is complementary to such an antibody population can establish idiotypic relatedness in functional studies, but cannot present a formal demonstration of the connectedness amongst members of an idiotypic series as this type of reagent has not been shown to discriminate clonally distributed receptors.

Recent evidence has been reported to suggest that different forms of the same antigenic determinant can trigger different B cell precursors (37-38). Balb/C mice immunized with the T-independent antigen dextran B1355s respond with the expression of two unique idiotypes in addition to the normal cross-reactive idiotype. Greater than 90% of these antibodies express lambda light chains. By contrast, spenic B cells stimulated with a T dependent form of antigen, dextran coupled to hemocyanin, carried a large proportion of kappa antibody, less than 25% of which expressed the cross-reactive idiotype. This selection for different idiotypic B cells may occur by means of T cells which recruit precursor B cells in an idiotypically specific fashion. Association of a given haptenic determinant with a variety of carrier determinants could thereby influence the selection of hapten specific B cells. By presenting such a haptenic determinant in an environment of variable T_H or T_S, idiotype expression may be modulated. This notion is germane to the chemistry of synthetic antigens which may vary in terms of molecular weight and conformation, and to the study of haptenic groups which must be coupled to large molecular weight carriers in order to become immunogenic.

The studies which are described in this thesis were undertaken with the aim of describing network interactions using a model system whose immunochemical properties were well defined. The ferredoxin (Fd) molecule is a small electron transport protein isolated from Clostridium pasteurianum (Figure 1). It has a

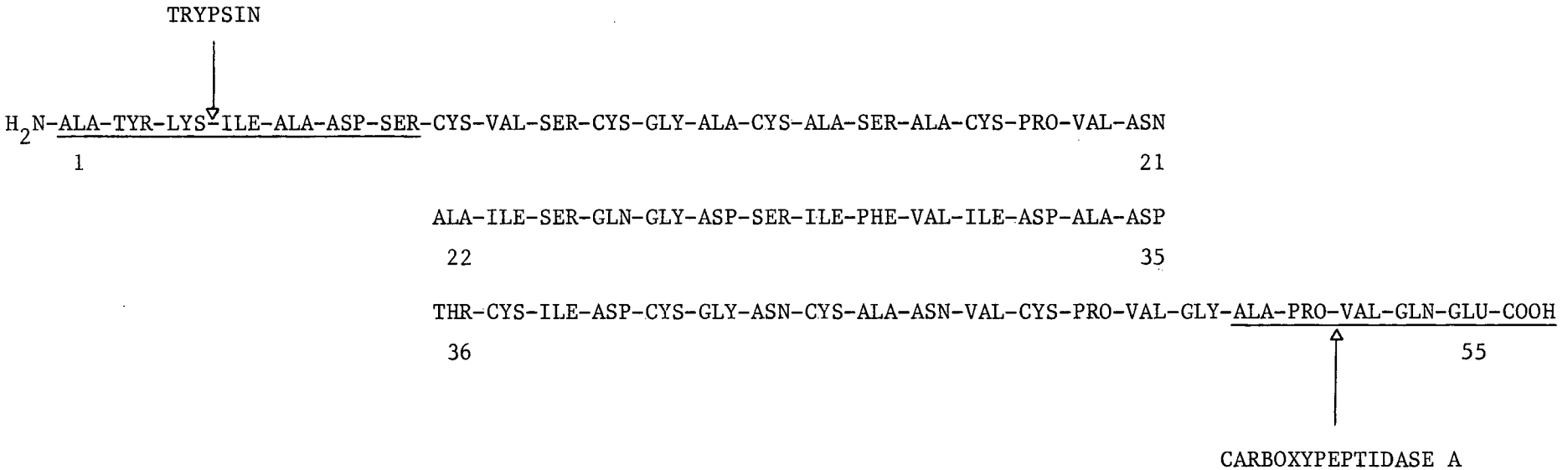

Figure 1: Amino acid sequence of ferredoxin. Underlined sequences indicate the two major antigenic determinants. Arrows demonstrate the point of trypsin cleavage in the N-terminal determinant and the point at which carboxypeptidase A activity is terminated in the C-terminal determinant.

total amino acid content of fifty-five residues all of which are present in a single polypeptide chain (39). In view of its molecular weight and antigenicity in several species, the molecule has been a convenient subject for immunological analysis. Earlier studies using synthetic peptides representing different regions of the molecule showed that the sequences located in the NH_2-terminal heptapeptide (NH_2-ala-tyr-lys-ile-ala-asp-ser) and the COOH-terminal pentapeptide (-ala-pro-val-gln-glu-COOH) constituted the two major antigenic determinants. In equilibrium dialysis experiments, it was shown that these peptides bound essentially 100% of the antibody synthesized in rabbits to Fd (40). The specificity of these assignments is also supported by chemical modification studies (41), and by the results of cellular studies which indicate that the NH_2-terminal determinant appeared to be the stronger of the two in terms of cell-mediated functions, while the COOH-terminal determinant was predominant in serological assays (42). These results are representative of cell cooperation models in immunology and have been the basis for genetic investigations of Fd responses in congenic strains of mice (43). At the molecular level, this antigen exhibits properties which lend themselves to immunochemical analysis. Located within each of those regions which have been identified as antigenic determinants are sites susceptible to hydrolysis by specific proteolytic enzymes. Digestion of ferredoxin with selected enzymes results in a molecule retaining 52 or 53 of its original 55 amino acid residues but possessing only a single functional antigenic determinant (44). Through the use of two selective enzymes trypsin and carboxypeptidase A, monospecific fragments having the individual characteristics of either an NH_2-terminal or the COOH-terminal determinant can be obtained.

CHAPTER 2. DETECTION OF SPECIFIC IDIOTYPE IN THE SERUM OF Fd-IMMUNE BIO.BR SERUM

Introduction

The ferredoxin molecule (mw 5500) is among the smaller antigens which have been utilized in immunological studies. Of currently available proteins such as myoglobin (45), lysozyme (46), and staphylococcal nuclease (47), and insulin, only insulin (48) has a comparable molecular weight. While Fd is immunogenic in mice, peak antibody titres are moderate in comparison to the levels of antibody produced in response to more complex antigens. Analysis of purified antibody by isoelectric focussing suggests that the repertoire of different antibody species is limited to a small number of detectable spectrotypes (44). An examination of idiotypy might provide a more accurate characterization of the degree of antibody diversity present in the anti-Fd repertoire. Customarily, researchers have produced anti-idiotype reagents by immunizing animals against the affinity purified antibodies pooled from a number of individuals. In view of the demonstrated variability in expression of inherited idiotypes between related individuals, however, studies based on this approach cannot be used to demonstrate absolute identity in idiotype repertoire but only overlap in certain species or between individuals.

Studies in other animal systems had shown that the immune response to Fd was limited to only two determinants of the molecule. Isolation of quantities of purified anti-Fd antibody sufficient to perform controlled idiotypic studies was precluded due to the fact that after normal immunization protocols, only small amounts of antibody could be obtained. This necessitated either the pooling of sera from large numbers of individuals or using serum from hyperimmunized donors. A decision was made instead to attempt to isolate monoclonal antibodies to ferredoxin. This approach presents the advantage of enabling one to first

determine the epitope specificity of the antibody before subsequently attempting to characterize its idiotype amongst the population of antibody molecules having similar epitope binding specificity in serum. Studies were therefore undertaken with the aim of producing anti-Fd monoclonal antibodies and determining their binding specificity for the two Fd determinants using immunochemical techniques and second, of raising antisera to these molecules in order to determine the role of these idiotypes in the immune response.

Materials and Methods

Animals

Female mice 6-8 weeks of age of the following strains were purchased from the Jackson Laboratories, Bar Harbour, Maine: B10.BR/OS_n DBA/2, C57BL/6, C3H, CBA, SJL, AKR, SM, C58, B10.D_2/OS_n, STB, M_aMY, CE. Young adult female New Zealand white rabbits were obtained from the University of British Columbia animal care centre breeding unit.

Antigens and immunization

Keyhole limpet haemocyanin (KLH) was obtained from Calbiochem, La Jolla, California. Ferredoxin (Fd) was purified in the laboratory from cultures of Clostridium pasteurianum grown and purified according to the procedures of Martenson (49). Briefly, crude Fd was isolated by acetone extraction of harvested cell pastes followed by precipitation with 90% saturated $(NH_4)_2SO_4$ and subsequent elution from DEAE Cellulose (Sigma). Fd was then purified from the crude preparation by molecular exclusion chromatography on Sephadex G-50 (Pharmacia) using the criterion for purity of A390/280 as described. Fractions exhibiting ratios of greater than or equal to 0.7 were used in the experiments.

Mice were immunized with 50 µg of specific antigen (Fd or KLH) emulsified

in complete Freund's adjuvant (CFA-Gibco) by intraperitoneal injection on day 0. Primary immune sera were obtained on day 21 by retro-orbital bleeding. Secondary immunizations were given on day 28 by intraperitoneal injection of 50 µg of antigen emulsified in incomplete Freund's adjuvant (IFA Gibco) and secondary immune sera were subsequently taken on day 35. In experiments involving adoptive secondary responses, animals were bled on day 35 as well as on day 49.

For cell fusion experiments, mice which had received secondary injections of antigen 4 weeks previously were given intraperitoneal injections of either 100 µg of Fd in CFA three days before using their spleens in fusion experiments.

Rabbits were used in the preparation of antiserum to normal mouse immunoglobulins (Pentex) and anti-idiotypic antisera to the monoclonal antibodies Fd-1 and Fd-2. Animals were initially immunized with 1.0 mg of the appropriate mouse immunoglobulin emulsified in CFA by intramuscular injection. Subsequent injections of antigen in IFA were given until antibody titres in serum reached levels of approximately 20 mg/ml. Antisera were obtained 7 and 14 days following the most recent injection by bleeding from the marginal ear vein.

Assay for specific antibody

Antibodies in immune serum were quantified by using the enzyme linked immunosorbent assay (ELISA) described by Voller et al 1976 (50). Conjugates of enzyme with anti-immunoglobulin were prepared in the laboratory using the following procedure. Rabbit anti-mouse immunoglobulin was purified from hyperimmune rabbit serum by adsorption to normal mouse IgG which had been covalently linked to Sepharose 4B-CL (Pharmacia) with cyanogen bromide (51). Specific antibody was eluted from the matrix in pH 2.3 glycine buffer and immediately neutralized with 3M NaOH. Those fractions containing significant quantities of protein (0.5 mg/ml) were pooled and dialysed against phosphate buffered

saline (PBS) solution overnight and concentrated to 5 mg/ml against polyethyleneglycol mw 20,000 (Fisher). Concentrated antibody was stored at -20°C until required. Five mg of alkaline phosphatase enzyme purified from calf intestine (Sigma type vll-s, Boehringer-Mannheim) was dialyzed against PBS (Sigma enzyme) or used directly without dialysis (Boehringer-Mannheim enzyme). Two mg of affinity isolated rabbit anti-mouse IgG (RaMIg) was combined with 5 mg of enzyme in a total volume of less than or equal to 1.5 ml in PBS. Ten microlitres of glutaraldehyde solution (25% EM grade, JB EM Services, Dorval) were added and the mixture left for 90 min at room temperature with slow stirring. After reaction with the cross-linking reagent, the conjugated material was dialyzed overnight against 0.05 M Tris HCL pH 8.0 and stored for use as a 50% solution in glycerol at 4°C in the dark. Conjugates prepared in this way were standardized against a fixed amount of normal mouse Ig which had been coated onto the solid phase of polystyrene substrate binding plates (Immulon I-Dynatech) by overnight incubation with 0.1 ml/well of carbonate coating buffer containing 100 mg/ml of mouse IgG (Pentex). After washing with PBS-Tween buffer followed by a brief incubation (20 min) with 0.2% bovine serum albumin (BSA) in PBS solution, coated plates were incubated with 0.1 ml/well of a dilution series of enzyme conjugate ranging from 1/100-1/10,000. Working dilutions of conjugate were chosen in order to yield an $O.D._{405nm}$ of 1.0 in a 30 min reaction time with substrate. Generally, optimum working strength of conjugate prepared using this procedure was obtained at a dilution of 1/1000-1/1500. No evidence of any decline in the working strength of conjugate was noticed over intervals as long as 24 months. The sensitivity of this assay for specific anti-Fd antibody has previously been calibrated using a radioimmunoassay (43). Under the conditions used, the lower detection limit of the ELISA for anti-Ferredoxin antibody is 10 ng.

Cell fusion

The conditions used in the production of hybridoma cell lines were essentially those of the protocol described by Oi and Herzenberg (52) with minor modifications. First as hyperimmunization does not appear to directly increase the likelihood of generating specific hybridomas an alternate immunization regime based upon adoptive transfer was used. Spleen cells were removed from donor mice 7 days following secondary injection of antigen (Fd or KLH) and adoptively transferred to syngeneic sublethally irradiated (500 rad) recipients, using $5x10^7$ cells per mouse. These animals were rested for 2 weeks during which time they received an antibiotic solution (Pfizer) in their drinking water. The mice then received a third dose of Fd (100 μg in IFA) intraperitoneally and their spleens were taken for fusion 3 days later. The myeloma cell line used, SP2/0 (53), was obtained from the Salk Cell Distribution Center, La Jolla, California. Prior to fusion, myeloma cells were cultured in 100 ml volumes in 250 ml capacity tissue culture flasks (Falcon 3013) at a starting density of 10^4 cells/ml and reaching a final density limit of $1-2x10^5$/ml. The culture medium consisted of freshly prepared (not more than 1 week) Dulbecco's Modified Eagle Medium supplemented with 2mM glutamine, 5mM sodium pyruvate, 100 μg/ml penicillin/streptomycin antibiotic solution, 2mM sodium bicarbonate (Fisher) and 10% by volume fetal calf serum (Gibco). Cell cultures at all stages were incubated in a humidified environment of 10% CO_2 at 37°C. Polyethyleneglycol (Serva-mw 4,000 purified for GLC) was prepared on the day of fusion by autoclaving of solid PEG followed by dilution with sterile PBS to a 50% solution (w/v). This solution was titrated to pH 7.4-7.8 using sterile 1N NaOH before use. Spleen cells were fused with SP2/0 cells at a ratio of 5:1 in 50% PEG using the procedure of slow dilution with warm (37°C) serum-free PBS. After dilution and pelleting by centrifugation, fused cells were incubated at 37°C

for 30 min in a small volume of tissue culture medium. The cells were then gently resuspended to a concentration of $5x10^7$ cells total/ml in medium containing 20% FCS and 10^7 mouse red blood cells/ml and dispensed into 96-well tissue culture plates (Costar 4596) using 0.1 ml/well of cell suspension. The plates were placed into plastic boxes with loosely fitting lids to retard evaporation and fed on the following day with double strength HAT medium. Hybridoma cultures were screened for antibody production between 7-10 days using the ELISA procedure described earlier. Subsequent details including cloning by limiting dilution, growth as ascites *in vivo* and purification on protein A Sepharose are as described in Oi and Herzenberg (52) with the exception that hybridomas were cloned on feeder layers of mouse red blood cells in place of mouse thymocytes.

Production of Anti-idiotype Serum

Anti-idiotype serum was produced in a New Zealand White rabbit by injection of monoclonal antibody (Fd-1) isolated from ascites fluid on Protein A Sepharose. For the primary injection, 1.0 mg of purified Fd-1 in complete Freund's adjuvant was given intramuscularly. Subsequent injections of Fd-1 were given as alum precipitate intraperitoneally at intervals of two weeks. Serum used throughout this study was taken two weeks following the fourth injection. Prior to testing serum for anti-idiotype activity, rabbit antibodies reacting with constant region determinants of Fd-1 were removed by adsorption onto a column of mouse immunoglobulins coupled to Sepharose 4B using the CNBr procedure (51). Rabbit anti-Fd-1 serum (8.0 ml) was passed through the column twice. Samples of adsorbed rabbit anti-Fd-1 serum used in anti-idiotype assays contained 10% by volume of normal B10.BR serum in order to preclude the possibility of detecting reactions toward non-idiotypic epitopes on mouse immunoglobulin.

Affinity Isolation of Anti-idiotypic Antibody and Preparation of an Anti-idiotype Immunoadsorbent:

Anti-idiotype antibody was purified from normal mouse Ig-adsorbed rabbit anti-idiotypic antiserum by using an affinity column of the monoclonal antibody Fd-1 coupled to Sepharose 4B. Anti-idiotype antibodies were recovered from the column by elution with .05 M glycine HCl pH 2.2 and immediately neutralized with 1 M NaOH. Purified antibody was then assayed for anti-idiotype activity against Fd-1 antibody as described below. Active material was coupled to CNBr activated Sepharose 4B at a concentration of 3 mg per 1 ml packed beads. Under the conditions used approximately 90% coupling efficiency was obtained.

Assays for Anti-idiotype Activity

Assays for anti-idiotype activity of rabbit antiserum to the monoclonal antibody Fd-1 were performed using the ELISA. Activity of inhibitors was measured against a constant concentration of a specific antibody which had been previously titrated to yield an absorbance at 405 nm of 1.0 after thirty minutes incubation in a standard system. Specific antibodies used in these assays consisted of serum from Fd immune B10.BR mice, serum from KLH immune B10.BR mice, monoclonal antibody to Fd and a rabbit antiserum to the hybridoma protein Fd-1. In stage 1 of an inhibition assay, doubling dilutions of inhibitors were mixed with a constant amount of the specific antiserum and incubated at 4°C overnight in microtitre trays. Aliquots of 0.1 ml for each sample were then transferred to ELISA plates bearing the appropriate antigen bound to the solid phase. From this stage onwards, processing of the plates was done according to standard ELISA technique. The tests were developed with alkaline phosphatase labeled sheep anti-rabbit immunoglobulin.

Determination of Combining Site Specificity of Anti-idiotype Serum

Interaction between rabbit anti-hybridoma protein and the hybridoma protein was measured in the presence of antigen as follows: Protein binding ELISA plates (Cooke Dynatech) were first coated with purified monoclonal antibody to Fd (Fd-1) by incubating them in the presence of 0.1 ml per well of carbonate coating buffer containing 20 µg per ml of protein A purified Fd-1 for 45 min. Following this step, Fd-1 coated plates were incubated with 500 µg/ml of bovine serum albumin in PBS buffer for 15 minutes in order to saturate unbound protein binding sites on the solid phase. This step was undertaken in order to reduce background colour development in the assay due to nonspecific adsorption. After washing the plates with PBS-Tween buffer, monoclonal antibody coated plates were incubated with varying concentrations of antigen, either Fd or KLH, for 1 hour. KLH served in this case as a non-specific control, to control for non-specific blocking by soluble proteins. The plates were then washed again and incubated with a solution of rabbit anti-idiotype serum at a dilution of 1/512. Binding of the rabbit anti-idiotype to the mouse monoclonal antibody coated plates was allowed to proceed for 30 min after which the plates were washed and the wells incubated with 0.1 ml of an alkaline phosphatase conjugate of sheep anti-rabbit Ig at a dilution of 1/2000 for an additional 30 min. After a further 1 hour incubation and final washing with buffer, the enzyme substrate reaction was allowed to continue for 30 minutes. Colour development was then determined spectrophotometrically using a Titertek Multiskan spectrophotometer (Flow Labs Inc).

Purification of Idiotype Bearing Antibodies from the Serum of Immune Mice

Idiotypic antibody was purified from the serum of B10.BR mice immunized 4 weeks previously with 50 µg Fd in CFA. One ml of pooled serum was passed over the anti-idiotype immunoadsorbent column, and bound antibody was recovered in the acid eluate. Antibody in the eluted fraction was then titrated for Fd binding activity by ELISA. Serum anti-Fd antibody eluted from the anti-idiotype column was tested in an inhibition assay as described above against measured quantities of whole Fd, its N- or C-terminal fragments, as well as affinity isolated anti-idiotypic antibody. Tests for the specificity of this antibody were devised in collaboration with Lydia Sikora (44).

Results

Typical antibody titres in B10.BR mice which are high responders to Fd, immunized with ferredoxin are shown in Table 1. Twenty-one days after a primary immunization with Fd, levels of specific antibody average about 3.0 µg/ml. In the secondary response, measured 7 days after injection of Fd this average titre rises to about 12.0 µg/ml. In comparison to typical antibody titres found after immunization with commonly studied antigens (NP, ARS) these titres are much lower. Given the molecules' low molecular weight and its limited number of antigenic determinants this moderate level of immunogenicity is not surprising. Anti-hapten antibody titres of up to 5.0 mg/ml have been obtained in mice after immunization with hapten coupled to large molecular weight carriers (17-18). Production of such large amounts of antibody presumably reflects the magnitude of T cell help provided by the action of carrier specific T lymphocytes. In comparison, the Fd antigen can be viewed as a molecule which consists of two covalently linked haptenic groups. According to the rules of hapten carrier interactions there can be only a single carrier moiety providing help for the alternate determinant and antibody responses to Fd may therefore reflect this

Table 1: Characteristic antibody titres in B10.BR (responder mice). B10.BR mice were immunized with Fd and bled following a primary (day 0) or secondary (day 28) antigen stimulus. Anti-Fd titres are expressed as a mean value for 6 animals.

Stage	Time of Bleed	µg/ml anti-Fd±S.E.M.
1° response	day 21	3.4±0.7
2° response	day 35	11.9±3.2

limitation.

Quantitative isolation of milligram quantities of anti-Fd antibody which would be required for the preparation of an anti-idiotype in either mice or rabbits was precluded by the low antibody titres obtained. It was also felt that a more definitive analysis of the idiotypic repertoire and the role of individual idiotypes in immune regulation could be obtained by isolating monoclonal anti-Fd antibodies using the cell fusion technique of Kohler and Milstein (17). In a preliminary study it was found that by using standard protocols, the frequency with which specific anti-Fd secreting hybrid clones could be obtained was considerably lower than was obtained using a more immunogenic antigen such as KLH (mw $3x10^6$). In order to increase this frequency, a different immunization schedule was designed. This protocol was based upon an adoptive transfer system in which mice were given a secondary injection 5 days before adoptive transfer into sublethally irradiated syngeneic recipient mice. Following spleen cell transfer, recipients were rested for 10-14 days. A third injection of Fd was then given, and the spleens were removed 3 days later for cell fusion. Using the adoptive transfer protocol, a marked increase in the yield of specific anti-Fd producing clones was obtained. The isolation and characterization of two hybridomas derived from an adoptive transfer as well as an earlier fusion have been described (44). Briefly, these two hybridomas, Fd-1 and Fd-2, were obtained in fusions between spleen cells from Fd immune B10.BR mice and the SP2/0 myeloma cell line. Because the parental myeloma does not synthesize a cryptic light chain, the antibodies produced by the two hybridomas contains no heterologous light chain and are therefore strictly monoclonal. An antigen binding titration comparing the two monoclonal antibodies with anti-Fd serum is presented in Figure 2.

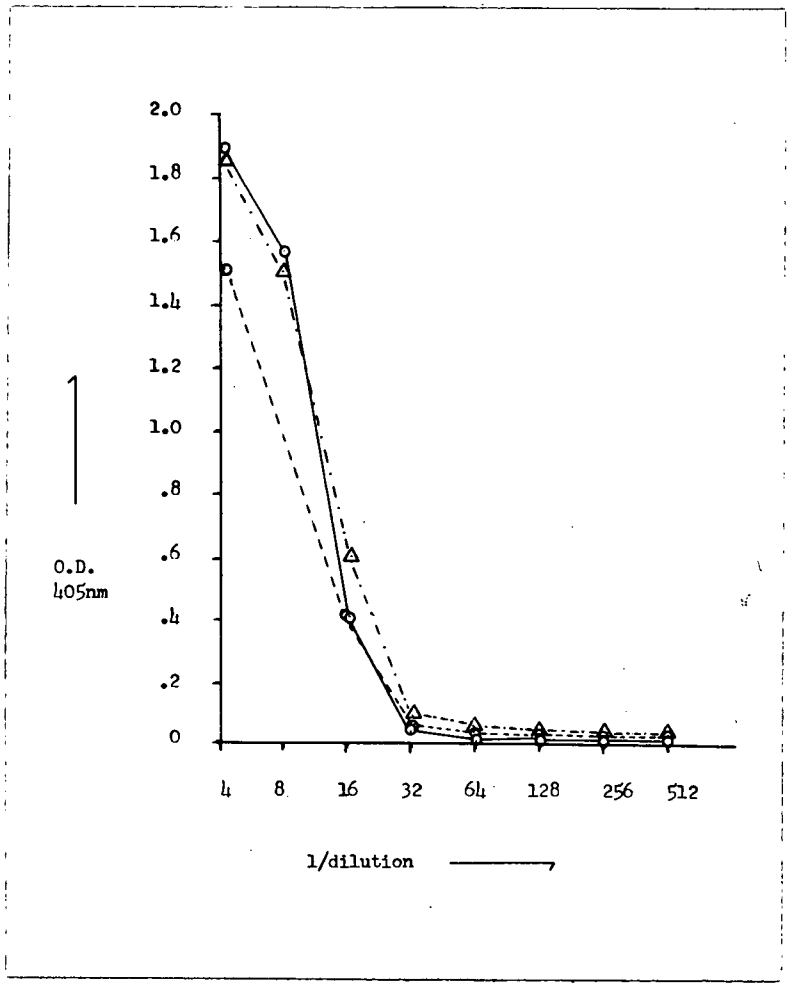

Figure 2: Titration of anti-Fd antibodies by ELISA. Samples of B10.BR anti-Fd serum (○—○), Fd-1 monoclonal antibody (△···△) and Fd-2 monoclonal antibody (○···○) were incubated on Fd-coated ELISA plates at the indicated relative dilution. The lowest dilution represents a concentration of 10% serum (v/v) or 16 μg/ml of purified monoclonal antibody.

Binding specificity of monoclonal anti-Fd antibodies

At the molecular level, Fd exhibits properties which are amenable to immunochemical analysis. Located within each of those regions which have been identified as antigenic determinants are sites susceptible to hydrolysis by specific proteolytic enzymes. As the molecule possesses a single lysine residue, it is susceptible to trypsin cleavage at only one peptide bond, between lys-3 and ala-4. Trypsin cleavage thus effectively destroys the NH_2-determinant while leaving the remaining 52 residues of the molecule intact. Similarly, carboxypeptidase A can remove the two COOH-terminal amino acids, but will not degrade the molecule past val-53 because of the presence of proline at position 52. This digestion process then effectively removes a significant part of the COOH-terminal antigenic determinant. Digestion of whole Fd with either of these selective enzymes results in a molecule which retains 52 of its original 55 amino acid residues, but possess only a single antigenic determinant (66). By using these monospecific fragments of Fd in an inhibition assay in the ELISA the Fd-1 antibody was shown to be specific for the NH_2-determinant and the Fd-2 antibody was found to bind the COOH-determinant Figure 3 and Figure 4. (These figures and the digestion protocol are included with the kind permission of Lydia Sikora).

Raising and immunoadsorption of antisera to monoclonal anti-Fd antibodies

Adult New Zealand white rabbits were immunized with protein A purified monoclonal anti-Fd antibody as described in Materials and Methods. Following the fifth injection, these animals were bled and tested for reactivity towards mouse immunoglobulins using a standard ring precipitin test against normal B10.BR serum. High antibody titres (2 mg/ml) were generally present and the rabbit antisera were first extensively adsorbed on an immunoadsorbent prepared with mouse IgG as described in Materials and Methods. The immunoadsorbent bed

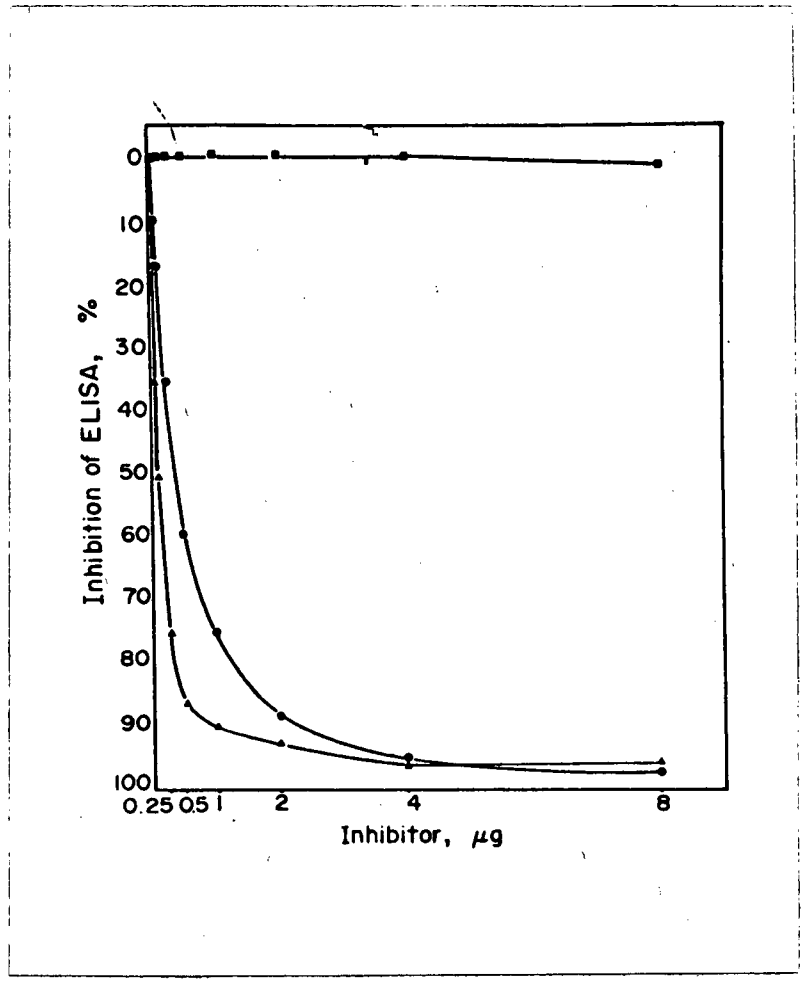

Figure 3: Blocking ELISA carried out with the Fd specific monoclonal antibody, Fd-1, at a concentration of 0.5 μg/ml of affinity purified antibody. The antibody was incubated overnight with dilutions of native Fd (△—△), N-fragment (○—○) or C fragment (□—□) and tested on ELISA plates coated with native Fd at 4.0 μg/ml. The test was performed in quadruplicate.

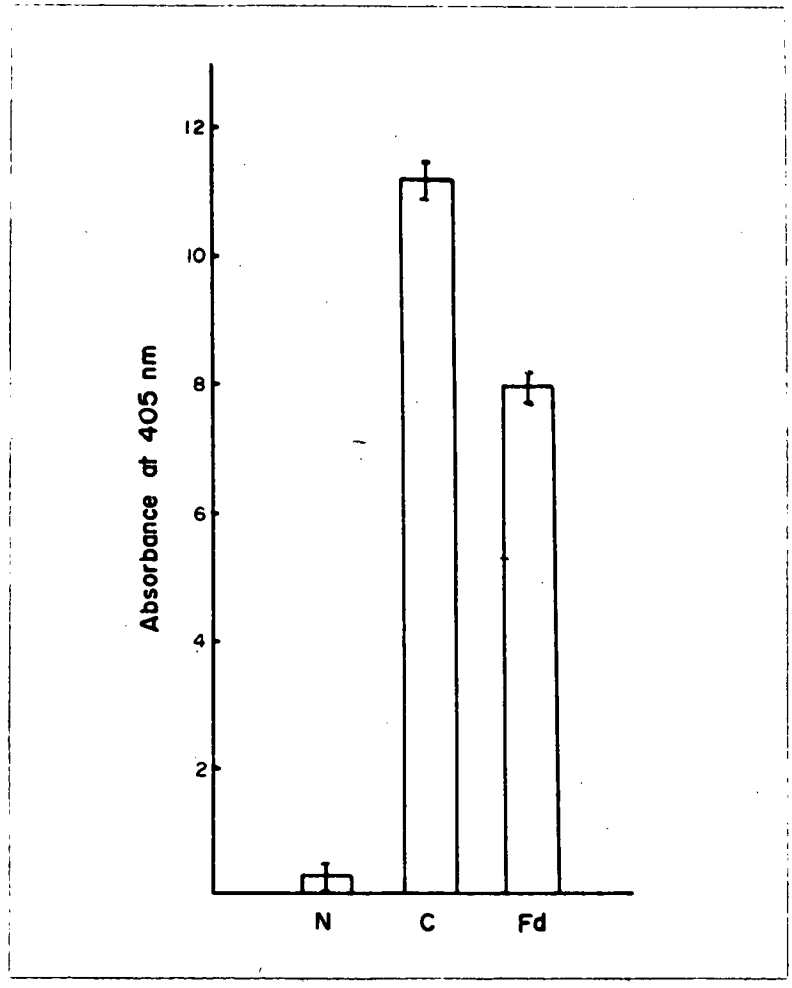

Figure 4: The binding of hybridoma Fd-2 to either the N- or C-fragment, or to whole Fd on ELISA plates. Antigen or fragments were affixed to plates at 4 μg/ml and monoclonal antibody was reacted for 30 min. at a concentration of 0.5 μg/ml. The test was performed in quadruplicate and demonstrates the inability of Fd-2 to bind the N-determinant, even at 5.0 μg/ml which constitutes levels of ten-fold molar antibody excess.

volume used was sufficient to remove 15 mg of anti-mouse IgG activity from hyperimmune serum. Antisera to the monoclonal antibodies were adsorbed three times on this column and all assays to characterize their anti-idiotypic specificity were run in the presence of 5% v/v of normal B10.BR mouse serum to preclude the possibility that the specificity of the antisera were directed towards non-idiotypic immunoglobulin determinants. Under these conditions, no reactivity of either adsorbed antiserum towards normal B10.BR immunoglobulins has been detected.

Differences in amino acid composition of hypervariable regions effect changes in combining site structure which are responsible for different binding specificities of antibody molecules. Structural evidence has indicated that amino acid substitutions are found in both combining site as well as areas adjacent to this portion of the molecule. Jerne recognized that since some of the idiotypic determinants (idiotopes) could be located within or immediately adjacent the antibody combining site, anti-idiotype antibodies (anti-idiotopes) should demonstrate the property of blocking the binding of antigen. A special term, paratope, was reserved for idiotopes which were located within the combining site. Combining site as well as non-combining site related idiotopes have recently been demonstrated using monoclonal anti-idiotype reagents (19). In the case of a polyvalent anti-idiotype anti-serum some degree of paratopic specificity would be expected as the antiserum should recognize a variety of individual idiotopes. This factor was therefore taken into consideration during the characterization of the anti-idiotypic antisera to the monoclonal anti-Fd antibodies.

Characterization of anti-idiotype antiserum to Fd-1:

The specificity of the anti-idiotype antibody was initially tested by assessing its ability to inhibit the binding of the monoclonal Fd-1 to Fd in

the ELISA. Controls were run using soluble Fd with Fd-1 and show that both Fd and the anti-idiotype inhibit binding of the monoclonal antibody Fd-1 to Fd coated plates (Fig. 5). To demonstrate the specificity of this inhibition, equivalent amounts of the anti-idiotype were tested with B10.BR anti-serum directed to KLH. No inhibition by the anti-idiotype was noted in these studies, indicating that the anti-idiotype was directed specifically towards anti-Fd antibody (Fig. 6).

To determine whether the anti-idiotype antibody was directed specifically to the antigen binding sites of the Fd-1 antibody, an ELISA was carried out in which Fd-1 was bound to the solid phase of ELISA plates and the binding efficiency of the anti-idiotype was assayed in the presence of increasing amounts of soluble antigen (Fd). As can be seen in Fig. 7, the binding capacity of the anti-idiotype was substantially blocked in the presence of antigen, indicating that a major portion (approx. 70%) of the anti-idiotypic activity is directed to regions of the Fd-1 antibody involved in or adjacent to antigen binding.

In order to establish a correlation between the N-determinant binding specificity of the monoclonal Fd-1 and its anti-idiotype, an anti-idiotype immunoadsorbent column was prepared and used to select antibodies from immune serum of B10.BR mice. The material isolated in this way was then analysed for both idiotype expression and determinant binding specificity. Results of this study are presented in Fig. 8. This experiment shows that the anti-idiotype column specificially selects N-determinant binding antibody from immune serum. That a small proportion of this antibody is inhibited at the highest levels of C-determinant may reflect the possibility either that trypsin digestion of this preparation may not have been carried to completion and therefore would conceivably be contaminated with small quantities of the N-determinant or, that the determinant recognized by Fd-2 extends somewhat beyond the lysine residue such that

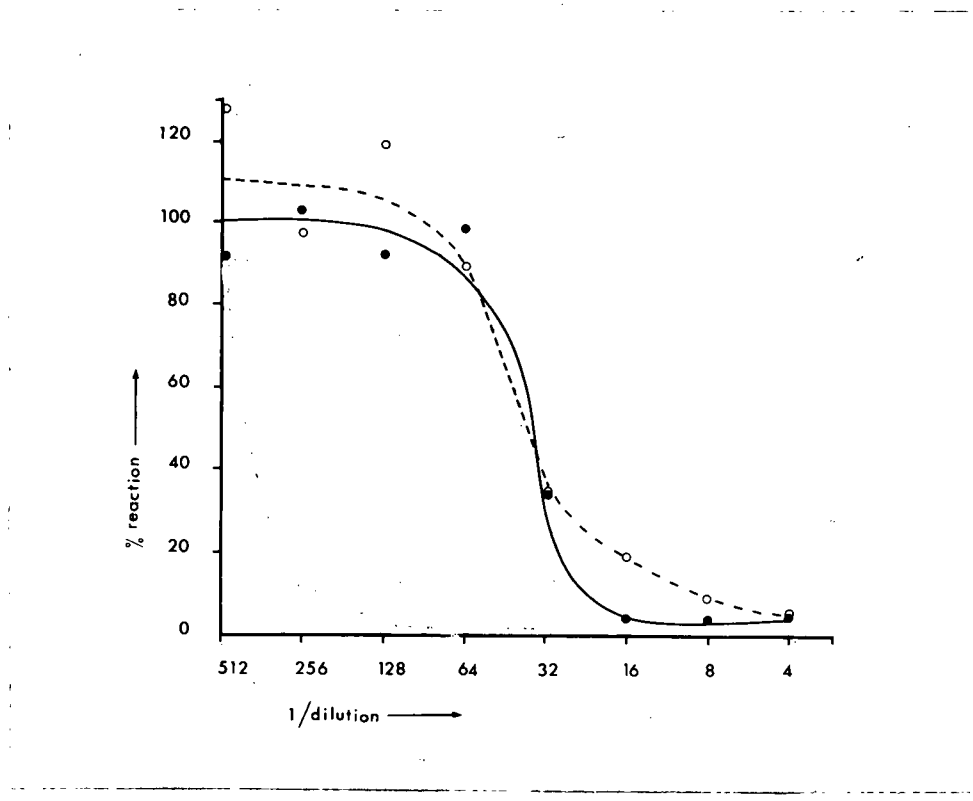

Figure 5: Competitive inhibition of monoclonal antibody Fd-1 by antigen and anti-idiotype. Inhibition of binding to Fd coated plates was measured in the presence of inhibitors, either Fd or rabbit anti-Fd-1, as described in Materials and Methods. Percent binding of Fd-1 in the presence of inhibitors is expressed relative to controls incubated in the presence of NRS.

Inhibitors: (●—●) rabbit anti-Fd-1 serum. (○···○) Fd 100 μg/ml.

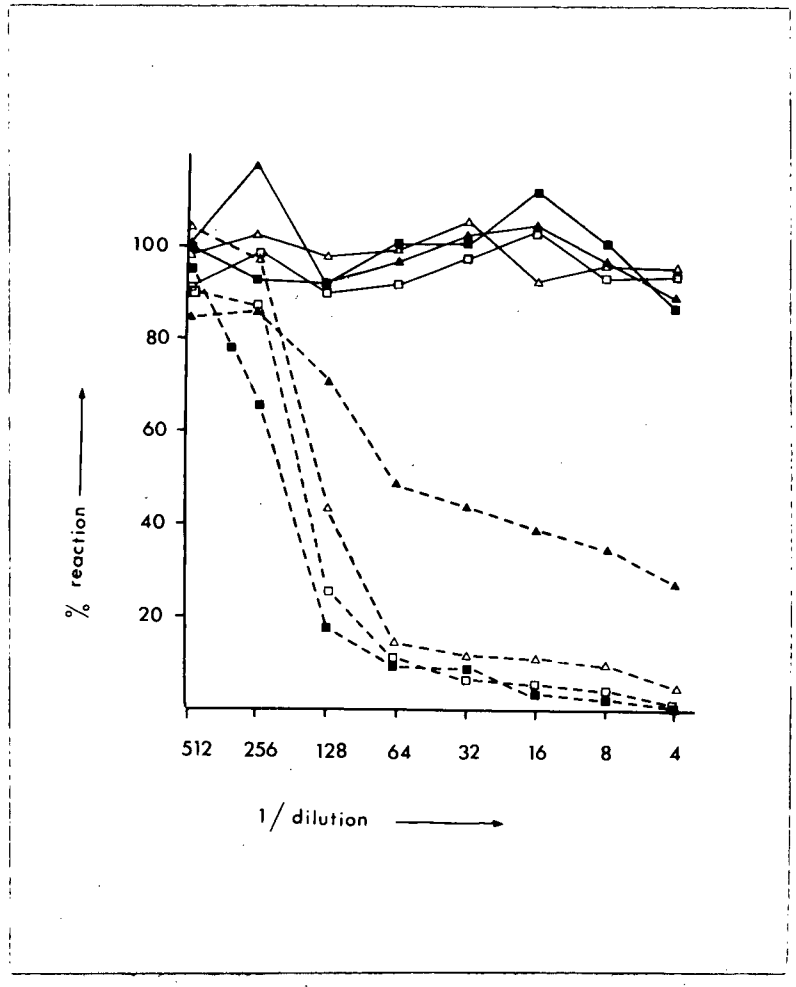

Figure 6: Lack of inhibition of serum and monoclonal antibodies to KLH by anti-idiotype serum anti-Fd-1. Inhibition of serum and monoclonal antibody binding to KLH was measured as described in Materials and Methods. Values plotted represent average of samples run in triplicate. (▲—▲) B10.BR anti-KLH serum; (□—□) B10.BR IgG_3 monoclonal anti-KLH; (■ ;△) B10.BR IgM monoclonals anti-KLH. Solid lines show results of the reaction of the various antisera or monoclonal antibodies in the presence of anti-Fd-1 in which no inhibition is seen. Dotted lines show results for equivalent antisera in the presence of increasing amounts of KLH.

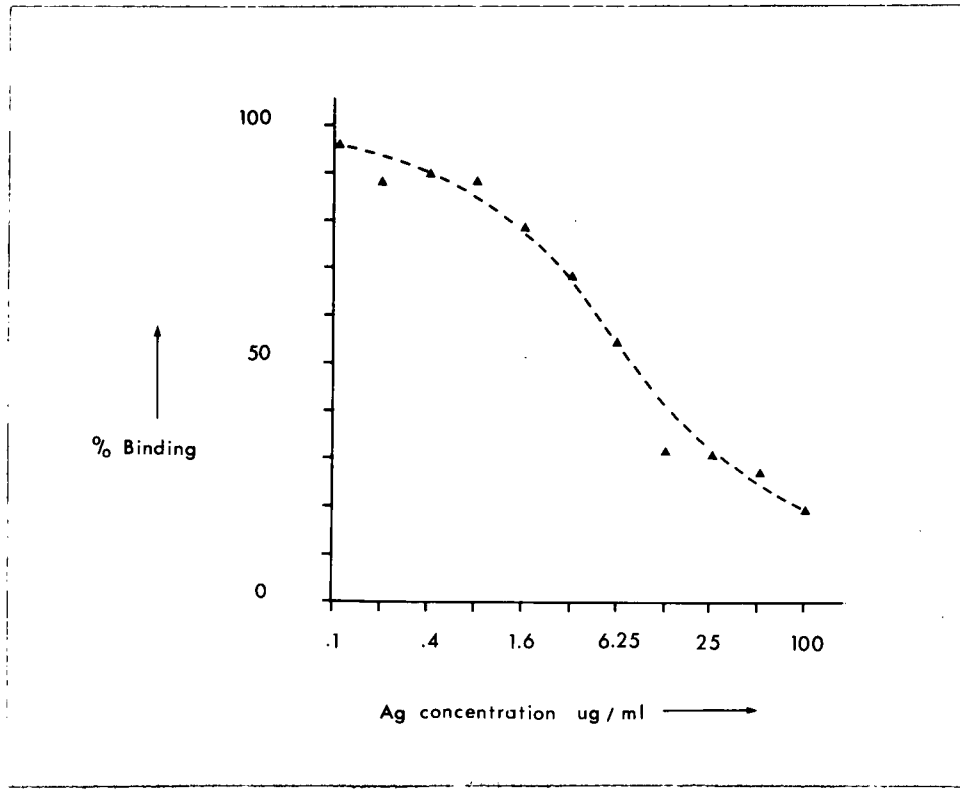

Figure 7: Competitive inhibition of anti-Fd-1 idiotype by Fd. Binding of anti-Fd-1 to Fd-1 coated plates was measured in the presence of Fd as outlined in Materials and Methods. Percent binding of anti-Fd-1 in the presence of Fd is expressed relative to a control incubated in the presence of equivalent concentrations of KLH.

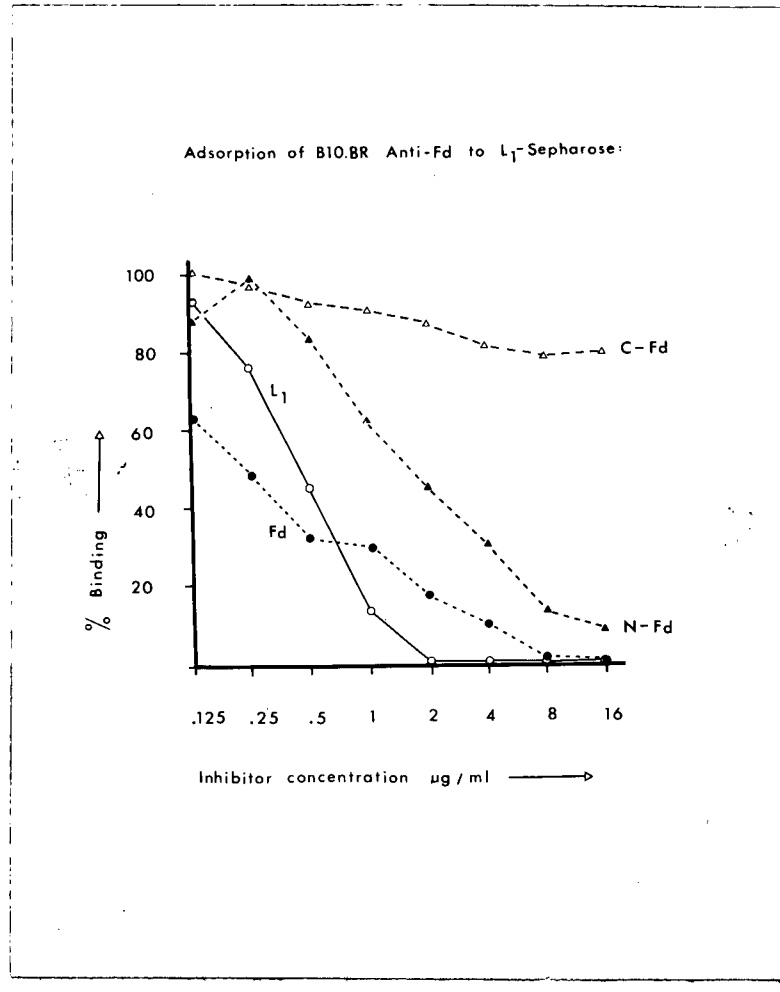

Figure 8: Specificity of anti-idiotype selected serum antibody. Serum antibodies to Fd were isolated over an anti-Fd-1 anti-idiotype column and assayed for percent binding to Fd coated plates in the presence of soluble inhibitors. Percent binding of antibody in the presence of each inhibitor was calculated according to a specificity control consisting of equivalent concentrations of purified normal rabbit IgG. Points shown represent the mean of triplicate samples. Standard deviations between replicates (not shown) were in all cases less than 2% (●--●) Fd; (▲--▲) N-Fd; (△--△) C-Fd; (○—○) Affinity purified rabbit anti-Fd-1.

even after trypsin digestion Fd-1 retains a small degree of affinity for this portion of the molecule.

Characterization of anti-idiotype antiserum to Fd-2:

The adsorbed rabbit antiserum raised to Fd-2 was also tested in an ELISA for its ability to inhibit the binding of Fd-2 to Fd coated plates (Fig. 9). Specificity of anti-Fd-2 serum for Fd-2 is indicated in Figs. 10 and 11. In Fig. 10, anti-Fd-2 was tested in an ELISA for its ability to block Fd-2 binding to Fd coated plates. Strong inhibition was seen in the presence of anti-idiotype serum as well as an affinity purified fraction of anti-Fd-2 antibody. In Fig. 11, anti-Fd-2 was tested in an ELISA to determine whether it specifically bound Fd-2 alone, or exhibited cross-reactivity with Fd-1 also. As measured in both the binding inhibition assay (Fig. 10) or in the direct binding assay (Fig. 11) no cross-reactivity of anti-Fd-2 with Fd-1 was detected. In order to determine if this idiotypic specificity of the antiserum recognized a combining site related determinant, the degree of competition between Fd and anti-Fd-2 for the Fd-2 antibody was measured in an ELISA. The results of this assay are presented in Fig. 12. Approximately 45% of the anti-idiotype is prevented from binding to Fd-2 in the presence of Fd, indicating that a significant proportion of the anti-idiotype detects determinants closely associated with the antibody combining site.

Discussion

This chapter has presented data concerning the immunochemical characterization of the anti-Fd response in mice. This antigen has previously been analyzed in both guinea pigs and rabbits (40). Studies using antigenic peptides synthesized by the solid phase Merrifield technique showed that individual antigenic determinants could be related to small peptides of defined sequence

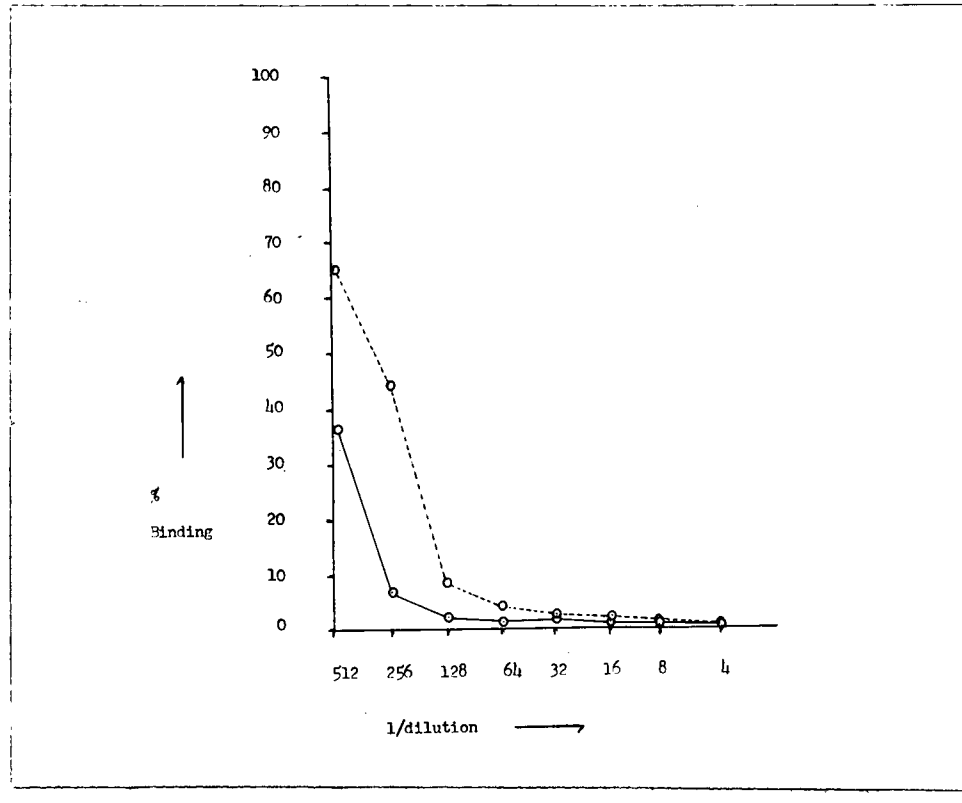

Figure 9: Inhibition of Fd-2 binding to Fd in the presence of anti-Fd-2. Dilutions of anti-Fd-2 serum (O—O) or affinity purified antibody (O---O) 16 μg/ml, were co-incubated with a standard concentration of Fd-2 antibody (2 μg/ml final) prior to loading onto Fd-ELISA plates. Percent binding of Fd-2 is expressed relative to an assay control.

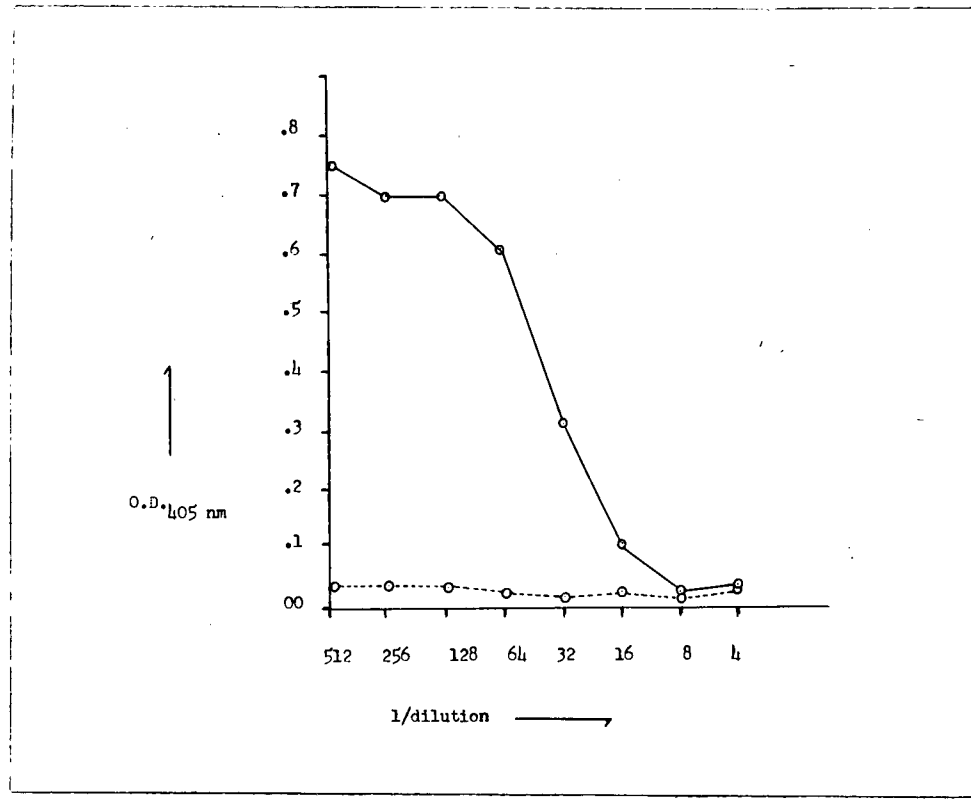

Figure 10: Specificity of anti-Fd-2 serum for Fd-2 antibody. Specificity was assessed by direct binding to idiotype coated plates. Dilution of anti-Fd-1 (O—O) or anti-Fd-2 (O---O) serum plus normal mouse serum were incubated on an ELISA plate precoated with Fd-2 antibody. Binding of rabbit antibodies was measured with a sheep anti-rabbit Ig enzyme conjugate.

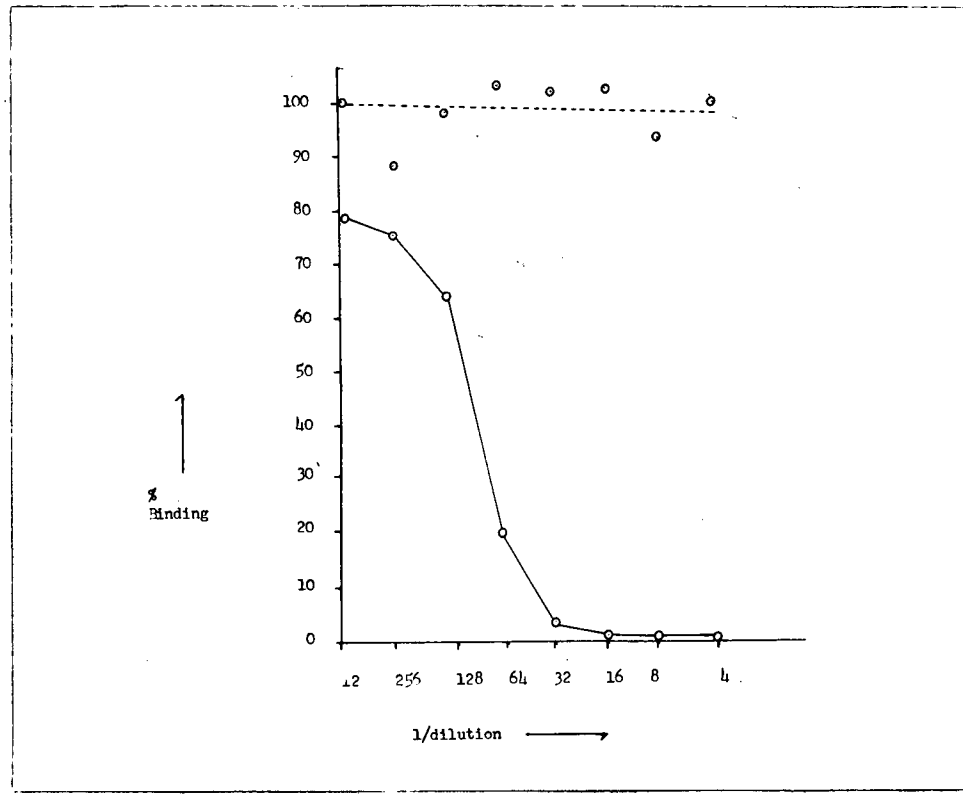

Figure 11: Specificity of anti-Fd-2 serum for Fd-2 antibody. Binding of Fd-1 antibody (2 μg/ml) to Fd coated plates was measured in the presence of anti-Fd-1 serum (O—O) or anti-Fd-2 serum (O···O).

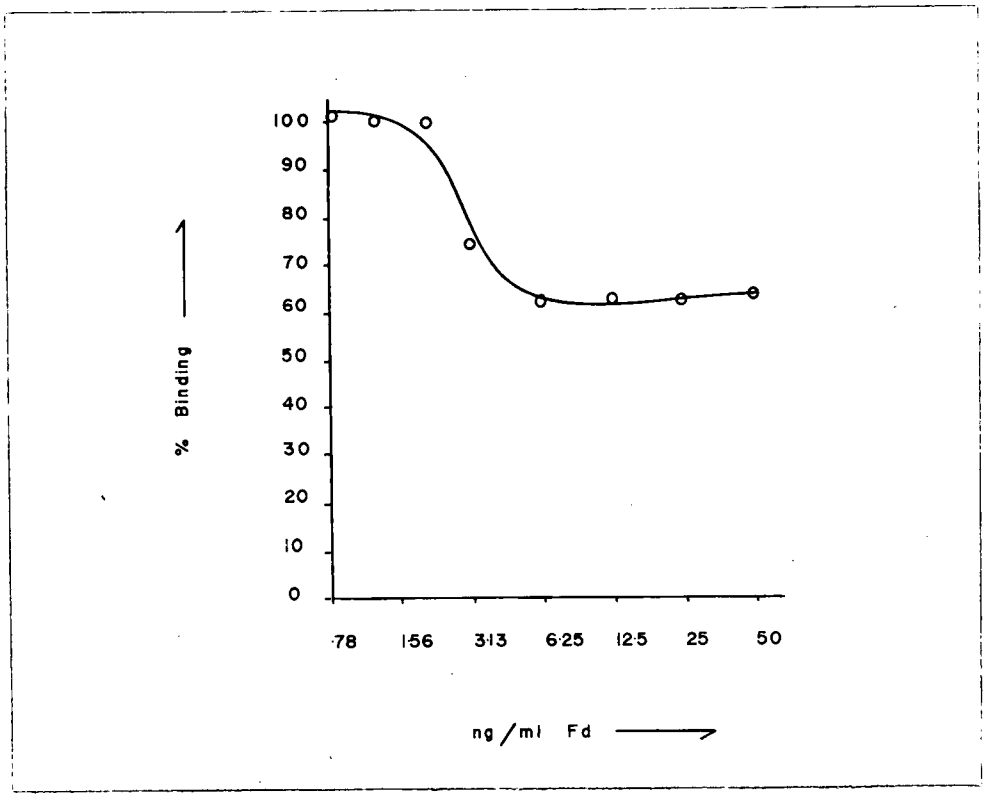

Figure 12: Competitive inhibition of anti-Fd-2 by antigen. Binding of anti-Fd-2 to Fd-2 coated plates was measured in the presence of Fd as outlined in Materials and Methods. Percent binding of anti-Fd-2 in the presence of varying quantities of Fd is expressed relative to a non-specific control (KLH).

(41). These conclusions were reached using the available techniques of antibody measurement and lymphocyte transformation assays.

Monoclonal reagents permit one to design highly specific assay systems. Using an ELISA the binding of proteolytically degraded fragments of Fd showed that the two Fd monoclonal antibodies described here were directed to separate determinants. The existence of unique N- and C-determinant binding antibodies confirms assignments which were made in earlier Fd studies. While the cell fusion technique yields antibody products which may not accurately represent the normal repertoire in serum and would therefore not rule out the existence of additional determinants, no evidence was found in previous studies to suggest that these might exist in significant quantities.

By using a conventional protocol, rabbit antisera were raised to each of these monoclonal antibodies. After the appropriate absorptions, it was shown that a portion of the remaining activity was specific for combining site related determinants of the hybridoma protein. These determinants were found to be unique to the hybridoma in question as no reactivity of anti-idiotype with antibody directed towards a dissimilar antigen was detected. The anti-idiotypes thus defined can therefore be used to investigate questions concerning the predominance of a particular idiotype in serum, and the presence if any, of similar idiotypes on receptors of cells involved in regulating the immune response to Fd. These issues are discussed in two chapters first from the point of view of the Fd-1 antibody (chapter 3), followed by studies based on the Fd-2 antibody (chapter 4).

CHAPTER 3: CELLULAR EXPRESSION OF Fd-1 IDIOTYPE

Monoclonal antibodies have proven to be useful as probes in the study and mapping of individual antigenic determinants on complex molecules. The specificity of these reagents has enabled researchers to obtain precise information on the location of specific epitopes on experimental antigens. Assignments of antigenic determinants have been made on the basis of reactivity of antibody with enzymatically cleaved fragments of the native antigen, cross reactivity of antibody for polymorphic forms of the same antigen or deductively, on the basis of cross reactivity of antibody for synthetic analogues (54-56). One factor which these studies fails to account for however, is the role of tertiary structure in the recognition of specific antigenic determinants by antigen reactive lymphocytes. The process by which B cells recognize antigen including the structure and genetics of the immunoglobulin receptor which it displays has been well characterized for many years. Although a large amount of work has, in the past decade, been directed towards elucidation of the equally specific receptor on the T cell, many questions remain unanswered. The most definitive work in this area has utilized small haptenic molecules as antigens rather than the more complex polypeptides. With use of these haptens it has been shown quite convincingly that specific T cells bear idiotypic determinants which react with anti-idiotypic antisera raised against the major idiotypes expressed in the serum of immunized animals (57,58). It would appear that when such sterically stable structures as these haptens are used as antigens, both reactive B and T cells share common idiotypy, presumably by way of similarities in the structure of their respective antigen receptors. While it is generally accepted that V_H markers can be found on T cells, no V_L markers have yet been detected. With regard to B and T cell recognition of more complex antigenic

structures, the information is not as clear cut. Immunoglobulin receptors on B cells are dependent for their interaction not only on the primary structure of an antigen epitope but also on its secondary and tertiary structure. At the T cell level of recognition, there are a number of examples in the literature indicating that secondary and tertiary structures may not be of great importance. Thus it has been found that antibodies raised to native lysozyme do not cross-react with its reduced and carboxymethylated derivatives, while cell-mediated immunity was cross-reactive regardless of whether animals were immunized with native or denatured material (59). Similar observations were made by Parish using native and chemically modified flagellin (60). More recently, studies on insulin and it's antigenic peptides have shown essentially the same results (61). There are at least two explanations for these data: either B cells (or Ig) recognize determinants which are conformation dependent while T cells recognize different determinants which are not configuration dependent and are distinct from those recognized by B cells, or the same determinants are recognized by both cell types but the T cell receptor is not as configuration dependent as is the B cell receptor.

Studies on the commonly used hapten systems have provided useful information regarding the role of idiotypic interactions between T and B cells recognizing the same determinant. In general, idiotypic interactions have so far dealt only with responses towards individual epitopes whereas in classical immunology, immune responses are primarily based upon a cooperative association between clones of lymphocytes specific for different epitopes of a complex antigen molecule. Where idiotypic regulation can so demonstrably regulate immune responses towards a given epitope, then it is conceivable that the effects may also influence the outcome of responses towards other idiotypically unrelated, but structurally associated determinants on the same

molecule. Associative recognition models of cell cooperation were originally based on the premise that T and B cells did not recognize the same determinants of an antigen (62). Idiotypic studies have since shown that this is not the general case, hence the distinction between haptenic and carrier determinants may only be a relative one. It would seem unlikely that idiotypic recognition could function separately, and in isolation of, interactions based on hapten-carrier recognition. These two modes of lymphocyte communication might be more closely interrelated. Predictable as this hypothesis may be, it has yet to be verified experimentally.

Chemical haptens have served as useful models of lymphocyte recognition although these are not intrinsic antigenic epitopes, and must be conjugated to large molecular weight carriers in order to become immunogenic. It is presumed that this provides sufficient T cell help for hapten specific B cells. In view of the demonstrated ability of T cells to recognize hapten according to idiotypic studies, this interpretation may be incorrect in the sense that the crucial interaction missing from this example may not lie in the absolute specificity of individual T and B cells (polarity) but in the manner in which they are connected. The emphasis of hapten research has been placed strictly on the outcome of idiotypic manipulation on the response to the hapten itself. Considering the complexity of the macromolecular immunogen however, responses to the specific hapten represents a small component of a potentially very heterogeneous immune response. Were it not for this fact, it might be possible to study the influence of idiotypic manipulations involving the hapten on the responses towards other determinants of the immunogen. The complexity of most protein antigens precludes fractionation of the entire molecule into defined antigenic portions particularly if the molecule were large enough for tertiary structure to play a significant role. Idiotypic probes on the other hand do constitute a highly specific marker against which

responses to the whole antigen may be monitored. This is especially relevant to the case of Fd as it consists essentially of two haptenic groups for which specific monoclonal antibodies have been defined.

Materials and Methods

Radioimmune assay for idiotype

Idiotype expression in mouse serum was measured using a solid phase radioimmune assay. Affinity-isolated anti-idiotype antibody was used at a concentration of 1 μg/ml in pH 9.6 carbonate buffer for a period of 1 hour at 37°C. Subsequently, coated plates were washed with PBS-Tween buffer and incubated for 15 minutes with PBS-Tween buffer containing 0.1% by weight of bovine serum albumin (Sigma) and normal B10.BR serum (.25%). Plates were then washed in PBS-Tween buffer prior to incubation with assay samples. Test samples of immune sera at a dilution of 1/40 in PBS-Tween buffer and control samples of non-immune sera containing added quantities of monoclonal Fd-1 antibody ranging from 1 ng - 1280 ng/ml were dispensed in triplicate to the wells of prepared plates. After 60 min incubation at room temperature, the plates were washed, and then incubated as before with 0.1 ml/well of a solution of ^{125}I-labelled Fd-1 monoclonal antibody labelled by the chloramine T procedure (63) using a total of 150,000 cpm/well. After 60 min incubation, the plates were washed, and the individual wells were cut out using a single edged razor blade and the amount of radioactivity bound per well was counted in a Beckman Biogamma radioisotope counter. A standard inhibition curve was constructed using cold Fd-1 monoclonal antibody as a competitor. Percent inhibition was calculated using cpm/well in the presence of normal mouse serum as zero, and taking cpm in the lower plateau portion of the curve as 100% inhibition. Percent inhibition of binding in the presence of test sera was then read directly from this standard curve. Values obtained were corrected

for dilution and are expressed as nonogram equivalents of idiotype per ml of serum.

In vivo administration of anti-idiotype

Biological effects of anti-idiotype were assessed in whole animals by injection of quantities of anti-idiotype intraperitoneally into non-immune mice. In early experiments the biological effects of anti-idiotype was assessed in groups of mice given injections of anti-idiotype or normal mouse Ig in comparison to mice whic h received no injections prior to antigen. One week following injection of anti-idiotype, mice were immunized with 50 ug Fd in CFA intraperitoneally on day 0. Twenty-one days after priming, they were bled and titres of anti-Fd antibody as well as relative amounts directed to the N- or C-determinant were measured. Specific idiotype was determined by ELISA and RIA. In later experiments quantities of whole anti-idiotype serum were substituted in place of affinity isolated antibody.

Adoptive transfer of anti-idiotype treated spleen cells

Spleen cells from either immune or non-immune B10.BR mice were resuspended in anti-idiotype serum at a concentration of 12 ug idiotype binding capacity per ml, or in an equal dilution of normal rabbit serum which had been adsorbed against mouse Ig for a period of 45 min. on ice. Cells were then washed three times with Dulbecco's modified Eagle medium (Gibco) and resuspended in rabbit complement (Lowtox, Cedarlane) at a final dilution of 1/5 in medium and incubated for 45 min. at 37^{o} C. Cells were then washed three times in medium, adjusted to a concentration of 2 x 10^{8} cells/ml and injected intraperitonally (0.1 ml/mouse) into normal B10.BR mice which had received 500 rad whole body irradiation from a gamma source (Gammacell 200, Atomic Energy of Canada) 24 hr. previously. Immediately after cell transfer, recipient mice were given an intraperitoneal injection of 50 ug Fd in CFA. In some experiments animals were also immunized

with KLH (100 µg) in CFA. Antibody titres of recipient mice were determined from sera taken 21 days post transfer when non-immune cells were used while titres of transferred immune spleen cells were determined from sera taken 7 and 21 days post-transfer. In some experiments, spleen cells were first fractionated before treatment with anti-idiotype or normal rabbit serum. T lymphocytes were enriched from suspensions of whole spleen cells using the technique of nylon wool adherence. Nylon wool columns were prepared according to the method of Julius et al. (64). A total of 3×10^8 spleen cells were passed over nylon wool (20g) packed to a volume of 20 ml into a 30 ml syringe barrel. Cell recovery using this procedure varied between 18-27%. T cell depleted spleen cell suspensions were prepared using treatment with a monoclonal anti-Thy 1.2 antibody (gift of Dr. H-S Teh) plus rabbit complement. Spleen cells were resuspended in 200 µg/ml of anti-Thy 1.2 antibody in culture medium at a density of 10^8 cells/ml and incubated at 4°C for 30 min. The cells were then washed, resuspended in the equivalent volume of medium containing a source of rabbit complement (Low Tox, Cedarlane) 20% v/v, and incubated for 30 min at 37°C. The cells were then washed three times in culture medium and counted. Viable cell recovery under these conditions ranged from 52-57%.

^{51}Cr release cytotoxicity assay

Two target cell lines were utilized in an assay designed to measure the complement dependent cytotoxic activity of anti-idiotypic antiserum anti-Fd-1. The lines in question were both hybridoma cell lines producing monoclonal anti-Fd antibody Fd-1 and Fd-2 as described in the text of the thesis. Target cells were obtained from the ascites fluid of mice in which the particular hybridoma cell line was being passaged. These cells were labelled using the following procedure: 2×10^6 cells were incubated with 250 µCi $Na^{51}CrO_4$ (New

England Nuclear) in a volume of 0.5 ml in 10% DMEM for 1 hr at 37°C with occasional shaking. The cells were then washed twice in medium and reincubated for a further 30 minutes. Labelled cells were then washed twice more, counted, and resuspended to a concentration of 10^6/ml in culture medium. At the same time, doubling dilutions of normal rabbit serum and anti-Fd-1 serum were prepared in culture medium in U-bottom 96 well microtitre plates (Dynatech). A total of ten microlitres of labelled cell suspension containing 10^4 target cells was then added to each well and the plates were incubated for 45 minutes at 4°C. The cells were then pelleted by centrifugation, washed once in .2 ml/well of ice-cold culture medium and resuspended in .15 ml of culture medium containing a source of rabbit complement (Low-Tox, Cedarlane Labs) at a final concentration of 10% v/v. The plates were incubated in the presence of complement for 45 minutes at 37°C and then centrifuged. Aliquots of the supernatant fluid (.1 ml) were removed for counting of soluble isotope in a radioisotope counter (Beckman Biogamma). Specific cytotoxicity was calculated in relation to two controls: (a) spontaneous release (complement only) and (b) 100% release (soluble in Triton buffer) using the following formula:

$$\% \text{ Specific cytotoxicity} = \frac{\text{Test} - \text{Spontaneous}}{100\% - \text{Spontaneous}} \times 100\%$$

Results

Preliminary characterization of idiotype expression

Previous immunogenetic studies have shown that the proportion of antibody in serum directed towards the N- and C-determinants of Fd varies between different strains of mice (66). Using sera from individual mice of two representative strains which had been titrated for N- and C-determinant reactive antibody, an attempt was made to determine the relative amounts of idiotype expressed in immune serum. Individual sera which had previously been

titrated and assayed for N-determinant antibody activity were tested with the anti-idiotype to determine the extent to which they could be inhibited. Varying quantities of the anti-idiotype or equivalent concentrations of normal rabbit serum were incubated with constant amounts of previously titrated antisera. These were subsequently tested for their ability to react with Fd in the ELISA. Percent inhibitions of N-determinant reactivity were estimated on previous calculations of N-determinant specific antibody present in each antiserum sample (44). Results are shown in figure 13. In B10.BR mice, in which N-determinant reactive antibody is relatively low (between 20 and 30% of total antibody), inhibition by the anti-idiotype serum ranged from 0-72%. In three of the six individual antisera tested, at least 50% of the N-determinant specific antibody was inhibitable by the anti-idiotype. In B10.S mice, in which N-determinant specific antibody in the anti-Fd population in whole serum is approximately 65%, a similar pattern is observed. This indicates that the rabbit anti-idiotype serum is reacting with a significant amount of N-determinant specific antibody in both these strains of mice.

The results show that the Fd-1 idiotype constitutes a variable, but significant proportion of N-determinant specific antibody in individual mice and that the idiotype is conserved between two strains of mice which share identity at the Ig-1 locus. While this form of competitive binding assay yields useful qualitative information on idiotype expression, it is not particularly suited to screening large numbers of samples. It was also felt that a more direct measure of idiotype expression could be obtained using a quantitative radioimmune assay.

Description and standardization of RIA

Anti-Fd-1 anti-idiotype was therefore used in a solid phase radioimmune assay in order to survey expression of the specific Fd-1 idiotype amongst individual mice of the B10.BR as well as in other related strains. Anti-

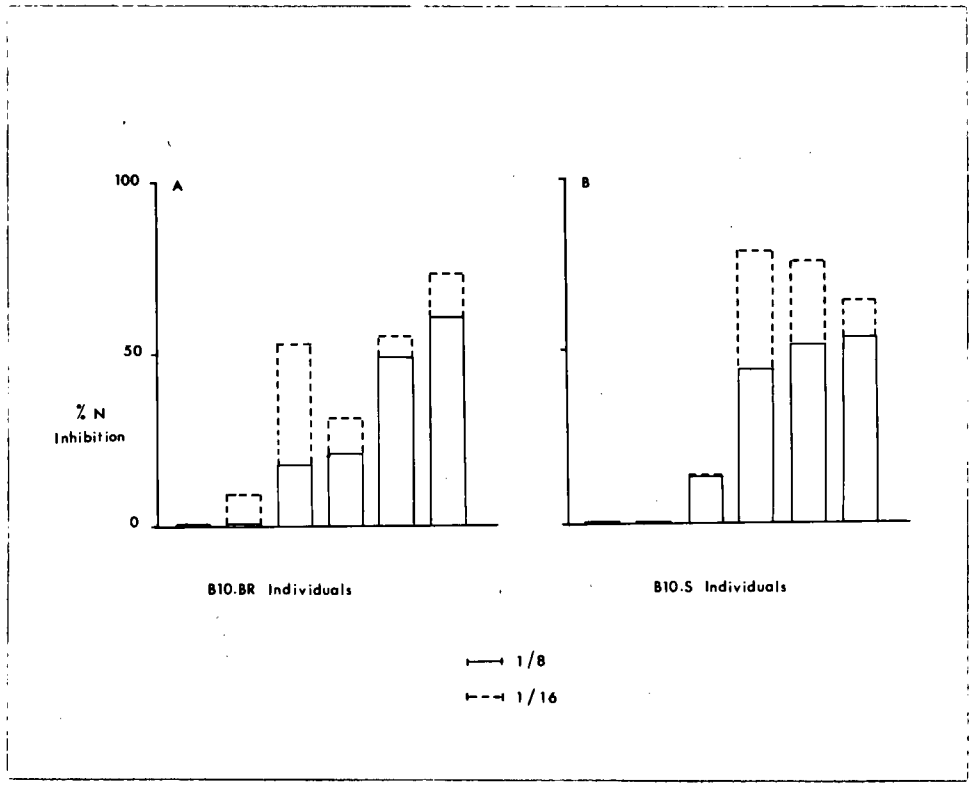

Figure 13: Inhibition of N-determinant directed antibodies by anti-idiotype to Fd-1. Binding of individual anti-Fd serum samples to Fd coated plates was assayed in the presence of rabbit anti-idiotype. Percent inhibition of N-determinant specific antibodies was calculated as described in Materials and Methods.

(A) Individual serum samples from B10.BR mice.

(B) Individual serum samples from B10.S mice.

Dotted lines indicate results obtained at serum dilutions of 1:8 and solid lines, results obtained at 1:16.

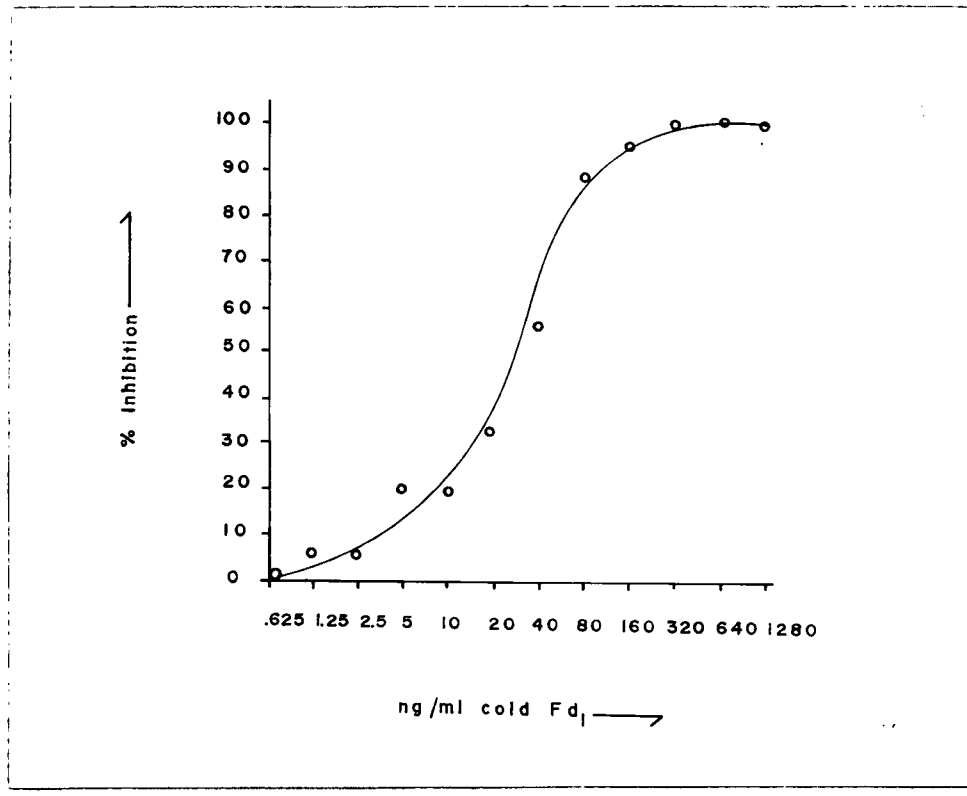

Figure 14: Radioimmunoassay for circulating idiotype in mouse serum. Varying quantities of unlabelled Fd-1 in normal B10.BR serum were incubated over anti-idiotype coated wells of a vinyl microtitre plate. After 1 hr. the samples were washed out and replaced with standard amounts of ^{125}I-labelled Fd-1. Percent inhibition of binding was calculated relative to control samples incubated with normal B10.BR serum alone.

idiotypic antibody which had been affinity isolated by passage of serum over a column of Fd-1 bound to Sepharose-4B-8L (Pharmacia) was used to coat the wells of polyvinyl microtitre plates. ^{125}I-labelled Fd-1 antibody will bind to anti-idiotype coated wells and this interaction can be quantitated by choosing a concentration of probe which falls within the range of 50-75% saturation of the solid phase, the point at which maximal sensitivity of this form of RIA is attained. Pre-incubation of the solid phase with set quantitites of unlabelled Fd-1 quantitatively inhibits the binding of labelled Fd-1. In this way a competition curve can be constructed which can be used as a reference in comparison with which concentrations of idiotype present in unknown samples may be determined. A sample competition curve is shown in figure 14. The lower limit of sensitivity attainable with this assay depends upon two principal factors. Firstly, the sensitivity is directly dependent upon the specific activity of the probe. While high specific activities are desireable in this assay, conditions of radioiodination were choosen in order to minimize the extent of damage done to the probe. Under the chosen conditions, it can be seen that concentrations of idiotype down to several nanograms per ml can readily be detected.

As it was necessary to perform simultaneous determinations of antibody titres, determinant specificity as well as idiotype on each of the individual serum samples tested it was necessary to economize on the quantitites of serum which were consumed in each assay. Ten microlitres of serum were diluted to a concentration of 1/40 so that each sample could be run in triplicate (3 x 0.1 ml). The effective lower limit of sensitivity for this dilution then becomes 40 x 1 ng/ml or 40 ng/ml of idiotype. Taking into account the range of variation observed between normal samples which presumably contain no idiotype, the statistical baseline was established at 45 ng/ml. Significance in this system is defined as 2 standard deviations about the mean

therefore concentrations of idiotype less than or equal to 45 ng/ml are listed as zero.

Results of serum survey for idiotype expression

Enzymatic degradation of Fd effectively destroys one of the antigenic determinants, generating a molecule which is immunologically monovalent. The derivatives which are obtained following either trypsin or carboxypeptidase A treatment can be used in an ELISA to determine the quantities of antibody in immune serum which bind to either the N- or the C-determinant. The proportion of N/C antibody is fairly constant within a given strain so that characteristic strain specific values can be calculated (66). Strains of different H-2 haplotype have been found to differ in this specific ratio, suggesting some form of MHC-linked control over the amount of antibody produced to the two determinants. As large numbers of serum samples which had previously been titrated for N- and C-antibody were available, these were selected in order to perform assays for idiotype content. In this way it is possible to calculate a figure which expresses idiotype as a percentage of the total N-specific antibody. Since this calculation takes into account the titre of anti-Fd antibody present, this figure can be used as a basis for comparison of idiotype expression between individuals and between strains. The results of a survey for idiotype in immune serum from several different strains are presented in table 2. Idiotype expression is very clearly limited to strains of mice which share identity at the Ig-1^b locus. This survey, involving strains of mice congenic at H-2 and representing different allotype linkage groups failed to reveal any association with other Ig-1 alleles. The expression of Fd-1 is apparently not influenced by genes within the H-2 region.

In vivo effects of anti-idiotype

As administration of anti-idiotype has been shown to have marked effects

Table 2. Analysis of anti-Fd sera taken from mice of various haplotypes and allotypes after secondary immunization with Fd.

Strain	Number of Animals	Haplotype	Allotype	Anti-Fd (μg per ml ± SEM)	Anti-N (μg per ml)	Idiotype Expression (ng per ml ± SEM)	% Idiotype Expression
B10.BR	12	k	b	9.0	1.8 ± 1.3	310 ± 37	22.9
B10S	Pool	s	b	1.2	0.7	88	13.3
C57BL/6	6	b	b	10.5 ± 3.8	2.2	237.4 ± 76.8	10.8
SJL	6	s	b	1.4	0.7	250	36.8
CE/J	6	k	f	14.0 ± 0.03	1.2 ± 0.2	0	0
AKR/J	6	k	d	10.8 ± 0.16	1.2 ± 0.2	0	0
ST/J	6	k	a	12.3 ± 1.0	2.4 ± 0.2	0	0
RF/J	6	k	c	10.7 ± 0.1	0.5 ± 0.2	0	0
C58/J	6	k	a	13.2 ± 0.04	1.1 ± 0.3	0	0

on the course of immune responses in vivo, a preliminary experiment was carried out to determine if anti-Fd-1 had any particular effects on the immune response to Fd in mice. Groups of unprimed B10.BR and DBA2 mice were given a single dose of purified anti-Fd-1 or normal rabbit Ig by intraperitoneal injection. One week later, these animals were immunized with 50 µg Fd in CFA and bled 21 days later. Using a single fixed dose of anti-Fd-1 (15 µg/mouse) injected 1 week before Fd, no direct effect on the primary anti-Fd response of B10.BR or DBA2 mice was observed (table 3). Similar results were obtained in mice treated with either anti-Fd-1 or NRS and immunized with KLH (data not shown). Because it has been shown in other systems that the effects of anti-idiotype may depend upon either the dose administered or the subclass (32), rather than attempt to fractionate the limited quantities of anti-idiotype available, it was felt that a functional characterization of the antiserum should first be undertaken. A ^{51}Cr release assay was therefore designed to test the cytotoxic potential of anti-Fd-1 on the appropriate target cells. Anti-Fd-1 was found to have a measurable cytotoxic titre at a dilution of 1/8 (table 4). The cytotoxic property of the anti-idiotype was then used under controlled conditions in which spleen cells were removed from immune mice and treated in vitro with either normal rabbit serum or anti-Fd-1 plus C' and then adoptively transferred into syngeneic irradiated recipient mice along with a 2° challenge of Fd. Control animals were immunized with KLH. These animals were then bled after 7 days and their individual antibody titres determined by ELISA. Data from this experiment is shown in table 5. Anti-Fd-1 plus C' treatment of spleen cells resulted in an enhanced titre of anti-Fd antibody in comparison to the control group, in which cells were exposed to NRS plus C'. When these sera were analyzed for N-determinant antibody it was observed that the amount of anti-N-antibody present had increased. This increase was found to be proportionate to the overall increase in anti-Fd

Table 3: Administration of anti-Fd-1 antibody *in vivo*. Small groups of B10.BR and DBA/2 mice were given an intraperitoneal injection of normal rabbit Ig (nRIg) or affinity isolated rabbit anti-Fd-1 antibody 7 days prior to immunization with Fd or KLH.

Experiment No.	Strain	Treatment	µg/ml anti-Fd±S.E.M.
1	B10.BR	nRIg	12.3±3.2
	B10.BR	anti-Fd-1	10.8±4.8
	DBA/2	nRIg	0.2±0.1
	DBA/2	anti-Fd-1	0.4±0.2
			µg/ml anti-KLH±S.E.M.
2	B10.BR	nRIg	2.0±0.8
	B10.BR	anti-Fd-1	1.4±0.3

Table 4: Complement dependent cytotoxicity of anti-Fd-1 serum on selected cellular targets.

		% Specific cytotoxicity			
Target cell line	treatment	dilution: 1/4	1/8	1/16	1/32
Fd-1 hybridoma	NRS +C'	8.1	6.2	6.6	0.0
	anti-Fd-1+C'	27.3	12.6	5.1	3.3
	anti-H-2^d+C'	37.4	38.4	15.6	17.1
Fd-2 hybridoma	NRS +C'	6.3	2.6	0.0	0.0
	anti-Fd-1+C'	1.3	5.0	0.0	0.0
	anti-H-2^d+C'	41.9	30.0	13.3	6.8
SP1/0 myeloma	NRS +C'	2.8	2.6	0.0	0.0
	anti-Fd-1+C'	6.6	1.0	1.7	0.0
	anti-H-2^d+C'	21.4	13.2	5.6	9.7

Table 5, Analysis of anti-Fd sera from an adoptive secondary response in B10.BR mice. Spleen cells from Fd-primed animals were treated with either NRS + C' or anti-Fd-1 + C' prior to transfer. Data are averages from 6 individuals per group.

Treatment	Antigen	Anti-Fd (µg per ml ± SEM)	Anti-N* (µg/ml ± SEM)	Idiotype** Expression (ng per ml ± SEM)	%*** Idiotype Expression
NRS + C'	Fd	1.0 ± 0.6	0.2 ± .02	87.2 ± 71.0	39.8
Anti-Fd-1 + C'	Fd	2.9 ± 1.3	0.6 ± .08	358.4 ± 297.0	56.5
NRS + C'	KLH	0 (4.85 0.70)+			
Anti-Fd-1 + C'	KLH	0 (4.36 1.92)+			

*Amount of N-specific antibody as calculated by specific reactivity on ELISA plates to which degraded fragments of Fd containing only the N- or C-determinant had been attached.

**Expression of the Fd-1 idiotype in individual antisera calculated by RIA.

***% of the N-specific antibody which reacted with anti-Fd-1 in the RIA.

+Anti-KLH titre (µg/ml ± S.E.M.).

titre, hence the actual percent of N-antibody remained constant. For this to be true there must therefore have been a corresponding increase in antibody directed towards the C-determinant. When sera were analyzed for idiotype content, it was found that idiotype expression had increased as well, but by a factor exceeding the general increase in anti-N antibody, indicating that the actual percentage of idiotype had risen.

In a second experiment, the role of complement in the adoptive transfer was examined. Recipient mice received 2×10^7 Fd primed spleen cells which had been treated with NRS plus C', anti-Fd-1 plus C' or anti-Fd-1. Immediately after adoptive transfer, mice were immunized with both Fd and KLH in CFA. They were bled 7 and 21 days after treatment. The results are shown in table 6 in which it can be seen that the anti-Fd-1 treatment enhanced the adoptive secondary response to Fd whether complement was added to the system or not. Neither treatment had any effect on the response to KLH.

Alterations in the magnitude of antibody responses have been induced in other studies by the action of anti-idiotype on regulatory T cells which express idiotype. The effects of anti-Fd-1 treatment are reminiscent of such results, and an experiment was devised in order to determine whether anti-Fd-1 was exerting an effect at the level of T cells or of B cells. Spleen cells were first fractionated using the principle of nylon wool separation of T cells and treatment with anti-Thy 1.2 plus C' for depletion of T cells. These T-enriched and T-depleted cell populations were then treated separately with normal rabbit serum or anti-Fd-1 as before and adoptively transferred into irradiated recipients following the combinations outlined in figure 15. These results demonstrate that anti-Fd-1 is primarily affecting T cells as an enhanced anti-Fd response is obtained only in combinations where T cells were exposed to the action of anti-idiotype independently of whether or not B cells were similarly treated. Treatment of B cells alone with anti-Fd-1 does not

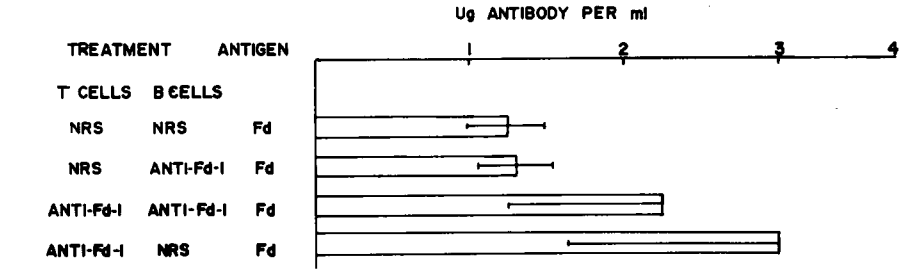

Figure 15: Treatment of T- or B-enriched cell subpopulations with anti-Fd-1 + C'. Cells were isolated as described in Materials and Methods and treated with anti-Fd-1 + C' in vitro. Treated cells were adoptively transferred to sublethally irradiated syngeneic recipients which were subsequently challenged with antigen. Animals were bled on day 7 and titres of anti-Fd antibody were determined by ELISA.

Table 6. The influence of anti-Fd-1 on the adoptive primary or secondary response of B10.BR mice to FD. Spleen cells from Fd-immune or naive B10.BR were treated with NRS plus complement or anti-Fd-1 ± complement prior to injection into irradiated B10.BR recipients and bled either 7 or 21 days later. Each experiment had 6 (expts. 1 and 2) or 10 (expts. 3 and 4) animals per group.

Expt. No.	Treatment	Time of Bleed	Anti-Fd (μg/ml ± SEM)	P* Anti-N	Anti-N (μg/ml ± SEM)	Idiotype Expression (ng/ml ± SEM)	P*	% Idiotype Expression
		primary						
1**	NRS + C	21 days	1.82 ± 0.95		7.28 ± 0.25	60.0 ± 32		8.3
	anti-Fd-1 + C	21 days	3.89 ± 1.83	NS	1.49 ± 0.75	121.0 ± 57	NS	8.1
		secondary						
2**	NRS + C	7 days	1.01 ± 0.57		0.24 ± 0.21	87.2 ± 41		39.8
	anti-Fd-1 + C	7 days	2.94 ± 1.34	NS	0.64 ± 0.34	358.4 ± 297	NS	56.5
		secondary						
3***	NRS + C	7 days	1.07 ± 0.53		0.36 ± 0.33	ND		
	anti-Fd-1 + C	7 days	2.12 ± 0.90	<0.05	0.56 ± 0.29	ND		
	anti-Fd-1	7 days	2.90 ± 0.95	<0.05	0.83 ± 0.44	ND		
		secondary						
4***	NRS + C	21 days	3.19 ± 1.92		0.97 ± 0.70	340 ± 192		17.4****
	anti-Fd-1 + C	21 days	13.27 ± 4.38	<0.025	4.24 ± 0.14	870 ± 4.76	NS	16.4
	anti-Fd-1	21 days	11.34 ± 4.77	<0.05	2.28 ± 0.85	380 ± 144	NS	14.3

*Differences in titres between anti-Fd-1 treated mice and NRS treated controls were analyzed by Students' t test to establish the significance of differences between the groups. NS indicates values p => .05.

**Six animals per experimental group.

***Ten animals per experimental group.

****Percentages are based on values obtained only from sera of mice which had detectable levels of N-epitope specific antibodies and therefore do not reflect a direct percentage of numbers shown in columns 6 and 7.

significantly change the response from that of the control group. It appears, therefore, that at least at the level of antibody production, that the Fd-1 idiotype is expressed on a subpopulation of T cells which may be susceptible to the action of anti-idiotype.

Owing to the historical development of these experiments, the sera from the original T plus B cell adoptive transfer experiment were not available for analysis so that a second experiment was performed utilizing only the combinations in which T cells alone were treated with anti-idiotype and substituting unprimed spleen cells. . . . While the antibody titres resulting from this repeated experiment were not as high as those observed in the initial experiment, parallel findings to the results of the whole spleen experiment were seen when the sera were compared for anti-N antibody and idiotype content (table 7). As before, the increase in N-antibody reflects the increase in total anti-Fd antibody, while the increase in idiotype exceeded this value. The fact that similar results were observed when these experiments were performed on unfractionated spleen cells or using a T-enriched cell population suggests that a common idiotype-expressing T cell may be the component which is responsible for these effects. In order to determine whether differences in titre between anti-Fd-1 treated and NRS treated T populations were maintained, a further experiment was done in which primed T cells were treated as described above. Recipients were immunized with both Fd and KLH in CFA, and bled 7 and 21 days later. Results are shown in table 8 in which it can be seen that anti-Fd-1 treated animals have significantly higher levels of antibody that do controls after 21 days. KLH responses were not significantly different between the two groups.

Studies using antibody feedback

In other systems, it has been shown that immune responses normally

Table 7. Analysis of anti-Fd sera in an adoptive primary response in B10.BR mice. Spleen cells of B10.BR mice were enriched for B and T cell populations using nylon wool or anti-thy-1 plus C. T cell enriched populations were treated with either anti-Fd-1 or NRS before mixing with B cells (NRS treated) and administration to irradiated recipients. Data presented are averages from 8 individuals per group.

Treatment		Anti-Fd	Anti-N	Idiotype Expression	% Idiotype
T cells	B cells	(μg per ml ± SEM)	(ng per ml ± SEM)	(ng per ml ± SEM)	Expression
NRS	NRS	0.72 ± 0.34	288 ± 35	60 ± 34	20.8
Anti-Fd-1	NRS	1.15 ± 0.34	435 ± 42	121 ± 46	27.8

Table 8. The influence of anti-Fd-1 on the adoptive secondary response of B10.BR mice to Fd. Spleen cells from Fd-primed B10.BR mice were separated by nylon wool or anti-thy-1 + complement to yield T or B cell enriched populations. These populations were treated with either anti-Fd-1 or NRS prior to transfer into irradiated recipients. In experiment 2, recipients were immunized with both Fd and KLH. Animals were bled 7 and 21 days later. There were 6 animals per group in experiment 1 and 12 per group in experiment 2.

Experiment Number	Treatment T Cells	Treatment B Cells	Time of Bleed	Anti-Fd (μg/ml ± SEM)	P*	Anti-N-epitope (μg/ml ± SEM)	Fd-1 Idiotype Expression (ng/ml ± SEM)	% Idiotype Expression
1	NRS	NRS	7 days	1.22 ± 0.88		ND		
	NRS	anti-Fd-1	7 days	1.26 ± 0.72	NS	ND		
	anti-Fd-1	NRS	7 days	3.05 ± 2.83	NS	ND		
	anti-Fd-1	anti-Fd-1	7 days	2.25 ± 2.20	NS	ND		
	T cells only							
2	NRS		7 days	1.40 ± 0.55		ND		
	anti-Fd-1		7 days	2.80 ± 0.80	NS	ND		
	NRS		21 days	3.93 ± 1.27		1.30 ± 0.80	627 ± 484	37.2
	anti-Fd-1		21 days	23.63 ± 12.00	<.005	3.32 ± 1.10	555 ± 264	16.7

*Differences in titres between anti-Fd-1 treated and NRS treated cells were analyzed by Students' t test to establish significance of differences between groups. NS indicated non-significance and p values of >.05.

involve the participation of anti-idiotype regulatory T cells in addition to the conventional role played by antigen binding, idiotypic T cells (67). Idiotype specific T cells have also been generated after immunization with idiotype bearing antibody, idiotype conjugated thymocytes and following administration of anti-idiotype antibody (68-70). This form of immunity to idiotype has been shown to significantly alter the normal course of immune responses. Induction of anti-idiotypic T cells under these conditions however, is not equivalent with evidence for their role during the normal response induced by antigen. In order to determine whether anti-idiotypic T cells played any role in the anti-Fd response, an experiment was designed in which non-immune mice were injected with the monoclonal Fd-1 antibody prior to immunization with antigen. Under these conditions anti-idiotypic lymphocytes would therefore be placed in contact with idiotype before antigen binding lymphocytes were first stimulated by Fd. This experiment is outlined in table 9.

Mice were given an intraperitoneal injection of 10 μg purified Fd-1 prior to immunization with Fd or a control antigen, KLH 7 days later. Two weeks after immunization, the mice were bled and their titres of antibody to Fd and KLH determined by ELISA. As seen in group 1, administration of Fd-1 alone did not induce synthesis of anti-Fd antibody, nor were appreciable quantities of residual Fd-1 detectable. When Fd-1 was administered followed by immunization with Fd the antibody titre which resulted was at least 3-fold higher than that obtained with Fd alone. In contrast, the specificity control groups 4 and 5 were not significantly affected by this same treatment. Pre-exposure of the mice in group 2 to idiotype has resulted in marked increases in their titre of anti-Fd antibody. Sera from groups 2 and 3 were also analyzed for the proportion of N/C specific antibodies present. The ratios between groups 2 and 3 are not significantly different. Thus, the increase in

Table 9. The influence of the monoclonal Fd-1 on the immune response to Fd in B10.BR mice. Seven days prior to immunization, animals received 10 μg of Fd-1. Animals were bled 7 and 14 days after immunization, were reimmunized on day 28 and bled again on day 35. In all instances, mice treated with Fd-1 alone showed no anti-Fd titre, and mice treated with Fd-1 and Fd had significantly higher titres of anti-Fd antibody than did controls. There were 12 animals in each experimental group.

Day -7	Day 0	Day 28	Time of Bleed (day)	Anti-Fd or anti-KLH (μg/ml ± SEM)	P*	Anti-N (μg/ml ± SEM)	Idiotype Expression (ng/ml ± SEM)	% Idiotype Expression
Fd-1	-	-	7			ND	182 ± 65	
Fd-1	Fd	-	7	1.65 ± 0.64	<.005	0.34 ± 0.87	112 ± 53	
Fd-1	KLH	-	7	0.92 ± 0.16**		ND	133 ± 64	
-	Fd	-	7	0.49 ± 0.26		0.15 ± 0.02	104 ± 61	
-	KLH	-	7	1.05 ± 0.25**		ND		
Fd-1	-	-	14	0				
Fd-1	Fd	-	14	3.33 ± 1.30	<.005	ND		
-	Fd	-	14	0.50 ± 0.40				
Fd-1	-	-	35	0			40	
Fd-1	Fd	Fd	35	27.18 ± 7.98	<.05	10.45 ± 3.78	386 ± 156	3.7
-	Fd	Fd	35	11.38 ± 4.63		5.30 ± 2.20	2450 ± 1644	46.2

*Differences between Fd-1 treated immunized mice and untreated controls were analyzed by Students' t-test to establish significant differences between the two groups.

**Titres were calculated to KLH in these instances. Differences are not significant between the two groups.

anti-Fd antibody clearly involves a marked increase in the amount of anti-C antibody in treated mice. When mice from groups 2 and 3 were tested after a secondary injection of Fd the same pattern of results was obtained. Animals which had received Fd-1 before antigen exhibited an increased titre of total anti-Fd antibody, and a decreased ratio of N/C antibody. The effect of passive idiotype in this experiment appears to be mainly on the production of anti-C antibody.

Discussion

In the mouse, there exists a system of allelic polymorphisms involving the constant regions of immunoglobulin heavy chains. These antigenic specificities or allotypes, are inherited in simple Mendelian fashion. Genes which code for the constant portion of immunglobulin heavy chains are located within a closely linked cluster located on chromosome 12 (30). The largest number of alleles occurs at the IgH-1 locus (IgG_{2a}). Linkage between the individual loci is so strong that few recombinants between loci have been documented. Most strains which have the same Ig-1 allele also share alleles at each of the other loci. IgGH genes therefore tend to segregate as haplotypes, hence linkage groups are generally listed according to their Ig-1 alleles only.

Unlike the clearly identifiable antigenic specificities of allotypic markers, idiotypic determinants result from the association of light and heavy chains and are theoretically dependent upon the segregation of at least two genes, In some cases genes linked to the light chain locus on chromosome 6 have been shown to govern expression of idotypic markers (ibid). In the majority of cases, however, idiotype markers of induced antibodies have been linked to the genes coding for heavy chain allotype.

In the Fd system, rabbit anti-idiotype antiserum was used to define

binding site related idiotypic determinants of the monoclonal Fd-1 antibody. When this anti-idiotype was used to determine the presence in immune sera of molecules sharing idiotypy with Fd-1, two observations were apparent. First, idiotype bearing molecules were detected only when the sera had been derived from mice sharing identity at the Ig-1 locus. This is in accord with similar linkage studies performed using defined idiotypes of either polyclonal (71) or monoclonal antibodies (72). Second, it was shown that idiotype was present in 50% of the sera tested and this expression represented a variable proportion (between 0 and 100%) of the total anti-N population present. This level of variability in the expression of a restricted idiotype is consistent with observations from other monoclonal systems.

Analysis of a large number of levan binding myeloma proteins has revealed the existence of two main categories of idiotypic determinants. Anti-idotypic sera prepared to 10 different levan binding myeloma proteins showed that each possessed its own unique individual idiotypic specificity (IDI) which was not shared by any other myeloma. In addition, most of the proteins carried a variable number of idiotypes which were shared by a number of other molecules (27). This second group of shared idiotype was termed a cross-specific idiotype (IdX). Private and public idiotypes are also present in the antibody response to the arsonate hapten (18). On the basis of defined monoclonal idiotype markers this response appears to be quite heterogenous. When sera from immune mice were screened for the presence of a particular idiotype belonging to the IdI category, only 50% of the sera contained detectable levels of idiotype, representing less than 2% of the total antibody present. Private idiotype determinants therefore appear to be present at very low concentrations in immune sera. Analogous findings were also observed in a system using monoclonal antibodies to the hapten 4-hydroxy-3-nitro-phenyl-1-acetyl (NP). Hybrid cell lines secreting monoclonal antibodies against idiotypes

of one selected anti-NP hybridoma protein, B1-8, were isolated and used to characterize the variable portion of B1-8 and other anti-NP antibodies all belonging to an antibody family which carries a predominant idiotypic marker (19). Monoclonal anti-idiotypes defined private, combining site related idiotopes which were expressed on 1-10% of serum antibodies, as well as public, non-combining site related idiotopes which were more widely expressed on 10-100% of serum antibody. Expression of private idiotypes was also shown to be linked to the Ig-1 locus. In this system, private idiotypic determinants also appear to represent a minority of the serologically detectable idiotypes.

On the basis of the restricted range of expression observed for Fd-1, the idiotype would appear to belong to the IdI category. In the absence of additional idiotype markers for the N-determinant however, this assignment must be considered tentative as the rabbit anti-idiotype would probably also be detecting a public specificity which is present in low concentration.

Effects of anti-idiotype on responding cells

When immune spleen cells were treated with anti-idiotype plus C' and transferred to irradiated recipient mice, the anti-Fd response in the recipients was found to be increased in comparison with control animals. When sera from these animals were examined for idiotype expression it was noted that while the relative proportion of N/C antibody was unchanged, the absolute amount of Fd-1 idiotype increased 2-4-fold. Despite the fact the idiotype bearing B cells may have been exposed to anti-idiotype plus C' they have clearly not been eliminated from the treated spleen cell population. The enhanced idiotype titre argues either in favour of a simple mechanism of B cell stimulation which is independent of complement or a more indirect mechanism acting upon idiotype bearing T cells which may or may not be

dependent upon C'. The observation that enhanced response to Fd was obtained either in the presence or absence of C' implies C' independence. However, it does not establish the mechanism of activation which could involve cell stimulation by antibody, or the elimination in vivo of antibody coated cells. The latter possibility appears to be the most favoured one at this time.

Because anti-idiotype treatment of adoptively transferred spleen cells did not appear to be eliminating idiotype bearing B cells, since increased idiotype expression was observed with treated cells, it was considered possible that the treatment was affecting a T cell population. When the appropriate experiments were performed it was found that an increased anti-Fd response was observed only in the case where the transferred T cells had been treated with anti-idiotype. This increase, again, was reflected both in level of anti-Fd antibody and Fd-1 idiotype. In view of the fact that the same treatment of B cells was without effect, it was concluded that anti-idiotype treatment selectively affected an idiotype bearing T cell population whereas B cells did not appear to be susceptible to its mode of action.

While the primary outcome of anti-idiotype treatment can be accepted as a strictly idiotypic effect, additional observations can be made. In the majority of hapten systems studied to date, anti-idiotype effects are usually restricted to changes in the expression of the corresponding idiotype while total antibody levels remain unchanged. As mentioned earlier however, these haptens are administered in the form of multideterminant derivatives of immunogenic carriers and usually result in the formation of substantial quantities of anti-hapten antibody. At the idiotype level this antibody has been found to be very heterogenous. When one considers the fact that hapten itself constitutes only one species on a large array of different epitopes, then the relationship of any single anti-hapten idiotype to the overall network of anti-immunogen idiotopes might actually be remote. In the case of

a more limited immunogen such as Fd where the immune response is confined to only two known epitopes, the repertoire of idiotopes may be smaller, hence the antibody network could be less stable with respect to any given idiotype. Idiotypic manipulations might theoretically perturb other members of the repertoire at a measureable level. In the case of Fd, idiotypic manipulation does measurably affect the level of total antibody. After allowing for relative increases in N-specific antibody it is clear that the increase in idiotype content does not account for the overall increase in total anti-Fd antibody. In other words, the observed increment in idiotype falls short of the actual increment in anti-N antibody. There is a similarity in this observation to the findings of Reth and co-workers who found that regulation by a given anti-idiotype antibody need not be restricted to the expression of the corresponding hapten binding idiotype but could extend to other hapten binding idiotypes as well (72). With Fd however, anti-idiotype treatment also resulted in an increase in production of antibody directed towards the unrelated C-determinant. It is clear, therefore, that alterations in the activity of T cells governing idiotype expression can affect not only the production of idiotype but can extend to other members of the repertoire as well.

Previous studies on Fd have shown that there are only two immunologically reactive regions present and these two epitopes are active at both the B and T cell level (41). Thus, in terms of hapten carrier interactions, it may be assumed that during the response to Fd, one epitope provides the help necessary to generate a response to the other. The idiotypic regulatory T cell influenced by anti-idiotype in these experiments is specific for the N-determinant. Since N-determinant binding T cells also provide the help necessary for the anti-C response, it may be that the idiotype bearing T cell affects the function of other N-binding T helper cells which may

or may not be idiotypically related. It is impossible at this time to define more precisely the nature of the cells involved in generating the increased help for the C-determinant response since treatment of primed T cells with anti-idiotype may preferentially affect one idiotypic population over another. Although B cells have been shown to participate in anti-idiotype regulation (73), the effects which are observed are the same whether the experiment was performed using whole or separated spleen cells and would appear to rule out any direct effects of anti-idiotype caused by bridging of idiotype bearing T and B cells.

It appears then, that the anti-Fd-1 antiserum is capable of affecting a population of immunoregulatory T cells, possible suppressor cells, which bear the Fd-1 idiotype. This treatment results not only in an increased level of antibody directed to the C-determinant (increased help from N-specific T helper cells), but also in an increased expression of the Fd-1 idiotype in N-specific B cells. In order for both these events to occur, it is necessary to postulate that the idiotype T immunoregulatory cells have a dual function: regulation of N-specific help, and regulation of idiotype expression by way of an anti-idiotypic population of, presumably, T cells. A model incorporating these observations is shown in figure 16.

Prior to contact with antigen the network of potentially reactive cells exists in a non-immune or ground state in which idiotype and anti-idiotype elements are in a state of equilibrium (74). This state of homeostasis can be easily distrubed by the introduction of antigen, idiotype or anti-idiotype. Normally, contact with antigen leads to a shift in the balance between idiotypic elements resulting in antibody synthesis. Prior contact with idiotype on the other hand, might potentially induce proliferation or elimination of idiotype binding cells (anti-idiotypic cells) biasing the existing equilibrium in a way which would result in an abnormal response to antigen.

This prediction was investigated by Forni et al. (75) who observed stimulation of IgM anti-SRBC plaque forming cells following passive administration of anti-SRBC IgM. The authors postulated a mechanism involving direct stimulation of anti-idiotypic T helper cells by the administration of idiotypic antibody. This procedure was applied to the Fd response where a marked enhancement in the titre of anti-Fd antibody was observed as a result of pre-immunization with monoclonal Fd-1 antibody. Unlike the former case however, the enhanced response was dependent on antigen as no anti-Fd response was detected after administration of Fd-1 alone. When the sera were analyzed for their N/C ratio it was found that the ratios remained unchanged and that the major shift in antibody production was again attributable to a significant increase in C-specific antibody. In order to accomodate these findings the model presented in fig. 16 includes linkage between idiotype and anti-idiotype bearing T cells.

In other studies, administration of idiotype in vivo has been shown to stimulate specific T cell functions, although the outcome is somewhat dependent on the mode of immunization (32). Conjugation of idiotype to syngeneic thymocytes has been used to induce anti-idiotypic helper and suppressor T cells depending upon whether they were administered intravenously or subcutaneously (69). One could logically extend this qualification to include the possibility that a single mode of immunization may induce multiple subpopulations of immunoregulatory cells which could have competing effects. The major interactions can only be defined by manipulating cells under highly controlled conditions. Several of the above mentioned factors may be contributing in some degree to the outcome of the Fd experiments. Also important is the fact that specific events involving B cells have not been explored in this study, so that idiotypic events have been largely interpreted from the point of view of T cell activity. Nevertheless, it has been demonstrated that the anti-Fd response

is sensitive to perturbations at the level of idiotype and anti-idiotype, and it is anticipated that these effects are most likely mediated through T lymphocytes.

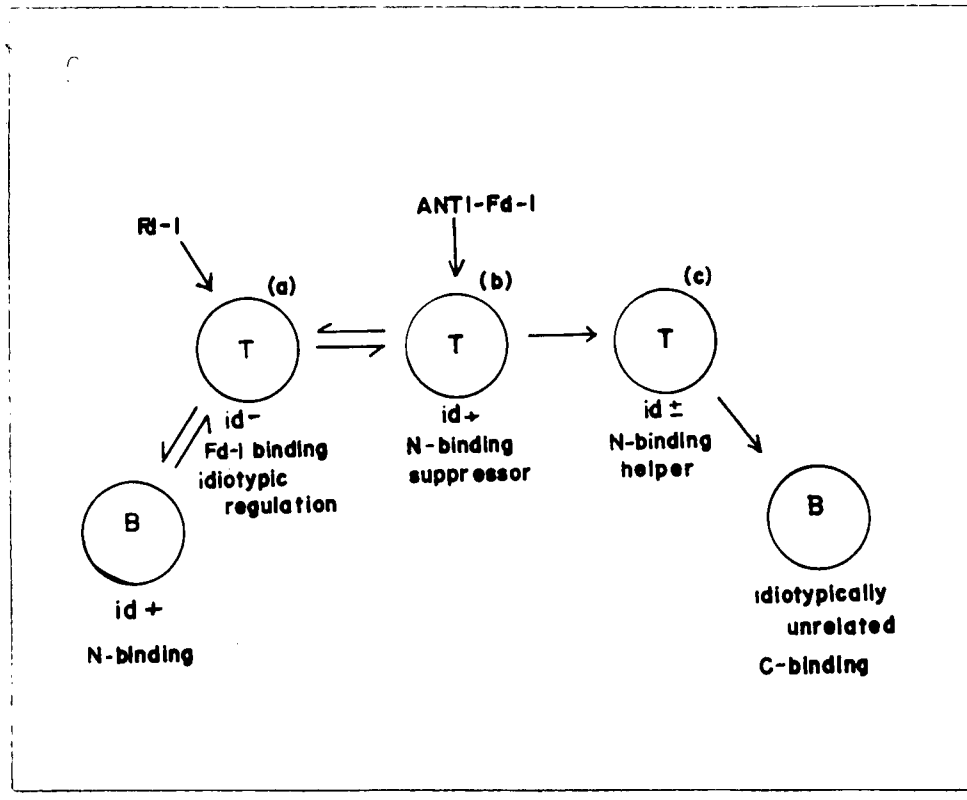

Figure 16: Model for idiotypic interaction in the immune response to Fd. A major id^+ regulatory T cell (cell b) controls the level of help generated by other N-epitope binding T-cells (cell c) which may or may not be idiotypically related but control the level of antibody produced by B cells reactive with the C-epitope. Elimination of this cell (cell b) by anti-Fd-1 increases the response to the C-epitope. Since the response to the N-epitope is also increased in this process, a second, id^- regulatory T cell is also postulated (this population (a) would be bound by the Fd-1 monoclonal since they are anti-idiotypic). Elimination of either of these cell populations destroys this level of network regulation.

CHAPTER 4: CELLULAR EXPRESSION OF Fd-2 IDIOTYPE

Evidence presented in the previous chapter showed that the Fd-1 idiotype was expressed on a significant portion of antibodies in serum which bound the N-determinant. Treatment of immune spleen cells with anti-idiotype affected the anti-Fd response by increasing the production of idiotype as well as other members of the anti-Fd repertoire. The second monoclonal antibody, Fd-2, is specific for the C-determinant of Fd. An anti-idiotypic antiserum was raised to this protein and could therefore be used to investigate questions similar to those which were studied using anti-Fd-1 serum.

Many studies have shown that the idiotypic repertoire can be quite extensive. Immune responses include the appearance of both shared, as well as private idiotypes. The immune system seems capable of mounting a response which while predictable in the case of more common idiotypes, involves numerous idiotypes which are not shared but nevertheless may constitute a sizable percentage of the expressed repertoire. It is not known whether the level of expression of any given idiotype in the serum reflects the specificity of specific T helper to T suppressor cells. This question has been investigated using the two anti-idiotypes which have been characterized in the Fd system. Earlier, it was shown that T cells bearing the Fd-1 idiotype exerted a significant influence over the expression of Fd-1 idiotype in serum antibody. The other idiotype in this system, Fd-2, provides an additional marker which allows for comparison of idiotypy at the serological and cellular level.

Materials and Methods

The following experimental methods and conditions used in this chapter are identical to those used in the Fd-1 studies which were described in detail in the Materials and Methods section for chapter 3.

1) Radioimmune assay for idiotype.

2) In vivo administration of anti-idiotype.

3) Adoptive transfer of anti-idiotype treated spleen cells. The protocol used in chapter 4 is identical except, where indicated, DBA recipients were not irradiated.

4) In vivo passive transfer of idiotype.

Results

Anti-idiotype serum to Fd-2 was prepared and characterized as described in chapter 2. This serum was used in a radioimmunoassay in order to look for the presence of similar idiotypes among serum antibodies to Fd. A sample standard curve of this RIA is presented in figure 17. As with the RIA for Fd-1, the assay is sensitive for idiotype levels of about 45 ng/ml. Unlike earlier findings with Fd-1 however, Fd-2 does not appear to be expressed as part of the normal anti-Fd repertoire in B10.BR mice. A small number of individual mice tested produced detectable levels of the idiotype however, even in those cases this expression constituted only a minor proportion of their anti-C antibody (table 10).

A pilot experiment using affinity purified anti-Fd-2 was undertaken to determine whether in vivo administration of anti-Fd-2 would affect the response of B10.BR mice towards Fd. As shown in table 11, prior administration of 15 μg of anti-Fd-2 to B10.BR mice markedly enhanced the Fd response in comparison to the control group which received the same quantity of normal rabbit Ig. When the experiment was repeated using a genetic non-responder strain DBA/2, anti-Fd-2 was able to induce a strong primary response. The antibody titres of these animals approached the values which are normally seen in responder strains.

According to genetic analyses, DBA/2 mice fail to respond to Fd due to a suppressor effect which appears to be inherited as a codominant trait

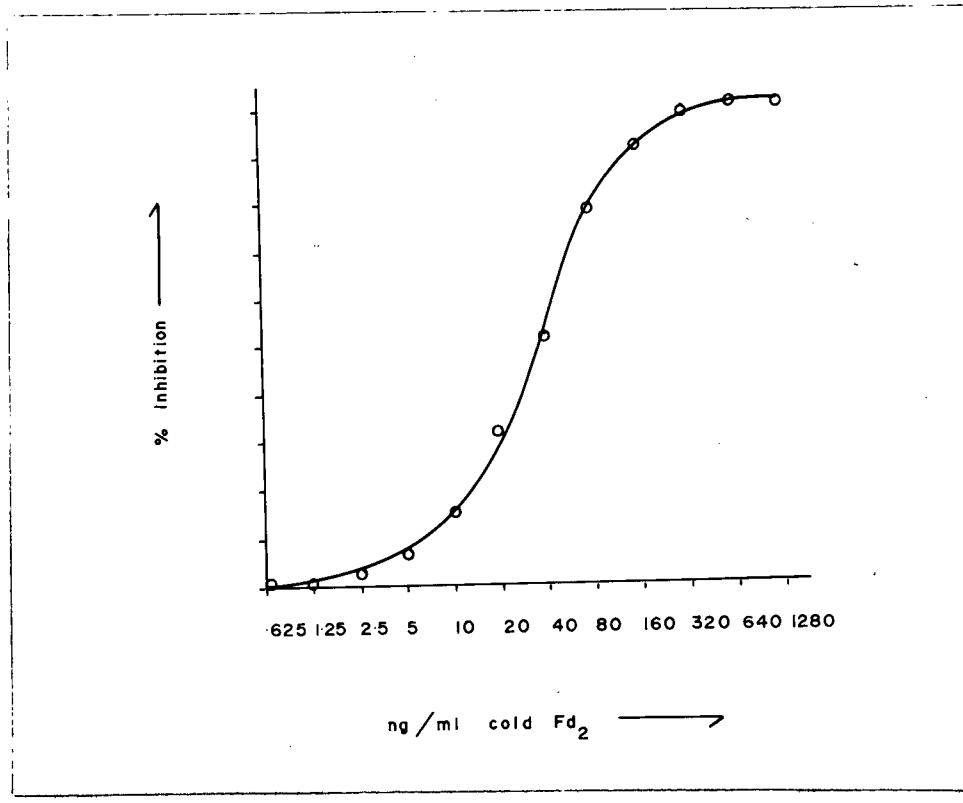

Figure 17: Radioimmunoassay for circulating idiotype in mouse serum. Varying quantities of unlabelled Fd-2 in normal B10.BR serum were incubated over anti-idiotype coated wells of vinyl microtitre plates. After 1 hr., the samples were washed out and replaced with standard amounts of ^{125}I-labelled Fd-2. Percent inhibition of binding was calculated relative to control samples incubated with normal B10.BR serum alone.

Table 10. Results of radioimmune assay for Fd-2 idiotype in immune sera of B10.BR mice. B10.BR mice were immunized with Fd and bled 21 days later. Sera from these animals were analyzed for idiotype content using a specific RIA for Fd-2 idiotype.

Anti-Fd (μg/ml)	Fd-2 (ng/ml)
1.6	0
2.4	120
1.8	0
3.2	75
4.4	0
2.2	0
3.0	0
2.7	0
2.1	140
3.1	0
4.5	0
5.3	0

Table 11: In vivo administration of anti-Fd-2 antibody. Small groups of B10.BR and DBA/2 mice were given a single intraperitoneal injection of 15 µg of rabbit Ig or affinity purified anti-Fd-2 antibody 7 days prior to immunization with Fd or KLH.

strain	treatment	µg/ml anti-Fd±S.E.M.
B10.BR	RIg 15 µg	12.3±3.2
B10.BR	anti-Fd-2 15 µg	31.5±6.1
DBA/2	RIg 15 µg	0.2±0.1
DBA/2	anti-Fd-2 15 µg	15.0±13.7

strain	treatment	µg/ml anti-KLH±S.E.M.
B10.BR	RIg 15 µg	2.0±0.8
B10.BR	anti-Fd-2 15 µg	2.4±0.6

controlled by the I region (43). Despite the small sample size, the effects of the previous experiment were felt to be sufficiently significant to warrant further investigation. Two experiments utilizing the ability of anti-Fd-2 to induce responsiveness in DBA/2 mice were devised. In the first experiment, DBA/2 mice were primed with Fd in the presence of two mitogens, LPS plus anti-Ig. Assuming that unresponsiveness resulted from a lack of appropriate T cell help, then coimmunization with mitogen might provide a missing signal sufficient to induce activation of Fd specific B cells. As indicated in table 12 however, each of these treatments failed to induce a response. The inductive effect of anti-Fd-2 in nonresponder mice might, alternately, have resulted from the stimulation of a specific helper population. An experiment designed to test this possibility was performed. Spleen cells from DBA/2 mice which had received in vivo anti-Fd-2 and responded in the initial trial experiment were adoptively transferred to non-immune syngeneic DBA/2 recipients which were subsequently immunized with Fd. The recipients were bled after 10 days to determine whether this resulted in the adoptive transfer of an anti-Fd response into DBA recipitents. As seen in table 13, the recipient animals did not however, respond. This result implies that the anti-idiotype may have acted by eliminating a population of suppressor cells rather than through the stimulation of helper cells. After anti-idiotype treatment, adoptive transfer of the responding cell population into normal unirradiated recipients serves to reconstitute the original environment in which suppression is dominant. Transferred cells would then fail to respond as a result of interaction with suppressor cells in situ.

This interpretation of events is based on an assumption that idiotype bearing suppressor cells are eliminated by anti-idiotype in vivo. This prediction was tested in an adoptive transfer system in which the donated lymphocytes were obtained from Fd primed DBA/2 mice. Donated spleen cells, the

Table 12: Lack of an anti-Fd response in nonresponder mice (DBA/2) co-immunized with mitogen. The indicated quantities of mitogen were administered by intraperitoneal injection 3 days prior to immunization with Fd.

Treatment	µg/ml anti-Fd
Fd	0
Fd + LPS (15 µg)	0
Fd + RaMIg (15 µg)	0

Table 13: Inability of the enhanced response of anti-Fd-2 treated mice to persist following adoptive transfer to normal, nonirradiated hosts.

strain	treatment of donor cells	µg/ml anti-Fd±S.E.M.
B10.BR	RIg	4.16±1.04
B10.BR	anti-Fd-2	3.52±0.48
DBA/2	RIg	0.13±0.30
DBA/2	anti-Fd-2	0.10±0.05

presumed source of suppressor cells, were treated in vitro with anti-Fd-2 plus C' and adoptively transferred to irradiated syngeneic recipients, which were then immunized with Fd. Antibody titres of the recipients were determined from sera bled 21 days after immunization. The results of this experiment are shown in table 14. As in the case of the in vivo experiment, exposure of cells to affinity purified anti-Fd-2 resulted in a strong primary response. Following secondary challenge with Fd this response is maintained.

Discussion

The results of anti-Fd-2 treatment of immune spleen cells differ in several ways from the effects which were observed using anti-Fd-1. The idiotypes defined by these reagents appear to be expressed in very different amounts during a normal immune response in B10.BR mice. Whereas the Fd-1 idiotype is a regularly expressed component of the anti-Fd repertoire, the Fd-2 idiotype is very infrequently expressed and always in very low concentration in immune serum. The two anti-Fd monoclonal antibodies were obtained in separate fusion experiments. Despite the apparently random nature of cell hybridizations, it cannot be concluded with any certainty that the products obtained via cell fusion are representative of the antibody repertoire existing at the time of fusion. Metzger and coworkers observed that the idiotypes of hybridomas derived at different times during the anti-lysozyme response differed significantly from those expressed on serum antibodies which were consistent throughout the response (76). These findings add evidence to earlier suspicions that cell fusion may be selective towards lymphocytes in a particular state of differentiation.

In contrast to their relative paucity in serum antibody, the effects of treatment of cells with anti-Fd-2 greatly exceeded those induced by anti-Fd-1. While administration of anti-Fd-1 in vivo had no short term effects, anti-Fd-2

Table 14: Ability of anti-Fd-2 to abrogate the nonresponder status to Fd in DBA/2 mice. Fd-primed DBA/2 mice served as donors of spleen cells which were treated with anti-Fd-2 serum and adoptively transferred to irradiated DBA/2 recipients together with Fd. Recipients were bled at day 21. On day 28 a 2° challenge with Fd was given, and recipients were again bled on day 35.

strain	treatment	µg/ml anti-Fd±S.E.M.	
		21 days	35 days
DBA/2	RIg	0.60±0.10	0.15±0.06
DBA/2	anti-Fd-2	3.28±0.53	10.10±2.60

administration markedly enhanced the anti-Fd response and was also active in adoptive transfer. In experiments designed to investigate the mechanism of this effect, no evidence for the induction of helper cells in anti-Fd-2 treated mice was observed. Rather, the effects of anti-Fd-2 treatment observed in the nonresponder strain are more consistent with the existence of an idiotypic suppressor cell. Anti-Fd-2 treatment of primed DBA/2 spleen cells resulted in a response which exhibited both the kinetics (peaking at 21d) and antibody titres which are seen in a normal primary anti-Fd response in B10.BR mice. The putative suppressor cell would be active in both the B10.BR strain, in which it may only be partially effective, and the DBA/2 strain in which it predominates. A potent suppressor cell with similar characteristics has been described in the immune response to lysozyme. Genetic nonresponsiveness of the B10 strain to lysozyme was found to be due to the activation of suppressor T cells by a restricted portion of the antigen. Removal of this epitope converted B10 mice to responder status (77). Anti-Fd-2 treatment may be an equally selective means of overiding this type of suppression in DBA/2 mice.

While the expression of FD-1 idiotype was found to be restricted to a particular allotype linkage group in B10.BR mice, this may reflect differences in idiotype production which are below the levels of detection afforded by the idiotype assay employed. Using a similar assay for Fd-2, only very low levels of idiotype were detected in the B10.BR strain. In contrast, in vivo and adoptive transfer experiments indicated that the Fd-2 idiotype may be expressed on a subpopulation of lymphocytes in two strains, B10.BR and DBA/2, which do not share allotype. Although this difference in allotype restriction may seem discordant, other experimental studies have furnished evidence which suggests that these findings may not be inconsistent with network principles. In studying delayed type hypersensitivity reactions with the arsonate hapten,

Hirai and coworkers found that idiotype specific T suppressor cells could be generated by immunization of mice with idiotype conjugated spleen cells (78). Interestingly, these idiotype specific suppressors could be generated in a number of strains of different allotype linkage groups which did not normally express the idiotype. The potential exists in these strains of recognizing an allotype restricted idiotype. In an unrelated study, it was observed that immunization of Balb/c mice with the T15 myeloma protein induced T cell helper activity which was specific for T15 as well as other PC binding myeloma proteins which were negative for the T15 idiotype (79). The authors proposed on the basis of this finding that T cell recognition of idiotypes was restricted to germ line specificites while B cells, having undergone extensive somatic mutation, would generate a spectrum of variant idiotypes. According to this proposal, T cells would recognize predominantly germ line encoded idiotypes while antibodies to B cell idiotypic variants might not completely cross-react with T cells.

Although this speculation might not be applicable to the xenogeneic anti-idiotypes used in this study, it does provide a possible explanation for the differences in allotype restriction of idiotype expression which have been observed in Fd as well as in other systems.

Summary Discussion

This thesis has presented experimental descriptions of effects which were induced as a result of the treatment of isolated spleen cells with, or administration to whole animals of, idiotypic and anti-idiotypic elements of a defined antigen system. Many of these results, while consistent with the logic of idiotypic interactions, cannot be used as a basis on which to propose a comprehensive model that clearly states the role of idiotypic interactions in regulating the immune response to ferredoxin. They do indicate, however,

that idiotypy is a significant mode of communication between lymphocytes as evidenced by the altered immune responses exhibited following the administration of idiotype or anti-idiotype. The cellular mechanisms responsible for observed shifts in antibody and idiotype production in this system have not however, been fully characterized. One dilemma which must be addressed in studies of this type is the difference between the goals of cellular immunology and those of network theory. Cellular immunology seeks to define specific temporal interactions between lymphocyte subpopulations of distinct functional activity. This notion is embodied in the widespread usage of phenotypic markers which are presumed to identify functionally mature T cell populations (80). In contrast to this cellular view, network theory avoids the use of functional terms and relies instead upon the more statistical concepts of homeostasis and symmetry in order to predict the behaviour of the immune system in response to a given stimulus (74). It would not be surprising, considering differences in their underlying logic, that the design and interpretation of experiments would not be mutually consistent between these two theories.

The experiments described in this thesis represent an attempt to explore the significance of both cellular and network theory within the confines of a simple antigen system in mice. In general, the results have been more amenable to a network interpretation, perhaps in part because idiotypes afforded a readily tested experimental parameter. Nevertheless, this situation became possible because the interactions described are real. Despite the fact that the experimental questions explored in this study do not constitute a formal test of network predictions, these investigations have raised questions and described phenomena that will, hopefully, prove useful in the course of future research.

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