THE IDIOTYPIC RESPONSE TO
FERREDOXIN IN MICE.

By

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B.Sc., The University of British Columbia, 1980

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

DEPARTMENT OF MICROBIOLOGY

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

July 1984

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ABSTRACT

These investigations define a new protein idiotypic system—that of Clostridium pasteurianum ferredoxin (Fd). Previous studies from our laboratory have shown that the response to Fd, a small, non-mammalian electron transport protein is controlled by H-2K/I-A linked loci in mice and is almost a unideterminant response in $H-2^k$ mice. Strains of mice varying in Igh, Igl, and H-2 loci were immunized with Fd. Their Fd-immune sera were tested by inhibition in the ELISA with four specific anti-(anti-Fd)- idiotypes produced in rabbits to the purified, pooled anti-Fd sera from four different strains of mice. Major idiotypes were observed reproducibly in the anti-Fd responses of AKR/J, RF/J, and B10.BR/SnJ mice, while the anti-A/J idiotype did not reproducibly inhibit a high frequency of A/J anti-Fd antisera. Significant differences were observed between the levels of inhibition of pooled or individually analyzed anti-Fd antisera from the same mice.

Further experiments showed that anti-Fd antisera from B10.BR mice have a high frequency of cross-reactivity with AKR/J anti-Fd idiotypes as do RF/J, C58/J, C3H/HeJ and CBA/J antisera. C58/J, C57BR/cdJ, and RF/J antisera also cross-react significantly with A/J anti-Fd idiotypes. Curiously, A/J and AKR/J anti-Fd antisera are not inhibited with anti-RF idiotype, and AKR anti-Fd antisera are not inhibited with anti-B10.BR idiotype.

The idiotype profile of monoclonal anti-Fd antibodies was studied. The results demonstrated interstrain idiotypic cross-reactivity that paralled the results obtained with the serum antibodies of the appropriate mouse strain. These data, with the data from experiments utilizing serum antibodies do not demonstrate a correlation between idiotypic cross-reactivity and Igh or Igl allotypes. H-2 genes were observed to vary the pattern of idiotype expression in B10 recombinant mice, but no correlation between H-2 genes and idiotype expression could be observed.

These results demonstrate that the anti-Fd response in H-2 mice is very complex with many intra and interstrain cross-reactive idiotypic families. Confirmatory results from 2-D electrophoretic analyses show the presence of many light chains in the anti-Fd response. These data are discussed in the context of existing hapten, carbohydrate, and protein antigenic systems, as well as in the context of current theories of idiotypic structure and antibody cross-reactivity.

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LIST OF ABBREVIATIONS

A5A	-	GAC idiotype
ARS	-	p-Azobenzenearsonate hapten
ARS-Br	-	ARS conjugated to _Brucella abortus_
B1-8	-	Monoclonal anti-NP antibody
BI	-	Bovine insulin
BSA	-	Bovine serum albumin
CGAT	-	GAT idiotype
CFA	-	Complete Freund's Adjuvant
C'	-	Complement
CRI	-	Cross-reactive idiotype in ARS system
DNP	-	Dinitrophenyl hapten
DTH	-	Delayed type hypersensitivity
FCS	-	Fetal calf serum
Fd	-	_Clostridium pasteurianum_ ferredoxin
GA	-	Glutamic acid, alanine polypeptide
GA-1	-	GA idiotype
GAC	-	Group A carbohydrate
GAT	-	Glu, ala, tyr random tercopolymer
GL	-	Glu, lys polypeptide
GT	-	Glu, tyr polypeptide
Gte	-	GAT idiotype
HEL	-	Hen egg-white lysozyme
Id I	-	Individual idiotype (various systems)
Id X	-	Cross-reactive idiotype (various systems)
IEF	-	Isoelectric focussing

IFA	-	Incomplete Freund's Adjuvant
Igh	-	Immunoglobulin heavy chain gene
Igk	-	Immunoglobulin kappa chain gene
Igλ	-	Immunoglobulin lambda chain gene
Igl	-	Immunoglobulin light chain gene
i/p	-	Intraperitoneal
i/v	-	Intravenous
J558	-	Dextran binding myeloma protein
KLH	-	Keyhole limpet hemocyanin
LPS	-	Lipopolysaccharide
MAb	-	Monoclonal antibody
MHC	-	Major histocompatability loci
MIg	-	Mouse immunoglobulin
M104E	-	Dextran binding myeloma protein
M460	-	DNP binding myeloma protein
Nase	-	Staphyloccal nuclease
NIP	-	(4-Hydroxy-5-iodo-3-nitrophenyl) acetyl hapten
NMS	-	Normal mouse serum
NNP	-	(4-Hydroxy-3,5-dinitrophenyl) acetyl hapten
NP	-	(4-Hydroxy-3-nitrophenyl) acetyl hapten
NRS	-	Normal rabbit serum
Ox-1	-	phOx idiotype
PAGE	-	Polyacrylamide gel electrophoresis
PC	-	Phosphorylcholine hapten
PFC	-	Plaque forming colonies
pGAT	-	GAT idiotype

PhOx	-	2-Phenyloxazolone hapten
RaMIg	-	Rabbit anti-mouse immunoglobulin
SRBC	-	Sheep red blood cells
SWM	-	Sperm whale myoglbin
S117	-	GAC idiotype
TGAL	-	Poly-L-(Tyr, Glu)-poly-D,L-(Ala)-poly-L-(Lys) polypeptide
T_sC	-	T suppressor cell
T_sF	-	T suppressor factor
T15	-	PC binding myeloma protein (major PC idiotype)
V_H	-	Heavy chain variable region
V_L	-	Light chain variable region
2-D	-	Two dimensional gel electrophoresis
6-90	-	Identical to Fd-1 Fd binding mAb

DEDICATION

This thesis is dedicated to my wife Lydia, and my parents Vic and Lois.

ACKNOWLEDGEMENTS

It would have been impossible to do many of the experiments, only some of which are presented here, that I did as a graduate and fourth year student without the help and moral support so readily available from the members of UBC Department of Microbiology. As well, I would like to acknowledge the support of Dr. P. Candido and the excellent assistance of Mr. J. Durgo in the modification of the Beckman 890 B sequencer to micro-sequencing capability -- the use of which, very sadly, could not be appropriately taken advantage of in the final form of my project. I wish to acknowledge my debt to Drs. J. J. R. Campbell and J. Levy for initiating and encouraging my interests in microbiology and immunology respectively, and to Dr. Julia Levy and the members of her lab who made my stay so academically and socially enjoyable. In addition, I would like to thank all of the members of my thesis committee for their careful reading of this manuscript. Finally I would like to thank Dr. Lydia Sikora for paving the way for my work, for her excellent help in the preparation of my thesis, and for her encouragement and company during my years in the Department of Microbiology at UBC.

Chapter 1. Introduction

1. Introduction

An important consideration to address prior to any scientific pursuit
is the reason for pursuit at all. The continued study of immune
responsiveness has the potential of yielding benefits in clinical fields
such as transplantation, infection, cancer therapy and preventative
medicine. As well, insights into fields such as biochemistry, molecular
genetics, cell biology and pharmacology have been made. Indeed, the study
of the regulation of the immune response has become a very large field of
study in itself.

The concept of idiotype was introduced by Oudin (1) and Kunkel (2) and
its possible role in immune regulation was postulated by Jerne (3).
Idiotype has been defined as the set of antigenic specifities displayed by
the variable regions of a set of antibody molecules produced by an
individual or group of individuals in response to a given antigen (3).
Idiotypes can be composed of two kinds of determinants. A paratope is a
determinant at the site of actual antigen binding, and an idiotope is an
idiotypic determinant that is not associated with antigen binding (3).
Aside from much data showing the importance of idiotypes in the regulation
of both the humoral (4) and cell mediated immune responses (5), idiotypes
have been used as genetic markers in the study of immunoglobulins at the
molecular level (6) and have been linked to certain clinical disorders such
as myasthenia gravis (7), Hashimoto's disease (8), rheumatoid arthritis (9)
and to the loss of immune responsiveness, due to aging (10). Currently the
study of the role of the idiotype in immune regulation is a topic of great
interest. In order to demonstrate where the investigations comprising this

thesis fit into the field, a review of the major systems involved in the study of idiotypic structure and regulation of the immune response is needed. Although a number of model systems have been investigated, certain systems have been extremely well characterized, sometimes to the molecular level of idiotype structure. These studies will be reviewed in depth.

II. Review Of Hapten Systems

A) p-Azophenylarsonate

The immune response to p-Azophenylarsonate (ARS) or p-Azobenzenearsonate hapten is one of the earliest and best characterized idiotypic systems available. When A/J mice are immunized with the ARS hapten coupled to the protein carrier keyhole limpet hemocyanin (KLH), they produce in their ascites fluid or serum, large amounts of anti-ARS antibodies. Of these, 20 - 70% bear a major cross-reactive idiotype (CRI). These idiotypes are detectable with a rabbit anti-idiotype (6), and have an iso-electric point in the range of 6.65-6.90 pH units (11). Antibodies bearing CRI were found to be of IgG_1 isotype (12), and studies linked the expression of the A/J CRI to the immunoglobulin heavy chain locus (Igh) (6). Thus, the CRI was proposed as a marker for heavy chain variable region genes (V_H) in A/J mice. Preliminary amino acid sequence studies (12, 13) showed that the heavy chain variable regions and the light chain hypervariable regions (14) of induced polyclonal anti-ARS antibodies in A/J mice were homogeneous in their respective primary structures. Further investigation using CRI positive monoclonal antibodies (mAb) did not corroborate these original observations. It was found that MAb exhibited a wide array of binding curves when assayed with anti-polyclonal CRI (15), implying structural heterogeneity of the original antibody population.

They were not usually of IgG_1 isotype (15,16) and differed extensively (16) from the earlier reported (13) heavy chain V region sequence. When individual anti-idiotypes were made by rabbits to each monoclonal, inhibition studies elegantly illustrated the concepts of "Public" or shared and "Private" or individual idiotypes (15,17). Protein sequence analysis corroborated the serological findings of heterogeneity and showed that there was microheterogeneity associated with both the heavy and light chain V regions. However, as might be anticipated, further work showed that even though CRI positive monoclonal heavy chain V regions exhibited some primary structure heterogeneity, the CRI negative heavy chains were much more diverse (19). Inspection of more protein sequence data revealed that the anti-ARS heavy chains could be classified into three families based on homology to a prototype monoclonal (20). One of these families, 91A3, possesses idiotypic determinants that are associated with all CRI positive anti-ARS antibodies (20). The 91A3 determinants were also found in some CRI negative anti-ARS monoclonals as well as polyclonal non-ARS binding antibodies (20). It was thus postulated that 91A3 encodes a single gene responsible for the CRI idiotype. This observation is interesting, since genetic studies had shown that the expression of the CRI idiotype was also linked to the K light chain loci (21). The situation was clarified when it was shown that indeed a specific 91A3 family light chain from either CRI positive or negative antibodies was required to form a CRI positive heavy chain (22). Thus, the expression of the CRI required both specific heavy and light chains, but only heavy chains from a CRI positive antibody could reconstitute the CRI (22). It is worthy to note, however, that the light chain had to be from the 91A3 family - none of the other prototype families could provide the necessary light chain.

Recent studies have analyzed the V_H gene DNA at the germ line level. The postulate that members of the 91A3 protein family are somatic descendants of a single germ line V_H gene (23) has been shown to be correct (24). It was concluded that the diversity seen among CRI positive antibodies was a result of somatic mutation processes. It is expected that only one or two genes will code for the necessary K light chain gene (24) but further work is in progress. The exact structural basis of the CRI idiotype is still unknown, although recent work utilizing monoclonal anti-idiotypic probes and isolated H and L chains (25) may eventually clarify idiotype primary structure in the ARS system.

In parallel with studies to determine the structure of the CRI idiotype, investigations of its role in immune regulation of the anti-ARS response was undertaken. It was found in A/J mice that T cells capable of suppressing the entire delayed type hypersensitivity (DTH) response to ARS could be induced by intravenous (i/v) immunization with rabbit anti-CRI antibody (5). This result was assumed to show that T suppressor cells had anti-idiotypic receptors, and that the T cell repertoire makes great use of idiotypes cross-reactive with B cells. It was also shown that the anti-CRI induced suppression was genetically restricted and linked with the same Igh allotypes as the humoral CRI anti-ARS response (5). Investigations, at this time, of the humoral response showed that A/J mice immunized i/v with syngeneic thymocytes conjugated with specifically purified A/J anti-ARS antibodies had a suppressed CRI positive response to KLH-ARS, although the total anti-ARS response was normal (26). Subsequent work showed that suppressor T cells were induced, which specifically suppressed the CRI response, and that these T cells could also suppress the entire DTH response to ARS (27). A flurry of investigative activity followed. Two

classes of suppressor T cells were found. One class, designated T_s1 was found to bear CRI determinants and was produced by immunization with ARS coupled thymocytes (29). The other class, T_s2, was found to be CRI-binding and was produced by immunizing A/J mice with soluble factors (T_sF1) derived from T_s1 cells (28,29), or produced, eventually, by immunization with rabbit anti-CRI (30). Other studies also showed that T_s1 cells produced T_sF1 factors that were ARS-binding, CRI positive and carried major histocompatability complex (MHC) markers. Also, T_sF2 cell factors did not bind ARS, but did bind CRI (28). It was concluded that appropriate anti-idiotypic and idiotypic interactions were essential for the manifestation of suppressor T cell function in ARS specific suppressor pathways (32). Recently, several T cell hybridomas have been produced (33). Suppressor cell hybrids producing factors binding both antigen and anti-CRI antibodies have been characterized (34), as have T lymphocyte hybrids that bind to CRI positive monoclonal antibodies (33,35). Unfortunately, an ARS binding CRI positive non-immunoglobulin molecule, thought to be a T cell factor but since found to be ubiquitous to almost all cells (and possibly a phosphatase), has complicated matters (36,37). Presumably, work on more carefully characterized T cell factors from hybridomas or cell lines will provide much more structural information on idiotypic determinants of T cells.

Recent experiments have complicated some of the earlier apparent simplicities of the anti-ARS CRI systems. Although the CRI produced in the anti-ARS-KLH response very clearly correlated to the A/J Igh[e] allotype (6), CRI is also produced by other mouse strains in IgM anti-ARS plaque forming cell (PFC) assays (38), when the mice were immunized once with ARS conjugated to Brucella abortus (ARS-Br) (a T-independent carrier). These

results clearly show that the hyperimmune response to a hapten linked to a T-dependent carrier does not represent the full B cell repertoire capable of hapten binding (38). Obviously, some regulatory phenomenon is occurring that has very striking and reproduceable effects. When mice are primed with ARS-KLH and boosted with ARS-Br, the results are similar to those seen when only ARS-KLH is used as an antigen (39). Thus, whatever phenomenon occurs, its effects direct antibody production on subsequent immunization. Other work has verified that different immunization techniques can result in CRI expression in supposedly CRI-negative strains (40). Also, in A/J animals the CRI idiotype is rarely seen at the B cell precursor level (41) and is magnified one hundred fold upon immunization with ARS coupled protein conjugates. This is probably not due solely to clonal expansion driven by antibody affinity, as CRI positive antibody does not have a particularly high affinity for ARS (42,43). Obviously, regulation of CRI production is very complex in mice (39,44) and depends heavily on the immunization protocol. To further complicate matters, it seems that some members of the CRI positive antibody family have no reactivity towards ARS (45).

Thus, investigations of anti-ARS idiotypes have yielded a lot of detail at the molecular level about CRI expression. In A/J mice, CRI positive antibodies are descendants of a single V_H gene (46); this gene is not found in inbred strains that do not express the CRI idiotype (24). T cells express CRI determinants, and anti-idiotypic modulation can produce large changes in the expression of CRI. Thus both genetics and regulation play a role in CRI expression in the A/J anti-ARS response. However, in spite of all of the investigation, the structural basis of the CRI and the mechanism of its regulation are largely unknown.

B) Phosphorylcholine

The study of the anti-phosphorylcholine (PC) response in mice began with the isolation of immunoglobulins produced by mineral oil induced myelomas in BALB/c mice. Some IgA mouse plasmacytomas were found to produce antibody which bound to bacterial polysaccharides (47). Characterization of one of the antigenic determinants revealed it to be phosphorylcholine (48). Eight of these monoclonal antibodies were characterized and injected into strain A mice (eg. A/J) to produce homologous anti-idiotypic sera. Five of these monoclonals (T15, H8, S107, M299, and S63) were found to cross-react extensively with one anti-idiotype (49). When BALB/c mice were injected with rough strain pneumococcus (R36A) cells, they produce a strong anti-PC response. Up to 98% of the anti-PC B cells reacted with anti-T15 idiotype as measured by a plaque assay (50). The serum antibody response to PC was studied in other strains, and large amounts of T15 idiotype were found to be present in BALB/c, C57L, C58, 129 and ST mouse strains. Other strains did not appear to express the T15 idiotype (51). Genetic studies established the linkage of the T15 idiotype to Igh loci (51).

Structural investigations of the murine anti-PC response showed that probably only 3 K light chains were involved in the entire anti-PC response. IEF investigations of isolated heavy chains could not be carried out, however, due to sialic acid charge heterogeneity (52). Other early work showed the anti-PC response to be quite homogeneous within some strains (51,53). This work also showed that some strains of mice which did not express the T15 idiotype (CBA, A, CE) produced antibody having similar binding specificities and L chain patterns as T15 positive antibody (52,53). These data strongly suggested the presence of a T15-like idiotype

in some T15-negative strains of mice. A binding myeloma protein from C57BL/6 mice (C3) was sequenced and found to have 96% homology to T15 (54). Homologous anti-idiotype to C3 very strongly inhibited T15 positive antibodies from binding to PC (55). It was also noted that anti-T15 idiotypes were strongly cross-reactive to the C3 idiotype (55). The C3 idiotype, like the T15 idiotype, was found to be linked to Igh allotype and was seen in the anti-PC response of C57BL/6, C57BL/10, SJL, A, and CBA mice. Surprisingly, C3H mice, which are very similar to CBA mice (56) did not produce C3 idiotype in response to PC (55). Since C3 and T15 idiotypes are strongly crossreactive, one cannot help but wonder why C3 positive strains were not found to be T15 positive in earlier experiments (51). Interestingly, antibodies from BAB.14 mice exhibited both C3 and T15 idiotypes (55). This might be due to unequal crossing over of mendelian (or true) alleles (55) in the generation of the BAB.14 V-C recombinant.

The use of monoclonal antibodies made from PC immunized mice has added greatly to the knowledge of the T15 idiotypic system. Though previous work (51,53) had indicated a homogeneous T15 response, closer scrutiny showed that the response was heterogeneous at the cellular (57) as well as serum antibody levels (58,59). Sequence analysis of monoclonal T15 positive antibodies confirmed this observation (60). Indeed, the PC response was theorized to comprise 3 distinct idiotypic families (61), all of which could have been somatically derived from T15 (60). The expansion of mutant clones was probably driven by antigen affinity, as more "mature" idiotypes have greater affinity for PC than T15 (62). The preferential expression of the T15 idiotype may be due to its association with affinity for PC (63), probably correlating with the presence of a glutamic acid residue at the 35 position of the T15 heavy chain in V_H (64). This residue has been

demonstrated to stabilize the hapten binding pocket (64) and its mutation to alanine destroys the PC binding capability, although T15 determinants are left intact (63).

Molecular genetic studies showed that a T15 V_H gene probe hybridized to 4 genes in germ line BALB/c DNA under stringent conditions (65). Sequence analysis of these genes revealed that 3 of them could be derived from the fourth, the T15 germ line gene (66). Studies of monoclonal antibodies showed that all known H chains sequences, but one could be derived from the T15 germ line gene. The only exception was encoded by one of the other 3 germ line genes (67). Thus, the entire immune response to PC in, at least, BALB/c mice is derived from one germ line V_H gene (66). It was also shown that somatic mutations of this gene were correlated with IgA or IgG isotypes, and not IgM which expressed the germ line T15 sequence (66). This corroborated earlier protein sequence studies, implying that somatic mutation is an event occurring after, or during, the class switch events and thus appears to be a regulated phenomenon (60). Genetic analysis of PC binding monoclonal antibodies showed that most utilized the J_H1 gene segment, exhibited D_H diversity, and utilized one of the 3 K light chain families (63). Unfortunately, though the T15 idiotype system represents one of the best structurally characterized systems, the chemical correlates of idiotypy remain undefined.

Regulatory studies of the T15 idiotypes have been very popular. As previously mentioned, when BALB/c mice are immunized with R36A vaccine, the anti-PC response is almost entirely T15 positive (51). When adult BALB/c mice are pre-treated with anti-T15, the subsequent anti-PC response exhibited a transient absence of T15 idiotype, which reaches normal levels within 2-3 weeks (68). When neonates, however, were treated with anti-T15,

the subsequent lack of T15 antibodies persisted for the life of the mouse (69) though the anti-PC response recovered after 15 weeks. In early studies, this chronic suppression could not be adoptively transferred to young BALB/c mice (70) although later it was shown that immunization with anti-T15 induced Lyt-2 positive T suppressor cells (T_SC) which could transfer suppression (71). When neonates were treated with monoclonal anti-idiotype, directed to various T15 idiotopes, a suppression of the T15 response was seen, although expression of the particular idiotope may still be quite detectable (72).

Hyperimmunization of BALB/c mice with R36A vaccine profoundly lowers the T15 expression (73,74). This "suppression" correlates with the appearance of anti-T15 antibodies in the sera of these mice (73,74). It should be noted that treatment of mice with strain A anti-T15 antibody suppressed the anti-PC plaque response in both C57BL/6 and BALB/c mice (72) even though C57BL/6 mice do not express the T15 idiotype (per se) in the anti-PC response. This result may be due to T15 cross-reactivity with C3.

Direct studies of T cells involved in regulating the T15 positive anti-PC response have been undertaken. At least two helper cells have been characterized. One is involved in MHC product recognition (75) and the other is involved in T15 recognition (76). Both are required to synergistically instigate a T15 positive anti-PC response (77). T suppressor cells have also been investigated. Immunization of BALB/c mice with PC-coupled syngeneic splenocytes caused the production of PC specific suppressor cells that could be neutralized with anti-T15 idiotype (78). A T cell hybridoma that stains with fluorescent anti-T15 idiotype and binds PC (79), produces a factor that has a PC binding site with T15 determinants, but does not express the T15 germline gene segment (80).

Recent work has analyzed T helper cells induced by PC-KLH priming, or monoclonal anti-T15 idiotype, and has shown that these cells cannot distinguish between T15 and M167 idiotypes (81). Such findings indicate that idiotype specific priming can induce non-idiotype specific T cells, implying that the idiotypic T cell network is based on a different selection of idiotypic determinants than the selection of the B cell idiotype network (81).

As with the anti-ARS CRI system, the hapten carrier may dramatically change the anti-hapten idiotypic response to PC. BALB/c mice immunized with Proteus morganii (Potter) produce an anti-PC response consisting of M603 idiotype (82) and not T15. This result may be due to the higher affinity M603 has for P. morganii (Potter) PC (82). Curiously, CBA/N mice bear an X-linked immune deficiency, such that F_1 females, carrying one copy of the gene, will respond normally, but F_1 males will not produce an anti-PC response (83). It has been shown that the F_1 males possess a T15 (or C3) specific helper T cell, but lack an Lyb-5 positive B cell population responsible for the production of anti-PC IgM (84). Another curiosity was observed when neonatal BALB/c mice were immunized with PC linked to a protein carrier. When the mice were boosted 30-40 days later, the anti-PC response was less in both amount and affinity than the placebo-immunized controls, and a lower ratio of T15 positive antibodies is observed. Apparently, immunization of neonates with PC coupled to protein carriers is associated with the production of low avidity, T15 negative antibodies (85). This phenomenon is not mediated by a T cell or auto anti-idiotype (85). Another complication is the fact that a monoclonal antibody to a V_K T15 determinant cross-reacts with a Thy-1 determinant (86).

Thus, the molecular genetics of the B cell T15 positive anti-PC response are well characterized. The T15 idiotype seems to be restricted to the Igh[a] allotype family, but a very closely related and possibly allelic gene is expressed by mice of different Igh allotypes. Various studies have evaluated the role and the characteristics of the T cell T15 response, and several studies have examined the regulation of the T15 idiotype. In spite of these investigations the structural character of the T15 idiotype is unknown, and the idiotypic regulation seems very complex and largely undefined.

C)   4-Hydroxy-3-nitropheny) acetyl (NP)

Another hapten system in which the idiotypic response has been extensively studied is that of the (4-hydroxy-3-nitropheny) acetyl (NP) hapten. When C57BL, 101 or LP mice are immunized with NP on a protein carrier, such as bovine serum albumin (BSA), or chicken serum globulin (CSG), the antibodies produced bear a major cross reactive idiotype (NP-b) in the primary response (87,88). These antibodies also have a peculiar binding specificity (heterocliticity) by which anti-NP antibodies actually bind to related haptens such as (4-hydroxy-3,5-dinitrophenyl) acetyl (NNP) or (4-hydroxy-5-iodo-3-nitrophenyl) acetyl (NIP) better than they bind to NP itself (89). Another idiotype (NP-a) has been linked to the Igha allotype (90). It was suggested (90) that the Np-a and NP-b idiotypes were allelic since both idiotypes are associated with λ light chain bearing antibodies, which are detected most easily in the primary response, are heteroclitic (91) and are mutually exclusive in all conventional and recombinant strains studied (90). Recent investigations have utilized monoclonal antibodies produced from the NP-a positive anti-NP response in BALB/c mice. These studies show that 50% of the monoclonal antibodies

express the K light chain -- the rest express λ, as seen in the primary serum response (92). After 3 weeks post immunization, or in the secondary response, the BALB/c antibodies express the K chain predominantly (92). Both λ and K bearing monoclonals were heteroclitic, but only λ bearing monoclonals expressed the NP-a idiotype (92). When NP-a and NP-b bearing monoclonals were compared, a subset of NP-b shared idiotypic determinants with NP-a (93). This cross-reactivity was also observed with immune sera of individual NP-b positive anti-NP responding mice (93).

One monoclonal antibody bearing NP-b idiotypic determinants has been very well studied. B1-8 is an IgM antibody that carries expressed germ line V_H and V_L genes (94,95) and possesses at least two idiotopes, corresponding to the specificities of two monoclonal anti-idiotypes, Ac38 and Ac146 (96). The B1-8 idiotopes are regularly expressed in the NP-b positive anti-NP response (97). Structural studies of B1-8 idiotopes have shown that both V_H and D_H regions are important in idiotype expression (98). Very small changes in primary structure can drastically affect idiotype specificity, such as the case of a mutant of B1-8. There, a single amino acid is altered in D_H causing the loss of seven parental idiotopes and changes in an eighth, but retention of NP binding (98). When Igh^b mice are immunized with monoclonal anti-B1-8 idiotypes, they produce B1-8 idiotype positive antibodies, some of which do not bind NP (99). The pattern of specificities produced is strain specific and it is thought that these observations provide evidence that the idiotypic repertoire is a result of V_H region polymorphisms (99) and that idiotypic and antigen binding residues are structurally separate. This proposal is reinforced by finding that an NP-b idiotype positive, non-NP binding antibody can be

generated by replacing most of B1-8 V_H gene with a neighboring V_H gene (100).

Recent studies have compared the V_H genes coding for the NP-a and NP-b idiotypes in mice (101). A V_H cDNA probe for B1-8 was used to screen C57BL/6 germ line DNA, revealing 7 bands on Southern blotting and hybridization under fairly stringent conditions (94). This same probe was used to screen BALB/c germ line DNA, and 5 genes were subsequently sequenced. The two that were not pseudogenes shared homology with the NP-b positive cDNA probe, but could not code for a protein binding NP (101). When the NP-b probe was compared to NP-a V_H cDNA, 28 (of 98) differences were noted (101). Thus the NP-b and NP-a V_H genes are quite different.

T suppressor cells that suppress the DTH response in Igh^b mice can be induced by immunizing C57BL/6 mice with NP-coupled syngenic splenocytes (102). These suppressor T cells are Ig V_H and MHC restricted (103). It was subsequently found that two populations of T cells were involved (104). One population was NP-b idiotype bearing and bound NP (104) but was probably not heteroclitic (102). The other population possessed anti-idiotypic receptors (104). Early results showed that NP binding, Ig negative molecules could be purified from sensitized T cells by antigen binding. These molecules were shown to bear the NP-b idiotype (105). More recent work has corroborated these findings. A T cell hybridoma from B10.BR has been isolated that binds to NP and possesses NP-b and framework Ig V_H determinants. This cell produces a T suppressor factor specific for the anti-NP response that is NP-b positive, I-J positive and is distinct from the cell surface receptor (106).

In vivo studies with anti-idiotypes have shown that monoclonal anti-NP-b idiotypes can suppress the response to NP-coupled sheep red blood

cells (SRBC) (107). This suppression could be mediated by IgG but not IgM or IgD monoclonals, or even $F(ab)_2$ fragments (107). Injections of small amounts of NP-b positive monoclonal antibody (B1-8) led to an increase in B1-8 idiotype expression in the anti-NP response (98). Recent work, however, has shown that virtually all of the NP-b idiotype of T cell preparations is due to conventional NP-b positive anti-NP antibody tightly bound to T cells (108). Such results make further interpretation of the T cell idiotypic response and control of idiotype expression difficult.

In summary, two major idiotypes expressed in the anti-NP response in mice have been characterized. These idiotypes are linked to different Igh alleles and are probably not allelic. Some structural information of the NP-b idiotype has been provided, both at the protein and gene levels. The regulation of the anti-NP response by idiotypic and T cell involvement has been investigated, but, unfortunately, some controversy is encountered in interpreting the results.

III. Carbohydrate Antigen Systems

A) Dextran

This T-independent system has been extensively studied and has revealed the most definitive information on structural correlates of idiotypy. Two idiotypic families of dextran binding myeloma antibodies have provided much of the information. M104E (IgM,λ) has optimal specificity for the α(1-3) linked dextran trisaccharide (109), while J558 (IgA,λ) has specificity for the pentasaccharide (110). The light chains of the two myeloma proteins are identical (111) and their V_H regions differ only at residues 100 and 101 (112). When mice of Igh^a allotype are immunized with B1355 dextran, they produce antibodies bearing light(λ) chain and sharing idiotypic determinants with J558 (113). This response is linked to

the Igh^a allotype (113). Further studies showed that over 50% of these induced antibodies share idiotypic determinants with both J558 and M104E (IdX) (114) and all of the serum antibodies have homologous light chains, as determined by IEF (115), to the myeloma antibodies. Thus most, if not all, of the information determining idiotypic specificity must lie in the V_H region. Structural studies of many monoclonal anti-dextran antibodies have been undertaken. The presence of arginine and tyrosine at positions 100 and 101 respectively, correlate with the individual idiotype (IdI) and binding specificity of J558 (112,116), while the presence of tyrosine and aspartic acid correlate with the IdI and binding specificity of M104E (112,116). The elimination of position 101 destroys both the J558 and M104E IdI determinants (112). IdX determinants correlate with residues at positions 54 and 55 in the V_H region. Since a carbohydrate moiety is attached to the asparagine at position 55, it is tempting to postulate that carbohydrate may have a role in defining IdX determinants (112). J_H and D_H sequences have been found to vary a lot without destroying either dextran binding or IdX specificity (112,116).

The dextran idiotype results are similar to those seen with anti-inulin idiotypes. Studies of this T-independent carbohydrate system show that individual idiotypes (IdI) are probably determined by two amino acids, though IdX determinants are more complex and involve residues inside and outside the hypervariable region. Some pairs of monoclonal antibodies differed by more idiotypic determinants than amino acid substitutions, implying conformational effects (inulin reviewed in 64).

Though the structural studies of anti-dextran idiotypes have utilized the T-independent response, a T-dependent response can be studied by using dextran attached to a protein carrier (117). When the T-independent and

T-dependent responses are observed, one finds IdX and IdI expression associated far more with the T-independent response (118). Thus, the two forms of the dextran antigen trigger different B cell precursor subpopulations (118). Interestingly enough, the frequency of T-dependent precursors is almost 3 times greater than the frequency of T-independent precursors (117).

In summary, the study of anti-dextran idiotypes has been very valuable in defining the role of primary structure in relation to idiotypic determinants, though the conformational aspects are still unknown. Unfortunately, the T-dependent anti-dextran response is rarely associated with T-independent idiotypes, making studies of regulation dependent on redefining anti-dextran idiotypes for the T-dependent response.

B) <u>Streptococcal Group A Antigen</u>

When A/J mice are immunized with group A streptococcal vaccine, they respond by producing antibodies of limited heterogeneity and bearing a major idiotype (119). This idiotype, designated A5A, was found to be linked to the Igh^e allotype (120). A/J mice were reported to make from 0 to 60% of all anti-streptococcal A (group A carbohydrate = GAC) antibody with A5A determinants (121), and these antibodies have limited, characteristic pI values (122). Such serum antibodies were initially found to be predominantly of IgG$_{2a}$ isotype (121), though now it is evident that they are usually of IgG$_3$ (123) or IgM (124) isotypes and express light chains that share pI valves by IEF(125).

Other anti-GAC serum cross-reactive idiotypes have been characterized. The S117 idiotype is seen in the BALB/c anti-GAC response, linked to the Igh^a allotype, and appears not to cross-react with the A5A idiotype (126). Also, in studies of hyperimmune A/J mice, it was found that monoclonal

anti-GAC antibodies share the common light chain spectrotype previously seen in serum (127). Anti-idiotype made to a monoclonal antibody possessing this light chain (anti-V_K GAC idiotype) reacted with all proteins possessing the V_K GAC light chain (127), even from mice outside the Igh^e allotype group (127).

Some investigators are very unhappy with the way in which antibodies bearing A5A idiotype have been characterized (127). It is thought that there is no dominant clone with A5A idiotype in the A/J anti-GAC response, because it is not a frequently occurring spectrotype (128). Though different anti-A5A reagents detect different determinants (125) it is not clear that they are on the same molecule (127). Since V_K GAC idiotype is present on 50% of A/J anti-GAC antibodies and reacts across Igh barriers, it is strange that A5A is only found in the A/J strain (121). The Igh allotype linkage of A5A may be a result of such loci affecting light chain expression, thus the A5A idiotype may not be a heavy chain marker (129). The anti-A5A reagents are known to react with some V_K determinants (125). Thus it was thought that in the anti-GAC response, diverse V_H regions are associated with a restricted number of V_K regions (127). Some interesting new experiments have shed more light on anti-GAC immunoglobulin structure. Multiple, closely homologous V_H gene segments contribute to the generation of anti-GAC antibodies (130). A common framework sequence, related to the V_K27 subgroup probably defines V_K GAC (130). As well, the A/J anti-GAC and BALB/c anti-inulin V_H regions are 95% homologous at the protein level and are likely encoded by overlapping V_H gene families (130). Furthermore, in the anti-GAC response, as in other antibody responses (24,66) somatic mutation events amplify the diversity of the multiple heavy chain genes

(130). At present, more experiments are in progress to determine the basis of the V_K GAC idiotype.

Results of experiments done to evaluate the regulation of the A5A idiotype, may be complicated by work implicating the anti-GAC response to be T-independent (123,124), work showing the response to be T-dependent (131), and work showing it to be both (132). The most convincing data indicated thymus dependence (132).

Guinea pig antisera were raised to purified A5A positive A/J anti-GAC antibodies. Mice receiving anti-A5A of IgG_2 isotype were suppressed for the production of the A5A idiotype without concurrent loss of anti-GAC activity (122). Mice receiving anti-A5A of IgG_1 isotype had slightly enhanced expression of A5A idiotype, again without affecting the total anti-GAC response (122). If A/J mice were immunized with the guinea pig IgG_1 anti-A5A idiotype, the mice could be shown to be sensitized to GAC without antigen (133). Strangely enough, this phenomenon could be observed in C57BL mice, which do not express the A5A idiotype in their anti-GAC response (121). Guinea pig IgG_2 anti-A5A idiotype was observed to induce idiotype specific suppressor T cells (134), probably bearing Lyt-2,3 antigens and susceptible to killing with anti-A5A and complement (C') (135). High doses of the IgG_2 anti-A5A idiotype were associated with short lived suppression (134) whereas lower doses were associated with a much longer lasting suppression (134) that could be adoptively transferred with as few as 10^5 cells (134). IgG_1 anti-A5A was associated with the production of idiotype specific T helper cells (133) that were found to respond only to anti-idiotypes prepared to idiotypes expressed by the donor strain (136). This phenomenon was illustrated by observing the A/J or BALB/c anti-GAC responses. Anti-A5A primed only A/J mice to GAC, whereas

anti-S117 primed only BALB/c mice (137), seemingly in contradiction to earlier results (121). The A5A helper T cells were only recognized by anti-idiotype having specificity for V_H region determinants to A5A (125). These experiments suggested that T cells carried B cell idiotypic determinants. Since there appears to be some controversy about the structure of B cell anti-GAC idiotypes, perhaps it would be better to wait for clarification before interpreting studies with T cells done with anti-B cell idiotypic reagents.

In summary, several anti-GAC idiotypes have been characterized, of which A5A has been the most important. Recent work has described another major A/J anti-GAC idiotype (V_KGAC) which is associated with a particular light chain family. Further work has correlated the V_KGAC idiotype with a V_K subgroup similar to V_K 27. Molecular genetic studies have shown the presence of multiple heavy chain genes in the A/J mouse genome that can provide the anti-GAC response. These gene products combine with a small number of light chains. These molecular geneticists in particular have been critical of the earlier definition of the A5A idiotype.

Regulatory studies have been concerned with the A5A system in A/J mice and the S117 system in BALB/c mice. Helper and suppressor T cells have been characterized and some elegant _in vivo_ experiments have provided data useful to theoretical immunologists (138,139). However, more investigations are needed to properly define the A5A idiotype. In this system, only the V_K structural correlates with idiotype have been discovered.

C) Lipopolysaccharid Antigens

Lipopolysaccharide (LPS) is a protein-free bacterial endotoxin composed of a lipid moiety (Lipid A) and a polysaccharide moiety,

determining the serological classification of the organism (reviewed in 140). Aside from its biological importance in instigating a strong anti-bacterial response, LPS is best known to immunologists as a potent polyclonal B cell activator (141); yet the specific immunogenicity of LPS is very strong (142). Recent work has shown that the immune response to LPS is genetically controlled by genes linked to the H-2 and to the X-chromosome (143). The memory response characterized by production of IgG antibodies to LPS has been mapped to genes linked to the Igh loci in mice (144).

Investigations of the idiotypic responses to LPS have commenced with the production of monoclonal anti-LPS antibodies from BALB/c mice responding to different carbohydrate residues and different bacterial LPS antigens, which share common idiotypes (IdX) (145). When antisera from mice immunized with LPS from E. coli 0113 were studied, the IdX was seen in individual immune sera from all strains of mice tested (146). These results demonstrate that no allotype linkage of LPS idiotypes is observed in the PFC anti-LPS response. It was also found that a minor idiotype (IdZ), not seen in the primary CE/J or C57BL/6 response, was seen in hyperimmune sera, demonstrating some kind of idiotypic maturation (146). It was thought by these investigators that shared idiotypes are common for antibodies specific for an antigenic family (145). Unfortunately, these LPS results are complicated because it is unclear whether IgG or IgM PFC are detected in the hyperimmune anti-LPS responses. If IgG plaques are being produced by all strains, earlier findings of memory linkage to allotype are contradicted and if IgM is found to undergo idiotypic maturation, then quite a lot of other earlier work is contradicted

(60,62,66). Obviously, more experiments have to be done to biochemically define LPS idiotype interstrain cross-reactivity.

IV. Polypeptide and protein Antigen Systems

A) (Glutamic acid ^{60} alanine ^{30} tyrosine ^{10}) Random Terpolymer (GAT)

The examination of the immune response to GAT may be thought of as the missing link between studies of hapten and protein systems. The response to GAT and related random copolymers has been extensively reviewed elsewhere (147), but some key points will be briefly discussed. GAT is a random copolymer of various proportions of glutamic acid, alanine and tyrosine. The response to GAT has been linked with H-2 loci (148), but non-H-2 linked gene influence was also observed (149).

Idiotype studies have been undertaken with GAT even though, if one assumes a pentapeptide-size determinant as seen with other studies (150,151,152) there could be at least $(3)^5$ or 243 theoretical determinants, ignoring epitope dominance or pH effects. In various inbred strains of mice, GAT immunization caused the production of antibodies bearing an interstrain cross-reactive idiotype. Those defined using a guinea pig anti-idiotype are referred to as CGAT (153) whereas those defined with rabbit reagents are referred to as pGAT (154). They may be assumed to be defining similar if not identical idiotypes (155). Both CGAT and pGAT can be found in rat antisera (153,156) and pGAT has been observed in guinea pig anti-GAT sera (156). CGAT can be observed upon immunization of mice with poly (glutamic acid, tyrosine) (GT) (157), but not with poly (glutamic acid, alanine) (GA) (158). Studies of antibody production (153,154) as well as IEF analysis (159) indicate that all inbred strains of mice produce CGAT/pGAT (153,154) and this may be due to conservation of common anti-GAT V_H and V_K germline genes, since "responder" and "non-responder" mice made

pGAT/CGAT positive antibodies. Monoclonal anti-GAT antibodies possessing CGAT idiotypic determinants were characterized (160) as well as some that share idiotypic specificities with anti-GA antibodies (GA-1 idiotype) (161). In addition, mice of Igh-1^a, Igh-1^c, and Igh-1^e allotypes share a common idiotype (srGAT-1) (162) as do mice of Igh^b allotype (Gte) (163). The Gte idiotype has been mapped to the Igh-V loci near Igh-NP, Igh-Nbp and Igh-Bgl markers, and its expression is not linked to Lyt-3 (Igk) loci (164). As well, antibodies uniquely specific to GAT share a cross-reactive idiotype with antibodies to poly (GT) or poly (glutamic acid, lysine) (GL) (165). As can be seen, many types of cross-reactive idiotypes are associated with the anti-GAT response.

The best structural characterizations have utilized the pGAT/CGAT idiotypes. These specificities require the interaction of both heavy and light chains to be expressed (166). The pGAT/CGAT determinants were also found to be associated with the antibody combining site, as idiotype-anti-idiotype interactions were inhibited with antigen (166). Amino acid sequence data from monoclonal anti-GAT immunoglobulins have shown that the kappa light chains from CGAT positive antibodies are identical and define a new V_K subgroup (167). Analyses of CGAT positive heavy chains indicate that hybridoma sequences are present in polyclonal anti-GAT immunoglobulins, pGAT/CGAT specifities are associated with heavy chains of limited heterogeneity and CGAT negative GA-1 positive heavy chains are similar to CGAT positive heavy chains, but are associated with a different light chain (155). It is thought that CGAT and GA-1 idiotypic determinants are germline markers for both V_H and V_K genes (155).

Recent data have been obtained at the level of the anti-GAT genes. The mRNA encoding the V_H regions (V-D-J genes) of 4 BALB/c pGAT positive monoclonal anti-GAT antibodies was sequenced, showing that 2 of the monoclonals were identical and the other two different in 3 and 8 positions respectively (168). When a cDNA V_H region probe from one of the homologous monoclonals was used to investigate germline DNA from 3 different strains differing in Igh allotype, hybridization, under stringent conditions, of Southern blot patterns made with various restriction endonucleases, showed 3-7 non-identical gene fragments (169). These results indicate that a small number of non-identical germ line genes (3 to 7) hybridize with the pGAT cDNA probe, suggesting that the V_H anti-GAT repertoire differs between at least 3 strains, even though their anti-GAT antibodies bear the pGAT idiotype (169). Recent work, comparing the V_H gene sequences of anti-GAT and anti-NP antibodies, shows that some heavy chains are derived from the same germline V_H gene - but are associated with different light chain isotypes (170), providing an elegant example of heavy and light chain combinational association determining immunoglobulin specifity.

Subsequently, the V_K genes were investigated, using the pGAT bearing monoclonals described previously (168,169). BALB/c, DBA/2 and C57BL/6 mice were shown to possess 3-5 identical Bam H1 or Eco R1 Southern blot patterns of germline DNA, hybridized under stringent condition to a pGAT positive V_K DNA probe (171). Further study of V_K regions (V-J genes) revealed that 3 germline genes are present in all strains, but that a given strain may not use all of them for the anti-GAT response. All of these V_K regions are very similar in primary structure (172).

The role of the CGAT/pGAT idiotypes in the T cell response has been studied. When mice of genetic "non-responder" status are immunized with

GAT, they produce T suppressor cells from which factor may be isolated (173). This factor binds to GAT, bears I-J but not Igh or Igl constant region determinants (174) and can stimulate the in vitro generation of T_s2 cells in "responder" strains (175). This T_sF has been shown to express the CGAT idiotype (176) and is now being produced by T cell hybridomas (177). Another T_sF, produced by a "responder" strain has been immortalized in a T cell hybridoma and possesses properties similar to T_sF produced by "non-responder" strains (178). The idiotypic profile of these elegantly purified monoclonal T suppressor factors (178,179) has not yet been published.

The presence of CGAT on helper T cell lines has recently been established. Syngeneic anti-idiotype that detects CGAT determinants produced by an anti-GAT monoclonal antibody will specifically inhibit help to an in vitro GAT-DNP (dinitrophenol) response by two T helper cell lines (179).

Molecular genetic analysis of 10 GAT-specific suppressor T cell hybridomas (6 of which secrete factors with CGAT specificities) and 3 GAT-specific helper T cell lines or hybridomas, has shown that T and B cells that recognize the same antigen do not transcribe similar heavy chain variable region gene segments, with a sensitivity of 1-2 mRNA copies per hybridoma cell and 5-10 mRNA copies per T cell line cell (180). In fact, one suppressor T cell hybrid producing antigen specific factor was found to produce below the minimum detectable limits (10 copies/cell) of any IgV_H gene, with a probability of detection greater than 99% (181).

In summary, the immune response to GAT is under the control of several genes, though all mice can produce anti-GAT antibodies. These antibodies exhibit idiotypic determinants, the best characterized of which are

CGAT/pGAT. Both B and T cells may express CGAT determinants. Molecular genetic analysis of B cells has shown that there are 3-7 anti-GAT-like germline V_H genes, that differ between strains. One of these may code for both NP or GAT binding, depending on its products association with a different light chain. There are probably only 3 anti-GAT light chain genes conserved between strains. T cells expressing CGAT/pGAT idiotypic determinants do not express V_H genes. As of yet, no structural correlation between anti-GAT idiotypes and amino acid sequence of either anti-GAT H or L chains has been shown.

B) Myoglobins

The mammalian myoglobins have been of immunological interest for the same reasons as the lysozymes. Myoglobins are small, easy to obtain and purify and nature has kindly provided many chemical variants whose structures have, in many cases, been determined (reviewed in 182). Studies with rabbit and goat antisera showed that there are 5 major antigenic regions and that antibodies are directed to conformational determinants (150,182). These studies were confirmed in mice at both B and T cell levels (183). Early work also showed that the antigenicity of a structural region of sperm whale myoglobin (SWM) is related only to the structural location and not the difference between SWM and other myoglobins (183). Further work showed that the response to different determinants of SWM does not change over the time course of the response (184). Thus, one would not expect to see a difference between epitope recognition by early antisera (such as used by Atassi to define SWM) (182) and antibodies produced later in the response. Surprisingly, when monoclonal antibodies were used, (185,186), the antigenic determinants recognized were vastly different from those observed earlier (150). These results were confirmed independently

with human myoglobin (187). All of the "new" B cell epitopes were topographical.

Experiments to characterize the T cell response initially revealed that T cells recognized the same structural regions of SWM as B cells, but that T cells recognized linear instead of conformational determinants (183). Later it was suggested, based on work with T cell clones, that T cells were incapable of binding conformational determinants (188), although this hypothesis was shown to be wrong, as some T cells were found to require a conformational determinant both in vitro (189) and in vivo (190). Thus, there may not be a separation based on epitope recognition of T cells and B cells in the SWM response. The genetic response to SWM has been carefully reviewed elsewhere (147). Early studies showed that both H-2 and non-H-2 genes controlled responsiveness to SWM in mice (191), but the H-2 linked regulation could be overcome with high doses of antigen (192). It also appears that the chemical properties of the antigenic site can determine the genetic control of the response to a particular myoglobin (193).

Idiotype has only recently been studied in the myoglobin system. Monoclonal antibodies were used to induce the formation of anti-idiotypic antisera to each monoclonal in guinea pigs. The results showed that high affinity monoclonal antibodies to different determinants, cross-reacted with respect to idiotype (194). Further data evaluating the idiotypic response in the myoglobin system are expected soon.

The myoglobin system (notably SWM) has been extremely well characterized with regard to epitope structure, genetics of responsiveness, T and B cell interactions and recent work has involved studies of the presence of cross-reactive idiotypes on anti-SWM antibodies.

Unfortunately, the determinants associated with myoglobin are almost all conformational and many exist. The genetics of responsiveness are complex, and the idiotypes of antibodies to different determinants cross-react. Thus, it may be difficult to obtain definitive data on the role of idiotypes in the myoglobin system.

C)   Lysozymes

The lysozymes exist in nature as a number of related, but distinct molecules. Such a situation is very useful immunologically, since response to molecules differing only in a few chemically (and sometimes conformationally) defined locations can be studied. Studies of the epitopes of hen egg-white lysozyme (HEL) originally revealed 3 antigenic conformational sites, as defined by rabbit and goat primary antibodies (although essentially all of them were IgG) (195). Subsequent investigations have discovered many other determinants associated with HEL as defined by heterologous (196,197) or monoclonal antibodies (198). These determinants seem to be predominantly conformational.

Genetic studies of the response to the gallinaceous egg-white lysozymes showed that H-2 linked genes control the response status, while non-H-2 linked genes control the magnitude of the hyperimmune response (199). T and B cells recognize several epitopes and the HEL response is regulated through an antigen bridging mechanism (200). Some T and B determinants are located on the same fragments of HEL (200,202,203,204) and helper and suppressor T cells were thought to recognize different determinants in non-responder mice (202). The model of antigen bridging between T and B cells recognizing different determinants has recently been independently confirmed (205).

Experiments with responder sera show that there is a major interstrain cross-reactive idiotype, whose expression does not correlate with H-2 or Igh allotype (206). Only serum antibodies specific for the NC-HEL fragment express this idiotype (IdX) (206), but monoclonal anti-HEL antibodies, specific for different epitopes, also express IdX determinants (207,208). Further studies showed that IdX determinant-bearing antibodies replace an IdX negative population during maturation of the anti-HEL response, implying a selective mechanism for idiotype expression (208). This observation was confirmed utilizing hybridomas from fusions carried out on HEL immune splenocytes at various times during the anti-HEL response (208,209).

Studies of IdX expression at the T cell level have provided several interesting observations. Utilizing several B10 recombinant strains, T suppressor cells in nonresponders specific for HEL may be induced by immunizing with anti-idiotype. Conversely, anti-HEL T suppressor cells may be killed with anti-IdX and complement (203). Thus, T suppressor cells and B cells can share IdX, and a T cell line producing a factor suppressing the primary and secondary anti-HEL responses in vivo has been isolated. The factor will not suppress the response to a very closely related lysozyme (ringneck pheasant lysozyme) nor the L_{II} fragment of HEL, thus demonstrating fine epitope specificity (210). Subsequently, the epitope specificity was narrowed to the phenylalanine residue at position 3 of HEL and the factor was shown to be both Igh and H-2 restricted, although the B cell response is not so restricted (206,211). Studies of the idiotypic nature of this factor are expected soon.

Thus, the lysozymes, most notably HEL, are small, related proteins that provide many conformational determinants for the B cell response,

although the T cells may recognize linear determinants (204). Careful studies have determined the genetic response and the role of T and B cells in the anti-HEL response. A major crossreactive idiotype that is seen in the mature anti-HEL response of many diverse strains, and is expressed by both B cells recognizing different HEL epitopes, and T cells has been characterized. Recent work, not yet available in the published literature, may shed more light on the anti-HEL IdX system (212).

D)   Staphylococcal Nuclease

Staphylococcal nuclease (Nase) is an enzyme isolated from cultures of Staphylococcus aureus, whose structure has been well characterized (213). Since Nase has an enzymatic activity, early studies used to assay antibody binding by the drop in Nase activity (214). Studies of Nase B cell epitopes showed that most were associated with the conformation of the antigen (215), making it very difficult to define the structure of determinants. T cells appear to be less restricted, and respond well to Nase fragments after Nase priming (214). The response to Nase in mice has been shown to be genetically controlled, at the level of the primary response, by H-2 linked genes (216), although the hyperimmune levels did not correlate with either the H-2 or Igh loci (217). Later work showed that the Ir genes were affecting the determinants specificity of the anti-Nase response (218) and non-H-2 linked genes controlled the magnitude of the response (217).

Further experiments characterized the genetics of anti-Nase idiotype expression. The presence of A/J H-2 linked genes was not necessary for the expression of idiotypes cross-reactive with A/J anti-Nase idiotypes, in studies with congenic recombinants (219); the H-2 genes were necessary for the determinant response to Nase (218). The A/J and SJL anti-Nase

responses both exhibited major intrastrain cross-reactive idiotypes, but the two idiotypes did not share specificities and were subsequently found to be linked to their respective Igh loci (219). BALB/c mice expressed both idiotypes in their anti-Nase responses (219). Further investigation characterized 5 separate idiotypes in the Nase response (220). These markers were shown to map to different V_H genes and were useful in detecting the V_H-C_H recombination event observed in the BAB.14 strain (220).

Various studies of the regulatory properties of anti-Nase antibodies have been undertaken. BALB/c mice treated with pig anti-BALB/c idiotype will produce idiotype positive antibody that will not bind Nase (221). These mice were found to contain antigen-specific helper T cells that were susceptible to anti-idiotype and complement, and were similar to those induced by priming with Nase (221, 222). A similar situation occurred in the A/J anti-Nase idiotypic system (223). Interesting experiments in the BALB/c system utilized recombinant strains. Pig (anti-BALB/c idiotype) antisera and complement killed only T helper cells from BALB/c and not B10.D2 mice (224). As well, B10.D2 animals did not produce anti-Nase antibodies of BALB/c idiotype. Yet B10.D2 mice given pig anti-BALB/c idiotype produced non-Nase binding, BALB/c idiotype positive antibodies, and idiotype positive, antigen specific T helper cells (224). Thus, idiotypes and binding sites could be distinguished at the B cell, but not at the T cell level.

In summary, the Staphylococcal nuclease system exhibits quite complex genetic mechanisms of response. T and B cell determinant recognition seem to be different, with B cells responding to a variety of conformational determinants. At least 5 different idiotypes are seen in the murine

anti-Nase response, which all map to regions linked to Igh loci. T helper cells, bearing some of these idiotypes have been characterized and some elegant experiments have shown different modes of idiotype expression between B and T cells. Unfortunately, the structures of B and T cell idiotypes in the Nase system are unknown, and idiotype regulation, which has not yet been studied to any great extent, appears to be extremely complex.

V.   Introduction Summary

A)   Hapten Systems

Idiotype analyses involving responses to haptens probably represent the simplest available systems. Haptens are small antigens and will have only one epitope. Also, the immune response to haptens is not usually controlled by H-2 linked genes. One may use haptens with T-dependent (11) and T-independent (38) carriers to control for carrier effects. Many important observations have been made about idiotypic structure and regulation, utilizing hapten systems. The best characterized of these are the ARS, PC and NP systems, and these have already been discussed. Pertinent work has been done with other haptens. 2-Phenyloxazolone (phOx) on a protein carrier causes the production of large amounts of antibodies bearing specific idiotype in BALB/c and DBA/2 mice (225) as defined by rabbit anti-idiotype and IEF. This marker (Ox-1) was linked to the BALB/c Igh allotype in studies with recombinant mice (225), though Ox-1 was expressed in the anti-phOx response of 5 strains of varying allotype (BALB/c, DBA, A/J, SM, RIII) (225). Monoclonal anti-phOx antibodies made at different times during the primary response were tested for idiotypic determinants recognized by conventional anti-idiotype (225) or sera recognizing anti-phOx heavy, and normal chain, recombinant antibodies.

Strangely enough, no monoclonal was recognized by the conventional anti-idiotype, whereas almost all day 7 but no day 14 monoclonals reacted with the anti- (reconstituted antibody) idiotype (226). mRNAs of the V_H and V_L regions of three idiotype positive and one idiotype negative monoclonals have been sequenced, and consensus V_{HOx-1} and V_{LOx-1} germline genes have been defined (226). More work in this system is forthcoming.

Another useful system is the study of the response to dinitrophenyl hapten (DNP). The presence of idiotypic determinants of the DNP-binding myeloma protein M-460 were seen in the sera of immune mice (227). The expression of M-460 idiotype was correlated with the Igh^a allotype using BALB/c recombinants (227). Closer scrutiny revealed that some M-460 determinant expression was linked to V_K loci (228). The expression of M-460 idiotype in the anti-DNP response is transient and regulated independently of the anti-DNP response (229). Pre-immunization of BALB/c mice with syngeneic anti-M-460 idiotype will enhance the production of M-460 determinants (230). Thus, the expression of the M-460 idiotype is probably associated with genes linked to both Igh and Igk loci. Studies of the T cell anti-DNP response will not be reviewed here.

In summary, the majority of idiotypic studies have been done in the ARS, PC, NP, ph(Ox) and DNP hapten systems. In the majority of these cases, idiotypic determinants appear to be Igh germline markers, although Igk certainly can play a role in idiotypic expression. Some of these germline genes are shared by mice of differing Igh allotype. Molecular genetic studies have shown that somatic mutation plays a major role in defining antigen binding or idiotype specificity of an expressed germline gene, but the structural correlates of idiotypy are poorly defined by hapten systems as of yet. Much data concerning immune regulation have been

obtained by the study of idiotype anti-idiotype interactions in hapten systems. Despite these investigations of idiotypy, even the simplest mechanisms of idiotypic regulation are largely undefined.

Some objections to the use of haptens may be that while being very useful tools, they represent quite abnormal antigenic structures (with the exception of PC), the immune responses to them may be too simple in comparison to the responses to more complex, natural antigens, and since they are small molecules (231), they may be readily bound by antibody specific for other antigens. Thus, while the information forthcoming from idiotypic studies may be very relevant in defining many aspects of idiotype expression and regulation, it should be kept in mind that any comprehensive conclusions regarding this element of immune responsiveness must include comparable data derived from more complex antigens.

B) Carbohydrate Antigen Systems

One reason that simple carbohydrate systems such as dextran, inulin and galactan are studied with regard to idiotypy is due to the early isolation of myeloma proteins with specificity for simple carbohydrate moieties. Carbohydrate determinants in polymers are natural, large enough to fill the binding site of antibodies (109,110), are easily available (except for GAC) independently of major regulatory influences from T cells in their native form. The responses to LPS or GAC constitute a natural and high level of response which is important for the host defence against bacteria. The more simple carbohydrates studied immunologically often are found on some part of the bacterial cell wall, outer membrane, or capsule.

The idiotypic response to simple carbohydrates is often very restricted, hence the interest in dextrans, inulins, and galactans. As has been shown, the dextran and inulin systems have provided very useful

information about the structure of idiotypic determinants at the primary structure or genetic levels. The galactan system has been used more to answer questions regarding antigen binding (reviewed extensively in 64). Very elegant computer simulations of changes in protein structure associated with antigen binding, and the different forms of anti-galactan antibodies have been utilized (232). Lately, anti-galactan hybridoma antibodies have been characterized in terms of their idiotypic determinants (233). The antibodies studied had virtually identical V_K regions, but differed at J_K with no obvious pattern. Thus, light chains probably contribute little to the actual structure of the defined anti-galactan idiotopes. Several correlations between idiotopes and primary sequence have been characterized (233) in the complementarity determining regions (CDR) of the H chain V region. The effect of these residues on the tertiary structure has not yet been defined. Thus, the simple carbohydrates have been very useful in the study of antigen binding and idiotypic determinants at the structural level.

More complicated carbohydrates such as LPS and GAC, as well as DNA have also been studied. As previously discussed, the LPS and GAC antigens stimulate a complex immune response, due to regulatory influences which have not been fully defined. In these systems structural work is progressing. In the GAC system, no concrete evidence defining idiotypic determinants is obvious, though the V_KGAC idiotype is associated with a specific V_K sequence (130). Multiple heavy chains encode the anti-GAC response (130). The LPS system has revealed some interesting data regarding an interstrain cross-reactive idiotype, but more work is needed. Anti-DNA idiotypes have been investigated in several autoimmune model systems. In at least three independent systems (234,235,236) autoimmune

anti-DNA antibodies share a dominant idiotypic marker. These systems may be very useful in studying idiotypic regulatory phenomena. Thus, in spite of some structural information, the complex carbohydrate systems have been more useful in describing regulatory phenomena.

In summary, carbohydrate systems have been provided much structural and regulatory information on idiotypes. Carbohydrates usually represent natural antigens of relatively well defined epitope structure and are frequently free of T cell directed regulation. The major disadvantage is that these systems often cannot be used to investigate T cell mediated regulatory phenomena, though one can conjugate a carbohydrate determinant to a carrier protein. In at least one of these systems (117) the idiotypic repertoire differs between the T-dependent and T-independent responses.

C)   Polypeptide and Protein Antigen Systems

Proteins have many epitopes and are usually T cell dependent. The disadvantage of using a protein in studies of idiotype expression is that the immune response to them is very complex. As a compromise, artificial polypeptides such as GAT, or poly-L-(Tyr, Glu)-poly-D,L-(Ala)-poly-L-(lys) (TGAL) have been used to theoretically limit the number and conformation of epitopes and to allow easy structural changes in the antigen. These polypeptides have yielded some interesting data on idiotypy. The study of GAT, as described, has provided structural and genetic information about anti-peptide idiotypes, as well as information on idiotypic regulation. The study of TGAL and other synthetic polypeptide antigens has yielded data concerning immune responsiveness and cellular cooperation (extensively reviewed in 147 and 237). TGAL has also been used in idiotypic studies. Two strain specific idiotypes have been found in the anti-TGAL response (B10 and C3H.SW idiotypes) (238). The majority of these idiotypic

determinants cross-react with those seen in the anti-GAT response (239). This was not observed earlier due to the poor sensitivity of the assay (238). Some monoclonal antibodies recognizing only TGAL have been isolated. None of these hybridoma antibodies (of 22) defined all of the serum idiotypic determinants, implying that no single structural gene is responsible for the predominant TGAL idiotypic family (240). Thus, of the synthetic polypeptide systems used to study idiotypes, the anti-GAT response is the best defined. Unfortunately, most of the idiotypes studied, even to different synthetic polypeptides, cross-react extensively.

Immunologists interested in a challenging occupation have studied the idiotypic responses to proteins. So many of these systems exist that it would be impossible to try to review them all. The most studied, or the systems with the greatest potential are those involving the responses to staphylococcal nuclease, hen egg-white lysozyme, sperm whale myoglobin and Clostridium pasteurianum ferredoxin (Fd). Ferredoxin will be discussed in depth later.

The studies of protein epitopes have been done in excruciating detail for SWM and HEL by Atassi (182,195). Many epitopes exist, even for these relatively low molecular weight proteins. The majority of these determinants appear to be conformational. As well, the immune responses to these proteins appear to be controlled by a number of relatively ill defined genes. There seem to be major idiotypes expressed in the immune responses to these proteins, but antibodies to different epitopes have been reported to bear the same idiotypic determinants. It is a very complicated business to accurately study idiotypic regulation when many protein epitopes may be involved. The idiotypic response to Nase is probably the best defined of the natural protein systems. The antigenic determinants of

Nase and the genetic control of the anti-Nase response have been studied, though not in the fine detail of the HEL or SWM systems. The idiotypic response has been relatively well studied. Major cross-reactive idiotypes have been produced in the response to Nase and five of these have been mapped to separate V_H loci. The molecular genetic studies have not yet been published. In addition, the regulatory properties of the anti-Nase idiotypes have been studied and show an interesting dichotomy between idiotype bearing T and B cell repertoires.

Recently, investigations of idiotypes produced during the immune responses to other proteins have added some interesting data. Such work has characterized idiotypes in the anti-H-2 antigen responses. Monoclonal antibodies specific for H-2^k molecules were produced and used to prepare either pig or rabbit anti-idiotypes (241). Mice preimmunized with anti-idiotype produced up to 65% idiotype positive antibodies when immunized with H-2K^k antigen, compared to undetectable levels seen in conventional BALB/c alloantiserum (241). This enhancement of idiotype expression is probably due to the production of idiotype specific helper T cells (242) by the _in vivo_ treatment with anti-idiotype. As well, the characterization of the monoclonal anti-H-2K idiotypes showed that most were associated with the H chain (243). This system has potential as an important model of idiotype-MHC immune regulation, but the potential for useful structural information on idiotypes seems limited.

Another useful system appears to be that of bovine insulin (BI). The epitope structure and responder studies of BI have been reviewed extensively elsewhere (147). IEF experiments have shown that individual mice responding to BI present complex spectrotypes that are not conserved between individuals (244). Monoclonal antibodies against BI were used to

immunize guinea pigs. Two of these were found to define public idiotypes (244). One was found in 17% of BALB/c sera and was Igh^a-linked, but could also be found in DBA/2 mice. The other idiotype is found in (Igh)a, b and c mice (244). Within each group, members could be found that bound to different determinants of BI (244). Thus, the insulin system appears to be quite complex with regard to idiotype expression.

In summary, the immune response to many proteins has been studied (for a partial list see 145), though only a few have been well characterized at the level of the idiotype. Since proteins induce complex responses, the best systems are those in which the genetics of response and epitope structure are well characterized. Such systems include the responses to GAT, Nase, HEL, SWM and now BI.

Ideally, the best system should utilize a protein that is: a) foreign to mammals so that no artifacts due to ubiquitiousness are observed; b) small in size; c) easily obtained or inexpensive, with no toxic properties; d) well characterized and simple with regard to immunogenicity. Such a system is the C. pasteurianum ferredoxin (Fd) system.

VI. Review of the Ferredoxin System

The ferredoxins are a large group of electron transport proteins found in bacteria and plants. The first ferredoxin was discovered in Clostridium pasteurianum in 1962 (245). The function of Fd is that of an electron carrier. Fd accepts electrons from an enzyme-catalyzed oxidation and is in turn oxidized by another enzyme involved in electron transport. As such, Fd can participate in many different reactions (246). C. pasteurianum Fd has 55 amino acids, including 8 cysteine residues used to chelate iron. Although many types of Fd exist, C. pasteurianum Fd is related to several other bacterial ferredoxins (247). The best studied of these is the

ferredoxin of Micrococcus aerogenes. Since it appears that C. pasteurianum Fd has not been studied at the level of the tertiary structure, but is similar to the M. aerogenes ferredoxin sequence (247), it will be assumed that its tertiary structure is also similar to that of M. aerogenes Fd. Structurally, this ferredoxin molecule consists of two similar Fe_4S_4 active sites which are about 1.2 mm apart. The active sites are buried in a hydrophobic environment, and communicate through the tyrosine residue to the aqueous environment (248). The molecule can be described as a prolate ellipsoid with radii of 2.2 and 2.7 nm. The major axis is parallel to the line between the two Fe_4S_4 centers. In the structure, an approximate 2-fold axis relates the two halves of the molecule, with the amino and carboxy termini in close proximity. Needless to say, the charges of the termini should ensure their position on the hydrophilic exterior of the protein (248).

Due to its small size, and relatively simple structure, C. pasteurianum Fd has been used as an immunological probe in our laboratory for many years. In order to determine the epitopes of Fd, several small peptides were synthesized and tested for their relation to epitopes of native Fd in a competition assay. These studies showed that the amino terminal heptapeptide and the carboxy terminal pentapeptide of the molecule constituted two major antigenic determinants of oxidized Fd (249). Further studies, using ^{14}C-acetylated peptides in equilibrium dialysis established that these two peptides accounted for essentially all antibody synthesized in rabbits to oxidized Fd (151). Subsequently, these results were confirmed with inbred mice (250). The primary structure of Fd is shown in Appendix 3. Further work showed that both the magnitude (251) and

determinant selectivity (250) of the anti-Fd response in inbred mice were linked to the genes of the H-2-K/IA region of the mouse MHC.

Idiotypes present in the Fd system have only recently been investigated. Two monoclonal antibodies have been produced, and experiments have characterized the expression of these idiotypes in the serum anti-Fd response. One of these antibodies, Fd-1 (amino determinant specific) was shown to represent idiotypic determinants shared by the amino determinant specific antibodies of about half of the B10.BR mice tested (252). Subsequently, this idiotype was found only in sera produced by mice of Igh^b allotype (253). Cell mixing studies also showed an influence of anti-Fd-1 on T cells (253). Another monoclonal antibody, Fd-B2, which is carboxyl determinant specific has been especially well studied. The idiotype of Fd-B2 is not represented in the anti-Fd response of B10.BR mice (254). Primed T cells treated with anti-Fd-B2 and complement were associated with a large increase in anti-Fd production when transferred to irradiated B10.BR recipients and boosted. There was no increase in Fd-B2 idiotype. Very interesting results were obtained when the same experiments were done with B10.D2 non-responder mice. The recipient mice were capable of responding to Fd, but again no Fd-B2 idiotype was observed in the response (254). Subsequent study showed that T cell idiotype anti-idiotype interactions were involved, and that idiotype-bearing molecules were neither H-2 or Igh linked. The anti-idiotype was shown to recognize an Lyt-1 positive T cell population (255). Further work is in progress.

In spite of the extensive study of the anti-Fd response in mice, essentially nothing is known about the serum idiotypic response. In order to define immune regulation in this system, such knowledge is required.

The Fd system is admirably suited for these studies, as the Fd molecule is very simple, possessing the minimum number of determinants necessary for an immune response, and the response is almost unideterminant in high responder strains, regardless of their Igh allotype (250). Therefore, studies to define major idiotypes in the serum anti-Fd response of several strains of mice were initiated.

Chapter 2. Materials and Methods

I.   Immunological Methods

A)   Ferredoxin and Keyhole Limpet Hemocyanin Antibody ELISA

1)   ELISA

The enzyme linked immunosorbent assay as described elsewhere (256) was used to detect the antibody responses to Fd and KLH in mice. This assay depends on the irreversible binding of the antigen to a polystyrene plate. Antibody is added and binds to the antigen on the plate. At this stage the antibody may be inhibited from binding to the plate by anti-idiotype which binds to the antigen binding site. The antibody that attaches to the plate may be detected by the addition of an enzyme labelled reagent specific for the bound antibody. When a substrate is added to the enzyme, the amount of bound antibody may be quantitated by the amount of enzymatic activity from the detecting reagent. The technical details of the use of this assay in this thesis are as follows:

Polystrene substrate plates (Dynatech Immulon 1) were coated with 0.1 ml per well of 1.0 mg/ml of antigen in carbonate coating buffer. The plates were incubated approximately 12 hours at 37°C in a humidified incubator. After washing at least twice with PBS-Tween, antibody to KLH or Fd, diluted in PBS-Tween, was added at 0.1 ml/well of solution. The plates were left at room temperature for 1-2 hours. 0.1 ml of a dilution of rabbit anti-mouse Ig (RaMIg) conjugated to alkaline phosphatase was added after 3 washes with PBS-Tween and the plates were left again 1-2 hours. After 3 more washes, the substrate (2 tablets of Sigma #104-105 per 10 ml of diethanolamine buffer pH9.8) was added and color allowed to develop at room temperature. Color development was quantitated at 405 mm with a

Titretek Multiscan (Flow). Fd was either a gift from Dr. L. Sikora (purified as described) (257) or purchased (Sigma F7629) and KLH was purchased from Calbiochem (374805). Sheep anti-rabbit immunoglobulin (SaRIg) conjugated to alkaline phosphatase was a gift from Agnes Chan (prepared as described) (256) and was used to quantify the amount of RaMIg in rabbit sera. The RaMIg - alkaline phosphatase used was gift of Rakesh Singhai (prepared as described) (256).

2)   Inhibition Assays for Idiotype Expression

a)   Pooled Sera

Pooled sera from mice immune to either KLH or Fd were titrated in triplicate. Tubes containing either control or normal rabbit serum, or dilutions of anti-idiotype (see below) in PBS-Tween were mixed with dilutions of mouse antisera at a dilution which had previously been determined to give an absorbance of 0.5 at 405 nm after 1 hour in a standard ELISA. The solutions were incubated overnight at room temperature, and tested for antigen binding by the ELISA. The normal rabbit serum control was used to define the 100% reaction in all assays.

b)   Individual Sera

0.2 ml of previously titrated individual sera at twice the final concentration needed were dispersed from a common stock dilution into tubes containing 0.2 ml of anti-idiotype or control normal rabbit serum, usually at 1:50. The final concentration of mouse serum was that necessary to give a reading of 0.5 in the ELISA after 1 hour, and final concentration of rabbit reagents was 1:100. The tubes were incubated overnight and assayed for antigen binding in triplicate, thus providing a convenient double check that both serum and anti-idiotype had been added to the tube. A positive control consisting of a dilution of pooled, appropriate anti-Fd serum was

used for each assay. The control in which normal rabbit serum was used for each individual serum defined the 100% reactivity.

B)   Preparation of Immunoadsorbents

1)   Ferredoxin - Sepharose

0.35 g of Thiopropyl Sepharose 6B (Pharmacia 17-0420-01) was reconstituted with distilled water for 2 hours at 22°C, then washed extensively by suction filtration with distilled water followed by PBS. The beads were transferred to a glass column (Bio Rad 737-2240) and washed with a solution of degassed 4M purified urea, 5mM sodium EDTA in PBS pH 7.5 (urea buffer). The beads were then incubated with degassed urea buffer containing supernatant from a saturated solution of 2,2 - Dipyridyl disulphide (Sigma D5767) for 15 minutes at room temperature. The column was then washed with 3 volumes of degassed urea buffer.

1.0 mg of Fd that had been lyophilized from distilled water was resuspended with 1.0 ml of degassed urea buffer and slowly washed into the column. The tube was rinsed with 0.5 ml of urea buffer, which was added to the column. The column was then flushed with a stream of nitrogen gas, sealed, and incubated at 37°C for 24 hours. At this time the Fd solution had lost its original brown color. The column was washed alternately with PBS or 0.1M HCl, and then the column activity was tested by absorption of anti-Fd antibody. The capacity of the column was estimated by titration of absorbed antibody by ELISA and by polyacrylamide gel electrophoresis (PAGE). This method of column preparation was found to be simple and reproduceable, as long as urea was used to denature the Fd.

2)   Immunoglobulin Sepharose

a)   Preparation of Immunoglobulin

5.0 ml of normal mouse serum was fractionated with ammonium sulphate at 4°C to a final concentration of 45% (257). The precipitate was collected by centrifugation at 10,000 rpm with an SS-34 rotor in an RC5B refrigerated centrifuge (Sorval) and resuspended in 2.0 ml of PBS. Dialysis for 24 hours at 4°C, with 2 l of PBS was followed by dialysis for 24 hours with 2 l of 0.2M Na_2CO_3, 0.5M NaCl pH 8.0. The absorbance at 280 nm of the solution was noted and the immunoglobulin containing solution was refrigerated at 4°C immediately before use, or stored at -30°C.

b)   Activation and Coupling to Sepharose

20.0 g of CL-Sepharose 4B (Pharmacia (7-0150-01) was activated with CNBr (Baker F946 only) as a variation of a method previously described (258).

In summary:

The fines from sepharose 4B were removed by decantation and the gel was washed with 100 volumes of distilled water on a suction filter. 20.0 g of the moist, packed gel was transferred to a 100 ml beaker containing a magnetic stirring bar and 20.0 ml of 0.5 M $NaPO_4$ pH 11.0. The beaker was placed in a 10°C water bath, on top of an external rheostat controlled magnetic stirrer in a fume hood. A thermometer and pH electrode was added to the beaker. When the solution temperature had reached 10°C, 6.0 g of crystalline CNBr (Baker gave twice the yield of Fisher or Eastman CNBr) was added and the rate of stirring was increased. The pH was kept to 11.0 +/-0.2 units with ice cold 5.0 M NaOH, while the reaction temperature was kept below 18°C with ice. The reaction was allowed to continue for 30 minutes (about 19 mls of 5M NaOH). At this time the contents were quickly rinsed by suction filtration in the fume hood with 50 volumes of ice cold distilled water. 5.0 g of packed beads were added to 4.0 ml of immunoglobulin containing solution in a 15.0 ml disposable plastic tube

(Falcon 2001) and the tube was rotated for 24 hours at 4°C on a Labquake rotator (Western 56264-302). The supernatant was then removed by suction filtration and the gel was washed with PBS and incubated with 5.0 ml of 10% monoethoanolamine, pH 9.0, for 24 hours at 4° as before. The efficiency of binding was calculated using before and after coupling A_{280nm} readings. After blocking of reactive groups was completed, the gel was washed with alternating rounds of PBS and 0.1 M HCl, transferred to a glass column (Bio Rad 737-2240) and stored at 4°C with PBS containing 0.5% sodium azide. Columns prepared in this way contained approximately 28 mg of protein per ml of beads.

c) Animals

1) Mice

Female mice between 4 and 8 weeks of age were obtained from Jackson Laboratories (Bar Harbor, Maine). Strains used in these studies were CBA/J, C3H/HeJ, C57BR/cdJ, B10.BR/SnJ, RF/J, A/J, AKR/J, ST/J, CE/J, and C58/J. Mice from our animal facility were random bred, aged 1-2 weeks and used as a source of thymocytes or aged 5-8 weeks and used to grow ascites fluid containing monoclonal antibodies. All mice were maintained in our facility.

2) Rabbits

Female New Zealand White rabbits weighing approximately 2 kg were obtained from the University of B.C. Animal Care unit and were maintained there or in our animal facility.

D) Production of immunological Reagents

1) Anti-Fd Antisera

Mice of strains CBA/J, C3H/HeJ, C58/J, CE/J, C57BR/cdJ, RF/J, A/J, AKR/J, B10.BR/SnJ, and ST/bJ were immunized with Fd (prepared by Dr.

Sikora). Each mouse was immunized with 0.1 ml total volume of a 50% solution of 1.0 mg/ml Fd in PBS and complete Freund's adjuvant (CFA) purchased from Difco (H-37Ra). Mice were immunized with 2 x 50 ul aliquots injected subcutaneously into each flank at intervals of 30 days from primary (1°) to secondary (2°) and 50 days from secondary to tertiary (3°) immunizations. The sera from these mice yielded immunoglobulin used to purify anti-idiotypic reagents. Another group of mice from AKR/J, A/J, RF/J and B10.BR/SnJ strains were immunized with commercial Fd as above but at 28 day intervals, and each of these mice were earpunched for identification.

Mice immunized with purified Fd, were etherized and bled from the tail at approximately 11, 15 and 21 days from 1° stimulus and 7 and 14 days following both 2° and 3° stimuli. Mice immunized with commercial Fd were bled at 28 days after initial priming and 15 days after both 2° or 3° immunization.

2) Anti-KLH Antisera

Mice of each strain were immunized as described with a 50% solution of CFA and 1.0 mg/ml of KLH. A separate group of B10.BR/snJ mice was immunized with KLH at 21 day intervals and individually marked by earpunching. All mice were bled for anti-KLH sera as described for Fd-immune animals. Normal mouse serum (NMS) was obtained by bleeding 10-15 unimmunized mice and pooling their sera.

3) Monoclonal Antibodies to Fd

a) Production of Hybridomas

Monoclonal antibodies RIO and R32 were produced from B10.BR/snJ mice and were a gift of Rakesh Singhai. Cloned cells of Fd-1 from B10.BR/snJ mice were a gift of Dr. M. Weaver and were recloned 5 times by limiting

dilution before ascites production of antibody. H-1, H-4, H-5, H-16 and H-32 hybrid lines were produced from C57BR/cdJ mice.

Hybridomas were produced by fusing hyperimmune murine splenocytes with myeloma cells in the presence of polyethylene glycol (PEG) in a manner similar to that described previously (252), but with significant modifications. Briefly:

Mice were hyperimmunized with Fd in CFA subcutaneously. At least 30 days after the last injection, C57BR mice received 50 ug of Fd i/p in 50% Incomplete Freund's Adjuvant (IFA) as well as 20 ug of Fd in PBS intravenously (i/v). Mice received 20 ug Fd i/v daily for the next 2 days and their spleens were fused 3 days after the last injection.

The parental myeloma line used for the production of Fd-1 was SP2/0 (252); all other hybridomas were produced with NS-1 (259). NS-1, a gift of Dr. F. Takei, was grown in Dulbecco's Modified Eagles Medium (Gibco 430-1600) supplemented with 0.29 g/l glutamine (Sigma G3126), 0.11 g/l sodium pyruvate (Gibco 890-1840), 2.38 g/l HEPES (Sigma H7006), 3.7 g/l sodium bicarbonate (Fisher S-233), 2.0 g/l D-glucose (Baker 1916) and 10.0 ml/l of penicillin-streptomycin (Gibco 600-5145) referred to as complete DME (or cDME) with the addition of fusion screened, heat inactivated fetal calf serum (FCS) from Gibco (200-G140). Cells were maintained at 37°C, 10% CO_2, and 95% of relative humidity in a Forma incubator. NSI was selected with 20 ug/ml and 40 ug/ml of 8-azaguaine (Sigma A1007) for resistant cells and grown for only one month after selection, before replacement with freshly selected cells from frozen stock. NS-1 cells were grown to a density of 1-2 x 10^5 cells/ml prior to fusion.

On the day of fusion, immune mice were killed by cervical dislocation, and a single cell suspension of splenocytes was prepared. The cells were washed 3 times with sterile, 37°C PBS by 5 minute centrifugations at 400

x g. NS-1 cells were harvested by centrifugation and washed in the same way. Cells were counted by trypan blue exclusion and mixed at a ratio of 4 splenocytes per NS-1 cell in PBS. This mixture was centrifuged at 400 x g for 10 minutes. Fusion was accomplished by decanting residual PBS, placing the tube in a 37°C water bath and adding 1.0 ml of 37°, 50% polyethylene glycol (PEG) (Baker 1540 or Serva 4000) in PBS prepared that morning. The PEG solution was added to cells over 1 minute while stirring with the pipette tip. The cells were stirred for another minute, then the PEG was diluted with 2 x 1.0 ml aliquots of 37 °C cDME (without FCS) with stirring over 2 minutes. PEG was further diluted with 5.0 mls of warm cDME added over 2 minutes. The suspension was then centrifuged at 400 x g for 10 minutes. The supernatant was decanted, and 10 ml of 37°C cDME was added without disturbing the cell pellet. The tube was capped and incubated at 37°C for 20-40 minutes while thymocytes were prepared from random bred mice.

Following incubation, thymocytes and fusion products were combined at a ratio of 2 thymocytes per original splenocyte in warm cDME containing 20% fusion selected FCS. To this was added sterile PBS containing 1.3 mg/ml hypoxanthine (Sigma H9377) and 0.4 mg/ml thymidine (Sigma T9250) termed HT stock, to a final dilution of 1:100. Sterile PBS containing aminopterin (0.2 mg/ml Sigma A2255) termed A stock, was added to a final dilution of 1:1000. HAT media allowed the selection of fusion products according to the method of Littlefield (260). 0.2 ml of the supplemented cell suspension was added to each well of a 96 well tissue culture plate (Flow 76-023-05). Generally 4.5 plates were used per spleen (10^8 cells) fused, with about twice that many thymocytes in 0.2 ml of 20% FCS and HAT cDME medium. Plates were incubated at 37°C, 10% CO_2 and 95% relative humidity with periodic inspection. Unless FCS was suspected of being deficient, the

the media was not changed until ELISA testing. By this protocol, 2 - 4 hybrids per well could be observed by day 5.

b)   Selection and Cloning of Hybridomas

Plates containing hybridomas were screened for antigen binding when colonies were very large, and at the point of outgrowing the media (about 10 days after fusion and 8 days after cloning). Cultures were tested by removing 0.1 ml of medium and adding it to sterile Fd coated ELISA plates. The media was replaced with 0.1 ml of cDME containing 1:100 dilution of HT stock and 20% FCS. Wells shown to possess Fd binding activity were transferred to 2.3 ml of medium containing 2×10^5 thymocytes/ml in cDME supplemented with 1:100 HT stock and 20% FCS, in 24 well tissue culture plates (Linbro 76-033-05). The cells were cultured until growth was confluent, and supernatants were tested for Fd and KLH binding activity. Cells from wells showing specific antibody to Fd were cloned from 10 to 1.25 cells per well by doubling dilution in cDME containing thymocytes and 20% FCS. Cells were usually cloned 3-5 times before they were assumed to be pure and stable. All cDME used in fusion and cloning was made within one day of use.

c)   Ascites Production

Mice were given 0.5 ml of 2, 6, 10, 14 - tetramethylpentadecane (Pristane) (Aldrich T2280-2) at least 3 days prior to injection of hybridoma cells. 24 hours prior to injection mice were irradiated with 500 rad using a ^{60}Co gamacell (AEC). 10^6 to 10^7 viable cells were given i/p in PBS to each mouse. Ascites fluid containing antibody usually developed within 14 days, and was drained every other day. It should be noted that random bred mice irradiated and given hybridoma cells were far superior to inbred pure or F_1 strains in ascites production.

Cells from cultures or ascites were frozen at -70°C in cDME containing 20% FCS and 10% DMSO (Fisher D-128) by standard procedure, and stored in cryopreservation vials (Nunc 1.8 ml). Cells were transferred to liquid N_2 after 24 hours at -70°C.

4)   Production of Anti-idiotypic Reagents

a)   Purification of Anti-Fd Antibodies

Anti-Fd antibody was prepared from the serum of AKR, RF, A, and B10.BR mice hyperimmunized with purified ferredoxin. Mice were immunized on day 0, 30 and 80 respectively, and blood pooled from 12-15 animals on days 101 and 104. The pooled sera was frozen until use.

Anti-Fd antibody was purified by immunoabsorption on Fd-Sepharose, and elution with 0.1M HCl into enough 3M Tris to neutralize the acid (35 ul/ml HCl). The eluted fractions were pooled and concentrated by N_2 pressure (Amicon YM 10) then dialyzed into PBS. The purified antibodies were stored frozen.

b)   Immunization and Bleeding of Rabbits

Female rabbits were hyperimmunized with 100 ug of purified anti-Fd antibody in 50% CFA. Each animal was injected with 0.1 ml subcutaneously in each shoulder and haunch (0.4 mls total). Animals were bled on days 7, 15 and 21 following tertiary or subsequent immunizations by either piercing the marginal ear vein, or by cardiac puncture. Blood from the three bleeds was pooled and stored frozen.

c)   Purification of Anti-idiotypic Antiserum

Anti-mouse Ig activity in the sera of rabbits immunized with anti-Fd mouse antibody was removed by repeated absorption of the rabbit antiserum over mouse immunoglobulin (MIg) immunoabsorbents as follows:

4.0 mls of PBS containing 0.5% sodium azide was added to 16.0 ml of rabbit serum. The serum was repeatedly absorbed over a 10 ml MIg column (a gift of Rakesh Singhai). Columns were washed repeatedly with 0.1M HCl to remove bound RaMIg. The RaMIg was saved and used to prepare enzyme conjugates for ELISA. After a variable number of passages over the heterologous MIg column, the rabbit serum was passed over a 5.0 ml immunoabsorbent containing MIg specific for the mouse strain against which the rabbit had been immunized. After numerous absorptions, the anti-idiotypic rabbit sera were assayed for RaMIg activity on the ELISA. In this way, anti-AKR/J idiotype, for example, was absorbed 20 times over the heterologous and 5 times over the strain specific columns.

d)   Concentration of Anti-idiotypic and Control Normal Rabbit Sera

Following immunoabsorption, the fractions containing absorbed sera were pooled (about a 3-fold dilution of original concentration) and concentrated by precipitation with a final concentration of 45% ammonium sulphate as described above. The dialyzed immunoglobulin in PBS was shell frozen and lyophilized. The lyophilized powder was resuspended in 10 ml of 50% glycerol containing 0.2% sodium azide to a final concentration of 50% glycerol in PBS/azide. To this was added 1.0 ml of NMS of the strain against which the anti-idiotype was produced. Control NRS was prepared by testing non-immune rabbit serum for anti-MIg activity, then adding an equal volume of glycerol. To this was added normal B10.BR sera and azide to a final concentration of 10% NMS and 0.2% azide. These reagents were kept at -20°C before and after use.

II.   Biochemical Methods

A)   Reagent Purification

Acrylamide (5521), methylene bis acrylamide (P8383) and TEMED (8178) were obtained from Eastman. Tris (T1378), silver nitrate (S-6506), SDS (L-5750), bromthymol blue (B-8630), Triton X-100 (X-100), sodium hydroxide (S5881), and glycine (G6761) were obtained from Sigma. Acetic acid (A-38-S), glycerol (G-33), sodium borohydride (S-678), sodium carbonate (S-263), formaldehyde (F-79), urea (U-15), phosphoric acid (A-260) and ammonium peroxydisulphate (A-682) were obtained from Fisher. Dithiothreitol (DTT-161-0610), agarose (162-0125), AG-11A8 mixed bed ion exchange resin (142-7834) and cellulose gel film (165-0922) were obtained from Bio Rad. Ampholines pH 3.0-10.0 were obtained from LKB. Only products from Bio Rad and LKB were electrophoresis grade.

30% stock solutions of acrylamide were purified by stirring for one hour with 10 g/l of AG-118 ion exchange resin at room temperature. The solutions were then filtered through a 0.45 micron filter and stored at 4°C.

500 g of urea were dissolved in 1 l of 70% ethanol at 50°C and stirred with 20 g/l of AG-118 ion exchange resin for one hour. The solution was suction filtered through Whatman 1 MM filter paper and left at -20°C overnight. The crystals were washed with 1 l of -20°C 95% ethanol followed by 500 ml of ether under suction. The crystals were packed and suction dried for 20 minutes then air dried overnight at room temperature (261).

1.5 M Tris pH 8.8, 0.5 M Tris 6.8, 10% SDS and gel solution containing 12% acrylamide for PAGE or 4% acrylamide for IEF were filtered through 0.45 micron filters. The gel solutions were stored at 4°C for up to a week before use. PAGE running buffer containing 9.0 g Tris, 43.2 g glycine and 1.5 g of SDS per 1.5 l was filtered through a 1.2 micron filter before use (262) and occasionally reused once without refiltering.

The above purification procedures and the use of 5mM DTT in the sample buffer, rather than 2-mercaptoethanol, dramatically reduced stained gel background, the presence of the 55K artifact, and the intense coloration around 20 K. These purifications, new electrophoresis plates, longer equilibration times for IEF tube gels in the sample buffer prior to running in the second dimension, and elimination of the agarose overlay did not eliminate the vertical line artifacts seen in the 2-D gels stained with the Sammon's Method (263). These lines were not observed in gels stained with either Coomassie Blue, or another silver staining method (264), and may be due to the greater sensitivity of the Sammon's silver stain.

B) Minicolumn Immunoadsorbents

1) Preparation

Mini-immunoadsorbents were produced as described by Pearson and Anderson (265). Columns containing 25 or 50 ul of Fd-Sepharose were packed into a 0.25 ml pipette tip (Bio Rad) and held with a wisp of cotton. This pipette tip was inserted into a 1000 ul pipette tip (Evergreen Scientific) which was placed within a 5 ml disposable plastic tube (Falcon 2003).

2) Use of Columns

Sera, ascites fluid, or fusion supernatants containing anti-Fd activity were added to the top of the mini-column and centrifuged at 200 x g for 3 minutes. The sera were cycled twice. The column was then washed as follows:

Once with 200 ul of PBS, followed by 200 ul of Buffer 1 (0.15M NaCl, 0.02 M Tris pH 7.5, 1% Triton X-100 - a gift of Rob Shipman), then 200 ul of Buffer 2 (0.15 M NaCl, 0.02 M Tris pH 8.8, 0.1% SDS, 1% Triton X-100 - a gift of Rob Shipman), followed by three washes with Buffer 3 (0.15 M NaCl, 0.02M Tris pH 6.8, 0.2% SDS, 1% Triton X-100 - a gift of Rob Shipman) and finally once with 200 ul of distilled water. The minicolumn was spun dry

at 400 x g for 1 minute, and then the column was removed from the tube and blotted dry. The column was then placed in a 1.5 ml plastic tube (Eppendorf) held within a 15 ml plastic tube (Falcon 2007). Bound antibody was eluted with 3 x 75 ul aliquots of 0.1 M HCl. The eluted antibody was immediately frozen at -70°C and lyophilized. The column was washed with PBS and stored wet in PBS containing 1% sodium azide at 4°C until reuse.

C)   SDS Polyacrylamide Gel Electrophoresis

Proteins were assayed by PAGE by a method similar to that described by Laemmli (266). Briefly:

Samples were diluted in sample buffer containing 2.3% SDS, 0.065M Tris pH 6.8, 10% glycerol, 5 mM DTT and bromthymol blue. Samples were heated at 100°C for 5 minutes in an oil filled block heater (Pierce 189000). Stacking was done in 3% acrylamide at 50V for 90 minutes, and the electrophoresis was performed for approximately 1100 V-h at either 75 V or 225 V with cooling at 4°C. All gels were 1.5 mm thick. Generally, less than 5 ug of protein was added to each sample well.

D)   2-Dimensional Electrophoresis

2-D electrophoresis was done by the method of O'Farrell (267) with modifications as described by Ingman-Baker and Candido (268) or Anderson (269) as follows:

Glass tubes: (Bio Rad 12.5 cm x 0.2 cm diameter) were filled with gel solution containing 4% acrylamide (30:1.8 mono:bis), 4.5 M urea, 2% Triton X-100, 2% pH 3-10 Ampholines, and polymerized with TEMED and ammonium persulphate. Polymerization was allowed to proceed for at least 3 hours and the tubes were covered in paraffin and stored at 4°C. Tubes were warmed at room temperature for 1 hour before use and prefocussed in 500 ml of 0.03 M NaOH (well degassed) at the anode, and 3 1 of 0.01 M phosphoric

acid at the cathode in a Bio Rad model 155 tube gel electrophoretic apparatus. Prefocussing was carried out at 200 V for 15 minutes, 300 V for 15 minutes and 400 V for 30 minutes. Anode buffer was replaced prior to sample addition.

The lyophilized sample was resuspended in 25 ul of SDS sample buffer without dye, and heated at 100°C for 5 minutes. Samples were well cooled and 25 ul of 4.5 M urea, 5 mM DTT, and 2% Ampholines 3-10 was added. The samples were vortexed briefly then centrifuged. 50 ul of sample was added to the tops of the IEF tubes through anodic buffer. Gels were focussed at 400 V for 17 hours then 800 V for 1 hour, with cooling at 4°C. Tube gels were carefully extruded with water pressure and stored in 5 ml of SDS PAGE sample buffer at -20°C.

Prior to running in the second dimension, gels were thawed by incubation for 45 minutes at room temperature. Tube gels were added to the top of the PAGE gel and sealed with a hot solution of 1% agarose, 2.3% SDS, 0.065 M Tris pH 6.8, 10% glycerol and 5 mM DTT. Gels were stacked and run as described above, until the marker dye had advanced 14 cm into the running gel (about 1200-V-h).

E) <u>Silver Staining</u>

PAGE gels were stained by the method of Sammon's (263). The only deviations from the published method were the reduction of fixing time in 25% and 10% ethanol to 1 hour, and staining time in silver nitrate to 1 hour. Stained gels were dehydrated from the sodium carbonate solution in 50% ethanol, 10% acetic acid, and 10% glycerol. Gels were preserved by drying onto cellulose membranes as per manufacturers instructions.

Chapter 3. Major Idiotypes in The Murine Anti-Ferredoxin Response

I.   Results

A)   Specificity of Anti-idiotypes

Young mice were immunized with either Fd or KLH and boosted as explained in Methods (Chapter 2). Sera from the 3° day 21 and 24 Fd-immune bleeds were pooled from B10.BR/SnJ, AKR/J, A/J, and RF/J mice strains. The pools were immunopurified on Fd-Sepharose and the purified antibodies were used to immunize rabbits. Serum from hyperimmune rabbits was purified to produce anti-idiotypic reagents and used for inhibition studies with antisera from various strains of mice (see Table I). The effectiveness of the anti-idiotypes was determined by inhibiting the anti-Fd binding capabilities of the pooled sera. It should be noted that this assay depends on the steric hindrance of bound anti-idiotype competing for antigen keeping the antibody from binding to the ELISA plate. Some idiotypic determinants may be too far away from the antigen binding site to interrupt antigen binding. Thus, the inhibition assay used here may represent a lower proportion of idiotype expression than is actually present. As can be seen from Table I, the reagents were prepared against mouse sera possessing different heavy chain and light chain allotype markers, usually interpreted to represent genetic divergence. All of these strains were high responders to Fd and produced over 80% C - determinant specific antibody (250). Consequently, one would expect most of the anti-idiotype to inhibit C - directed antibodies. The ability of the anti-idiotypes to specifically inhibit the immunizing antibodies to which they were raised is shown in Figure 1. As can be seen, the anti-idiotypes are quite specific to the anti-Fd response and did not inhibit the multistrain anti-KLH pool to a significant degree. The relatively steeper

Table 1

Characteristics of Mouse Strains Utilized in the Experiments Described in This Thesis

Table 1 consists of data that have been known to affect idiotype production in other systems (as reviewed previously). All mice used produced more than 20 ug of anti-Fd antibody per ml of serum, hence their high responder status. Igh allotypes are determined by the IgG_{2a} allotype and IgK allotypes were determined by iso-electric focussing. As can be seen, all mice produce antibodies predominantly directed to the C determinant of Fd.

Table I - Characteristics of Appropriate Strains

Reference Strain	H-2	Igh	IgK Ef1	IgK Ef2	Fd response	N:C Ratio
C3H/HeJ	k	j	b	a	High	5:95
CBA/J	k	j	b	a	High	15:85
AKR/J	k	d	a	a	High	10:90
B10.BR/SnJ	k	b	b	a	High	15:85
A/J	a	e	b	a	High	ND
RF/J	k	c	a	a	High	10:90
CE/J	k	f	b	b	High	10:90
ST/bJ	k	g	b	a	High	20:80
C57BR/cdJ	k	a	b	a	High	ND
C58/J	k	a	a	b	High	10:90

Data from references 56, 250, 251, 271 and 277.

Figure 1:

Specificity of Anti-idiotypic Sera on

Pooled Anti-Fd or Anti-KLH Sera

Anti-Fd or anti-KLH serum was pooled
from 12-15 individual mice and
assayed in quadruplicate for
inhibition of binding to antigen by
anti-idiotype. The anti-idiotype was
used to inhibit the anti-Fd
antibodies from the mouse strain to
which it was directed. In this way
the specificty of idiotype binding is
illustrated. The standard errors of
inhibition of binding of Fd (•) or
KLH (▲) were never above 10 percent.

62

anti-AKR idiotype

anti-RF idiotype

anti-A/J idiotype

anti-BIO.BR idiotype

PERCENT INHIBITION

DILUTION X 10⁻²
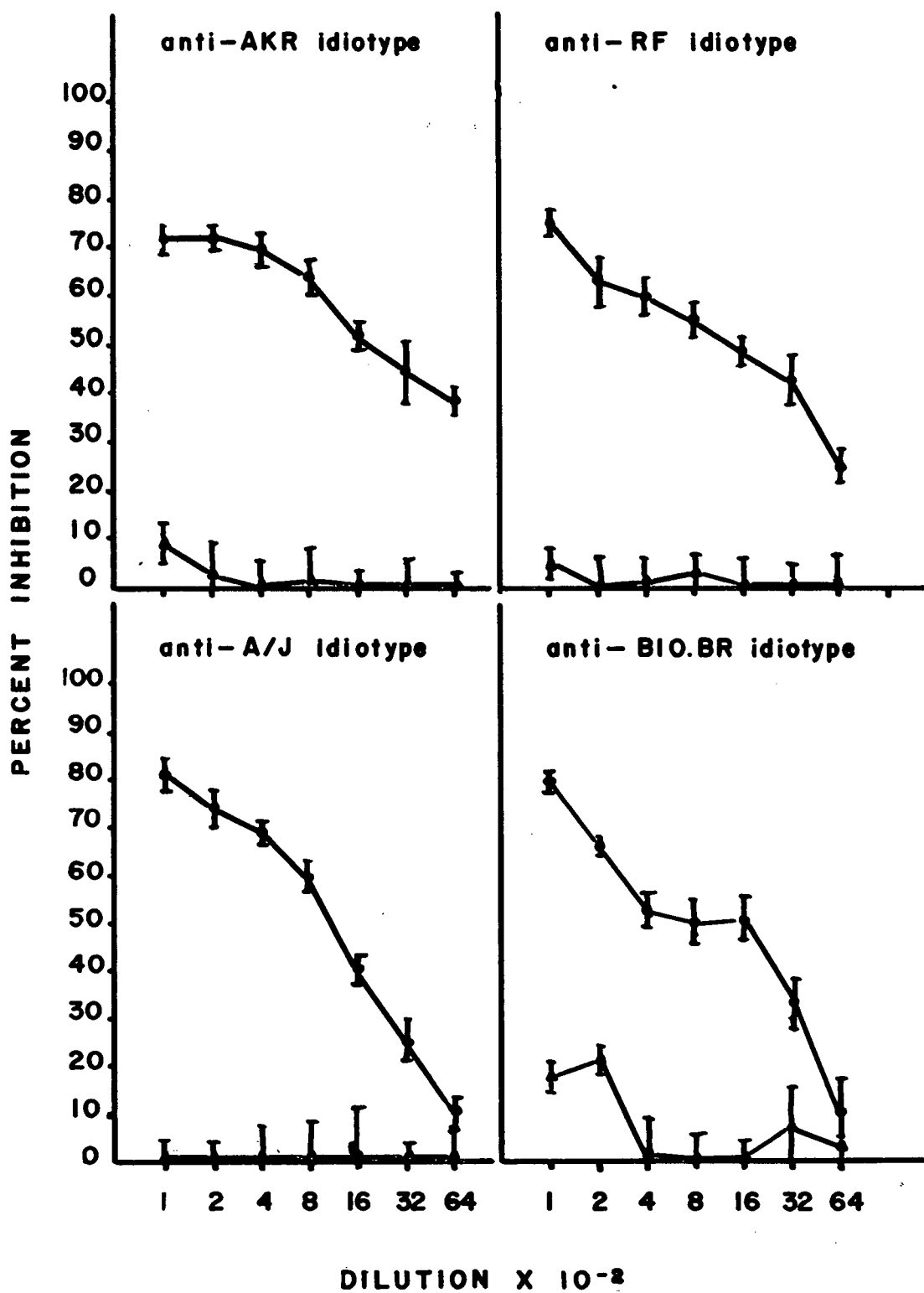
titration curves seen with the anti-A/J and anti-B10.BR idiotypes are probably due to the use of anti-idiotypic sera obtained from an earlier response (3° - 4°) than that from the anti-AKR or anti-RF anti-idiotypic antisera, which were obtained after 6 or 7 immunizations. From ELISA data (not shown), it was found that greater than 99% of the anti-mouse Ig activity was removed from all of the anti-idiotypic antisera following the multiple absorptions on MIg columns, as described in Methods.

The amount of mouse antiserum used in the inhibition assay was that required to give an approximate A_{405nm} in the ELISA of 0.5 in one hour at room temperature. This value was usually in the middle of the titration curve, but not necessarily the titration point. It was thought that in this way a constant amount of Ig would be bound to the plate, affinity effects would be minimized since there was still antigen excess, and the value of 0.5 was near the middle of the linear range of sensitivity of the spectrophotometer, permitting more accurate calculations or inhibition. It should be noted that in over 15 independent assays of the control sera, the standard error was in the range of 2% inhibition, demonstrating the reliability of this method (data not shown). In these inhibition assays, all negative values have been regarded as zero percent inhibition.

A very important observation arises from the data on Figure 1. The maximal inhibition values seem to plateau near 75%. Thus, even after 7 immunizations, the rabbit does not respond to up to 25% of the immunizing idiotypes. The most obvious rationale is that there are many poorly represented idiotypes in the pooled sera, possibly as a result of the N-determinant (10 - 20% of anti-Fd response) specific response. Since these idiotypes are present in small concentrations, there may not be enough of any particular idiotype to appropriately immunize the rabbit. At

two of possibilities may account for these observations. The individual may express a few major idiotypes and many minor ones, or a few immunoglobulins from high titre sera may dominate the anti-Fd binding of the pool, and the idiotypes available to immunize the rabbit, thus biasing the observations. The different possibilities may be distinguished by observing the inhibition of individual antisera.

B)   Inhibition of Anti-KLH Activity of Individual Sera by Anti-Fd Idiotypes

Mice immunized with KLH were bled on day 15 after 2° immunization, and the antisera were used in individual inhibition assays. These results are shown in Figure 2. As can be seen, the maximum inhibitions are less than 25%, with most inhibitions being less than 10%. The high background of anti-B10.BR idiotype observed in Figure 1 is not seen at the individual level; in fact, none of the anti-idiotypes have a disproportionate background, as the mean backgrounds are within 2 - 5% inhibition (see Appendix 1). The C57BR/cdJ mice appear to be more sensitive to inhibition, but the small sample size limits conclusions.

Since the levels of inhibition were so small, it was assumed that the inhibition of anti-KLH binding was due to non-specific factors, and these data were used as the non-specific background for statistical purposes (see Appendix 1).

A second experiment was done in which B10.BR/SnJ mice were individually marked and followed through their response to KLH. These data are shown in Figure 3. As can be seen, the inhibitions are again quite low, and do not change greatly over time. There was no significant difference between the B10.BR anti-KLH inhibitions and background, and no significant change occurs over time. In conclusion, the anti-idiotypes have been shown to poorly inhibit anti-KLH sera, from at least two independent experiments.

Figure 2:

Specificity of Anti-idiotypic Sera on

Individual Anti-KLH Sera

Individual secondary anti-KLH sera from different strains of mice were inhibited from binding to KLH in triplicate by four anti-Fd idiotypic reagents. The percent inhibition is represented on the vertical axis and the results for each mouse are represented on the horizontal axis. Vertical alignment of inhibitions with different anti-idiotypes define the idiotypic profile of a single mouse serum. The standard errors of inhibitions were never above 10 percent.

Figure 3

Inhibition of Anti-KLH Sera from

Individually Marked B10.BR Mice

Sera from the anti-KLH response of

B10.BR mice were tested for

inhibition of binding to KLH in

triplicate at various times in the

immune response. Percent inhibitions

for each anti-idiotype are

represented on the vertical axis

while the results for the individual

mice at various times in the response

are represented horizontally. The

vertical alignment of the bars

represent the inhibition of a single

animal with the four anti-idiotypes.

Standard errors of inhibition are

less than 10 percent.

INDIVIDUAL BIO.BR anti-KLH SERA
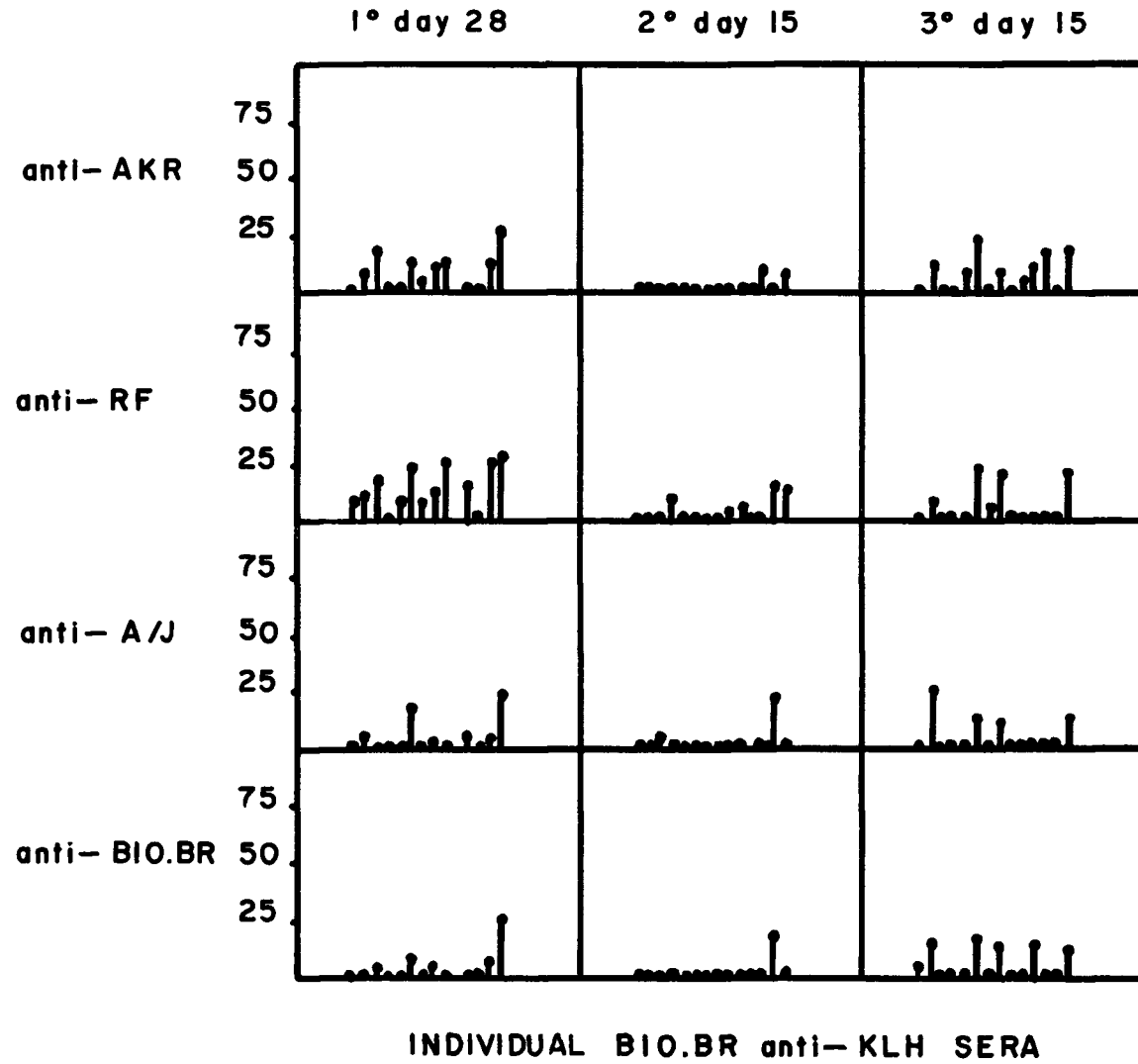
C) Inhibition of Anti-Fd Individual Sera

Individual antisera, taken at various times in the immune response from the group of mice used to immunize the rabbits, were tested with the anti-idiotypes. These data are shown in Figure 4. The scatter plots show that a majority of the mice produced significant amount of idiotype. No apparent change over time was observed. As well, the amount of inhibition does not appear to correlate with the anti-Fd titre of AKR, RF, or B10.BR mice (data not shown). A/J mice, on the other hand, that expressed high proportions of inhibitable idiotype, produced high titre sera. In this case only the large amounts of individual idiotypes associated with high titre sera were capable of eliciting an immune response in the rabbit. Thus, only AKR, RF, and B10.BR mice produce a major cross-reactive idiotype that is expressed in most sera, and no correlation between idiotype expression and titre were seen, whereas high titre sera of A/J mice reacted better with A/J anti-idiotype than low titre sera. It should be noted that some antisera did not react with anti-idiotype. Presumably, these sera were composed of a variety of either minor intrastrain, or individual idiotypes.

It is interesting to compare the results of individual sera with the results using pooled sera (see Table II). At a 1:100 anti-idiotype dilution, the pooled sera showed about 75% inhibition. If the means of the individual inhibitions are taken, the result is sometimes less than half of the pooled results. The titre of the sera (determined both by amount and affinity) probably has a major artifactual effect on the pooled sera results. Thus, inhibitions using pooled sera should not be used to quantify the behavior of a population of individual antisera.

Figure 4

Inhibition of Individual Anti-Fd Sera

with Appropriate Anti-idiotype

Individual anti-Fd anti-sera from the four strains of mice to which anti-idiotypes were prepared were inhibited from binding to Fd in triplicate by their appropriate anti-idiotype. Percent inhibition is represented vertically and the results for individual mice at various times in their anti-Fd response are represented horizontally. Vertical alignment does not represent the same antibody. Threshold values of inhibition for each anti-idiotype (as defined in Appendix 1) are indicated on the horizontal axis. As before, the standard errors of inhibition were less than 10 percent.
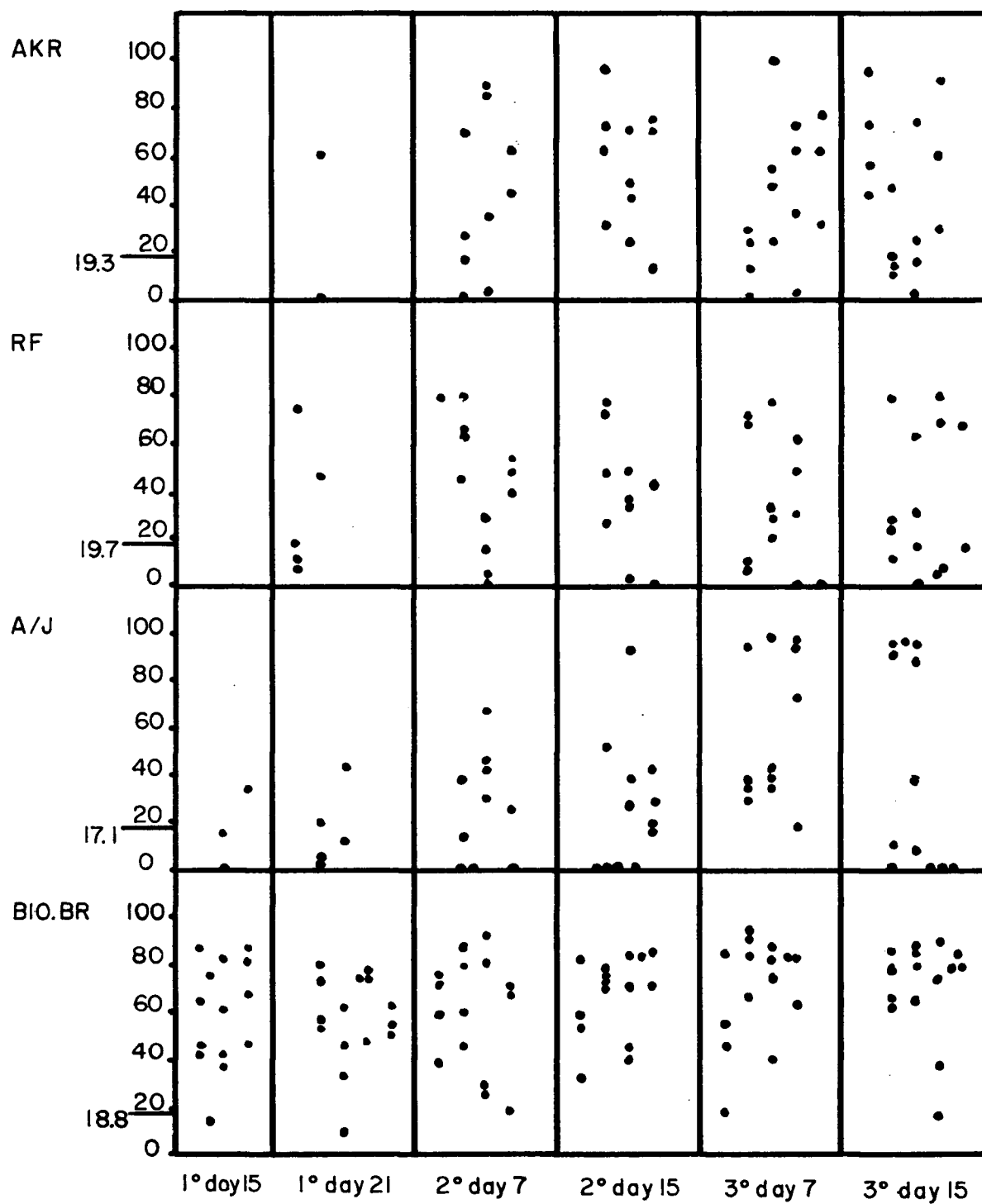
Table II

A Summary of Pooled Versus Individual Antibodies Inhibited
by Various Anti-idiotypes

This table indicates the difference between the pooled and mean individual serum inhibitions of the anti-Fd antibodies tested with their respective anti-idiotypes at the same dilution of anti-idiotype. These data were obtained from Figures 1 and 4 respectively. The data are expressed as percent inhibition.

Table II - Summary of Pooled Versus Individual Anti-Fd Inhibitions

Strain	Anti-id. Dil.	Pooled Serum	Mean Indiv.	No. Ind.
AKR	1/100	71.6	39.4	14
RF	1/100	74.9	36.1	14
A	1/100	81.0	44.0	11
B10.BR	1/100	79.7	71.9	15

The results of Figure 4 show that most mice of the groups used to immunize rabbits possess anti-Fd sera bearing idiotypic determinants, recognized by rabbit anti-idiotype. These determinants are observed in primary sera, and the frequency of their expression appears to be constant throughout the response. Since the mice were not individually marked, nothing can be said about the kinetics of each individual mouse. As well, one cannot recognize, safely a major cross-reactive idiotype in these mice since each serum may have produced antibodies to its own individual profile. This is unlikely since many sera are uninhibitable with anti-idiotype. A new group of mice well marked and followed individually throughout their response would add some very useful information, but the best experiments would involve anti-idiotype directed against the anti-Fd response of a single mouse, which may not be possible due to the low amounts of anti-Fd produced even in high responder animals (251).

D) Kinetics of Idiotype Expression

New mice of each idiotypic representation were marked and immunized with a different preparation from that used previously. These mice were bled at intervals during their response and tested as before for inhibition of binding to Fd. Thus, even though the mice used were immunized with a different Fd preparation, ELISA assays were done using the same Fd preparation as used previously. The results of these experiments are shown in Figure 5. One can observe that the frequency of sera containing idiotype in AKR, RF, and B10.BR mice is quite high, while very few strain A mice are inhibited with anti-A/J anti-idiotype. Thus, AKR, RF and B10.BR mice definitely produce major cross-reactive idiotypic families but strain A mice appear to make many anti-Fd idiotypes, very few of which are shared between the two independent groups of mice.

Figure 5

Kinetics of Inhibition of Anti-Fd
Sera from Individually Marked Mice with
Appropriate Anti-idiotype

Individual anti-Fd anti-sera from
four strains of mice were tested in
triplicate with their appropriate
anti-idiotype at various times of the
anti-Fd response. Percent inhibition
for each stage of the response is
represented on the vertical axis
whereas the individual results of
each individual idiotype-anti
-idiotype pair of the four strains is
represented on the horizontal axis.
The inhibition results of the same
mouse at various times in its anti-Fd
response can be followed vertically.
As before, the standard errors of
inhibition were less than 10 percent.
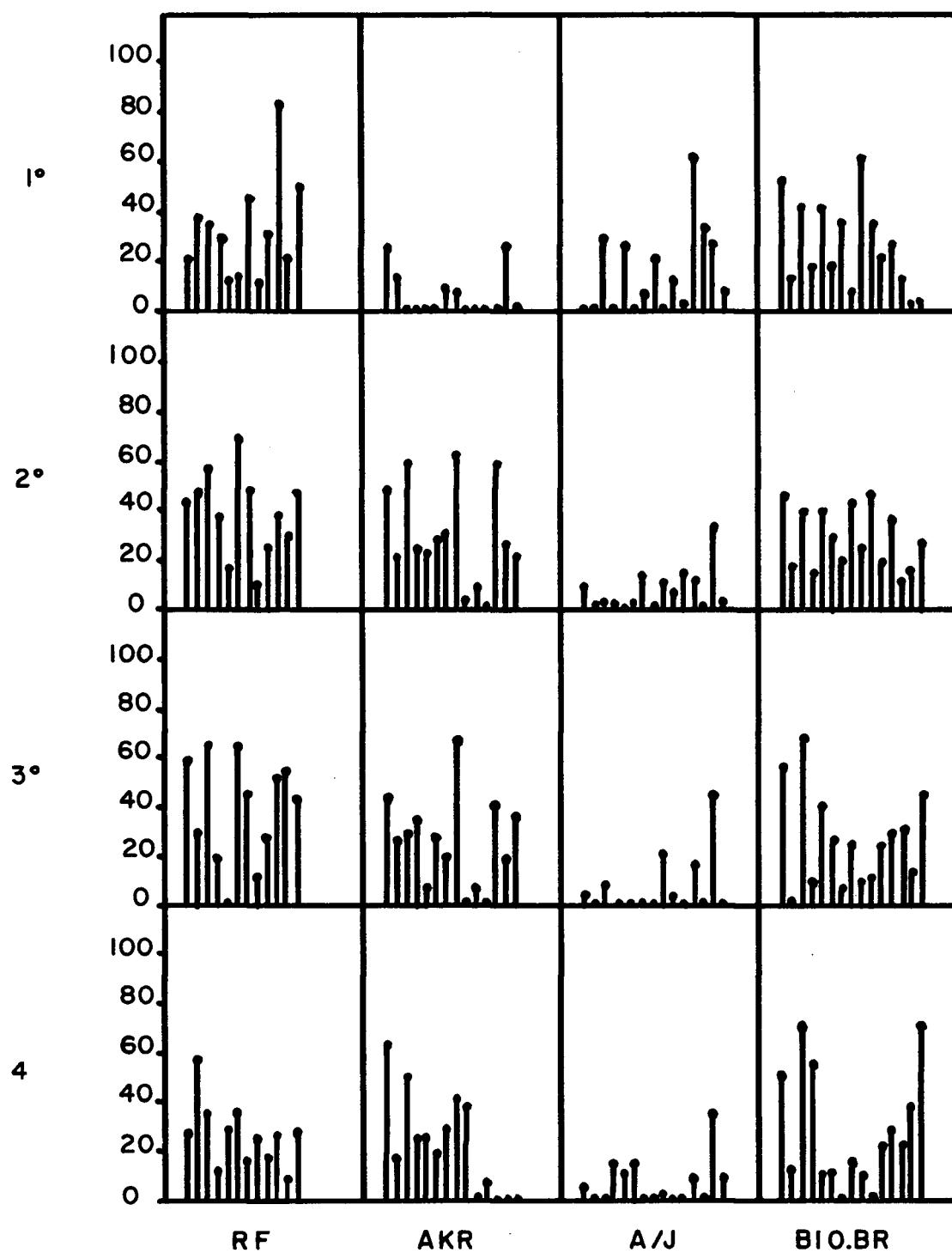
There appears to be a rise in the frequency of AKR animals producing idiotype which is very highly significant (see Chapter 4). The other responses do not vary significantly over time. This phenomenon not seen in the earlier experiments (Figure 4) may be attributable to the different batches of Fd immunogen. The first batch contained many aggregates, while the commercial preparation was quite homogeneous. Thus, the form of the antigen may have influenced development of the repertoire in AKR mice. As well, the form of the antigenic Fd may have caused the production of different idiotypes in the second experiment, lowering the amount of idiotypes recognized by the anti-idiotypic reagents. Such a phenomenon would account for the A/J results. Equally possible is the theory that many idiotypes exist in the anti-Fd response in mice, and it is unlikely that all of the idiotypes present in the first group of mice will be expressed by the second group of mice, regardless of the form of the antigen. The most likely explanation for the differences between figures 4 and 5 is probably a combination of these possibilities.

II. Discussion

In order to investigate idiotypic populations in mouse sera, it is important to have specific anti-idiotypic reagents as probes. Several ways to produce these have been documented in the literature. One may immunize another species with either pooled pure antibody (219, 272, this work), pure antibody from a single animal (110, 203, 273) or monoclonal antibody (194, 274). As well, one may immunize another strain of mice with the antibody (51) or even try to raise anti-idiotype in normal, syngeneic mice (119). Once obtained, these reagents must be rendered free of any non-idiotypic anti-immunoglobulin activity by absorption (273) or

appropriate selection of a monoclonal anti-idiotype (275). The anti-idiotype may be assayed by direct binding to idiotype (16), by inhibition of binding of labelled anti-idiotype from idiotype (276) or by inhibition of binding of idiotype to antigen (194, 203, 219, this work). It was comforting to see that almost exactly the same immunization protocol (272) and assay for idiotype used in this work (194) have been previously used by other investigators. The rationale for the immunization protocol was that the xenogeneic determinants of mouse Ig would provide help to the idiotypic determinants, yielding a high titre, fast response to even poorly represented idiotypes. The disadvantage was that exhaustive (and tedious) absorptions were required to remove the interfering, non-specific RaMIg activity. Some slight (1%) activity could not be absorbed and was probably due to the response to denatured MIg, which could not be removed by absorption, and was amplified as a consequence of the nature of the ELISA assay. This activity was presumably mopped up by the added NMS, and did not seriously affect specificity. The assay for inhibition of binding of anti-Fd to Fd was chosen for its simplicity, relatively low background and theoretical specificity for determinants in the vicinity of the antigen binding region of anti-Fd antibody.

The data in Chapter 3 show that four specific anti-idiotypic reagents were successfully prepared. Mice of AKR, RF and B10.BR strains produce an idiotypic family that is restricted in variety enough to be present in two independent samples of Fd immune animals. The anti-idiotype produced from A/J anti-Fd sera seems to react well with the idiotypes represented by high titre sera, and does not fully represent the idiotypic repertoire of the anti-Fd response of this strain.

Inspection of the kinetic data (Figure 5) shows that the frequency of mice expressing AKR idiotypes change over time, compared to RF, B10.BR, or A/J. Probably some degree of idiotypic maturation exists, such as will be discussed in Chapter 4.

One can see differences in the maximal inhibition of pooled sera, and the mean inhibitions of the individual sera (Table II). This is not surprising, as no account had been made of the affinity or concentration of the individual antibodies in the pool. Consequently, one would expect a large difference between the pool and individual serum mean inhibitions, since low affinity or concentration anti-Fd antibodies, standardized individually, would be swamped out in the pool. Thus, one should use individual sera rather than pooled sera to look at complex idiotypes, or for quantification, therefore properly representing the serum characteristics of the strain. Ideally, one could immunize 15 rabbits with antibody from individual mice, then, using standardized, equivalent amounts of anti-idiotype, make a pool, which would be used to test the idiotypic repertoire of the strain. Needless to say, this would be both time consuming and probably not even possible in the Fd system due to the well documented poor production of antibody even in high responder strains. A quick way to determine if antibody concentration of responding mice is a factor in determining inhibitions is to look for a correlation between titre and inhibition. Such a correlation was observed only in A/J results.

In conclusion, the data support the possibility that multiple idiotypes exist in the anti-Fd response. Multiple idiotypes in the immune response to macromolecules have been already discussed in Chapter 1, and will be further reviewed in Chapter 4.

Chapter 4. Interstrain Cross-reactive Idiotypes in the

Anti-Ferredoxin Response

1) Results

A) Interstrain Cross-reactive Idiotypes in the Pooled Anti-Fd Response

Tertiary antisera of mice immunized with Fd were pooled for each strain and tested for inhibition of Fd binding (see Table I for strains) with each of the four anti-idiotypic reagents discussed in Chapter 3. A rough estimation of idiotype sharing between strains may be made by comparing inhibition curves of pooled sera. The data from Figures 6, 7, and 8 show that there is indeed some cross-reactivity of idiotypic repertoire between strains.

AKR antisera express B10.BR idiotypic determinants, C58 antisera express AKR and A/J idiotypes, B10.BR antisera express AKR idiotypes, and C57BR antisera express A/J idiotypes. RF/J antisera probably express some AKR idiotypes, though not very many. Thus, a strong indication of cross-reactivity of idiotypic repertoires between strains exists, although at least three possibilities may account for this observation. Many idiotypes may be present in each response, of which some cross-react between strains. A second possibility may be that a small number of idiotypes exist of which only one or two cross-react, but these are dominant. Finally, a small amount of high titre sera may be cross-reacting, and artificially influences the level of pool inhibition, as discussed in Chapter 3. As before, one must test individual sera to determine the true idiotypic picture - despite the massive increase in complexity compared to that of working with pooled sera.

Figure 6

Inhibition of Pooled Tertiary Anti-Fd

Sera from AKR/J, RF/J, A/J and

B10.BR/SnJ Mice

Tertiary anti-Fd sera from the
indicated mouse strains were pooled
from 12-15 animals and tested in
triplicate for inhibition of binding
to Fd by the indicated dilutions of
anti-idiotype. The standard errors
are represented by the vertical bars.
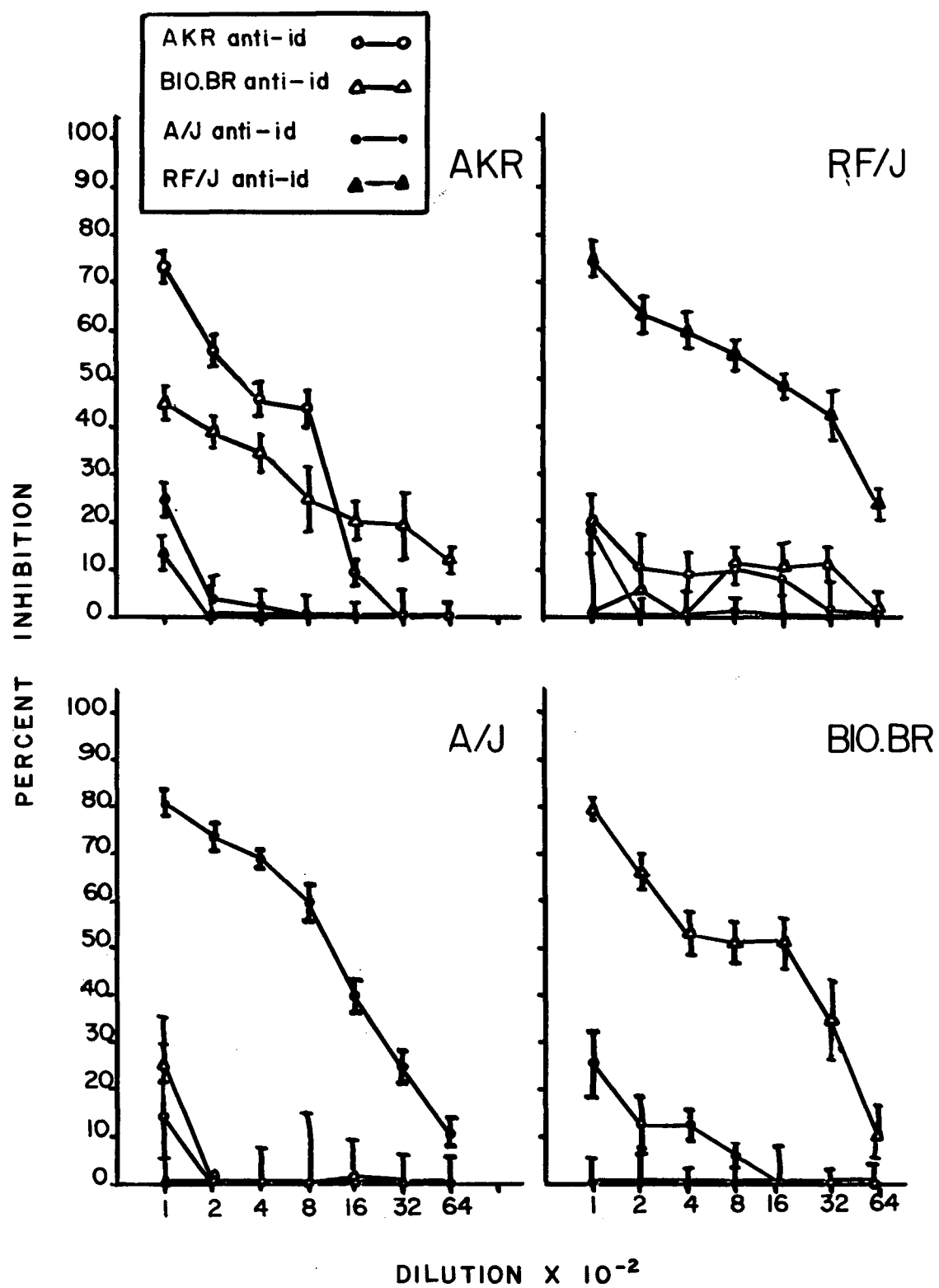
Figure 7

Inhibition of Pooled Tertiary Anti-Fd

Sera from C3H/HeJ, ST/bJ, C58/J

and CE/J Mice

Tertiary anti-Fd antisera were pooled from 12-15 individuals of the indicated mouse strain and tested for inhibition of binding to Fd in triplicate with various dilutions of the indicated anti-idiotype. The standard errors are represented by the vertical bars.
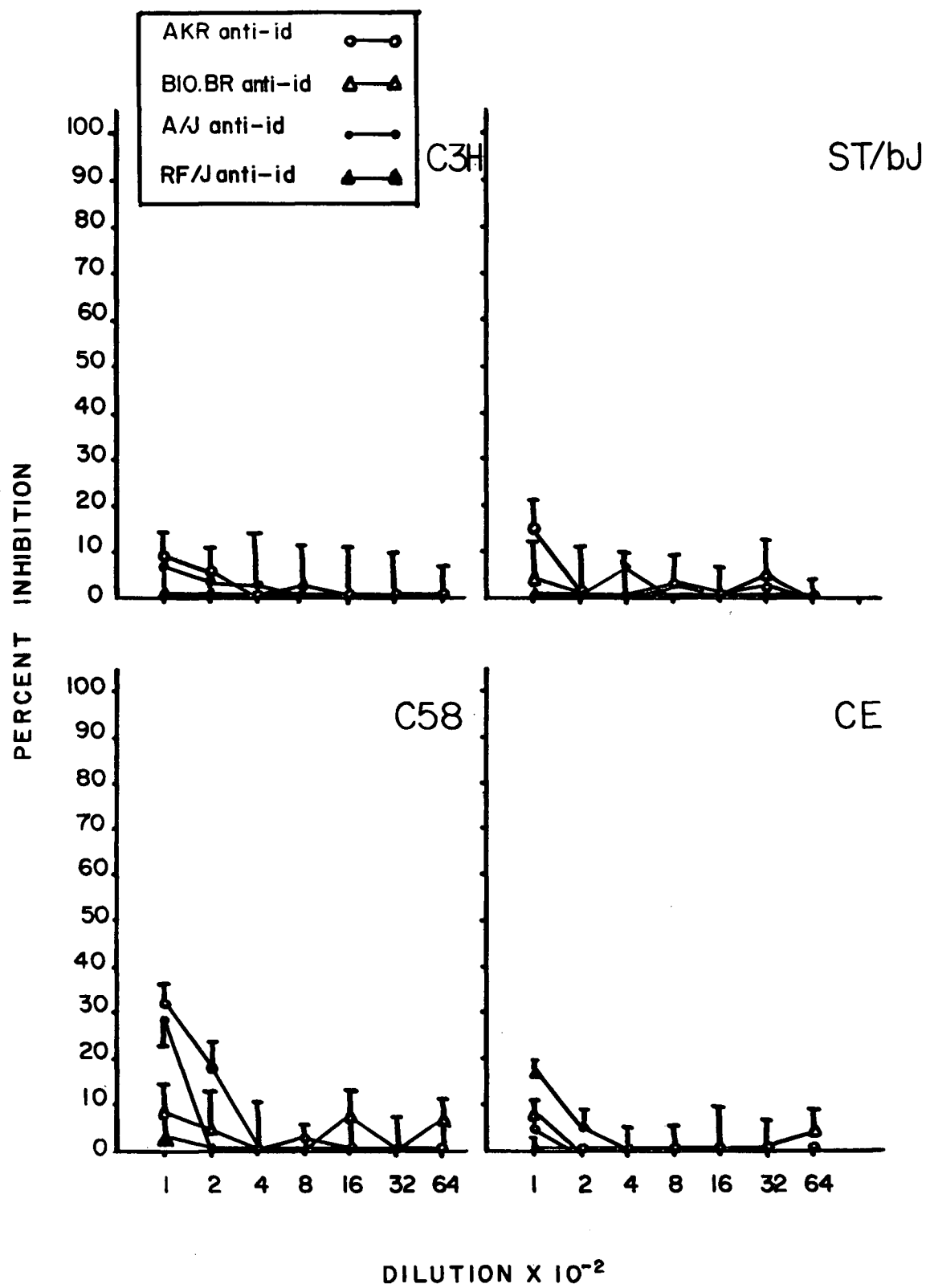
Figure 8

Inhibition of Pooled Tertiary Anti-Fd

Sera from C57BR/cdJ Mice

Tertiary anti-Fd antisera, pooled
from 9 C57BR/cdJ mice, were tested in
triplicate for inhibition of binding
to Fd by various dilutions of the
indicated anti-idiotype. Percent
inhibition is represented on the
vertical axis. The standard errors
are represented by vertical bars.

C57BR

Legend:
- AKR anti—id ○—○
- BIO.BR anti—id △—△
- A/J anti—id ●—●
- RF anti—id ▲—▲

DILUTION X 10⁻²
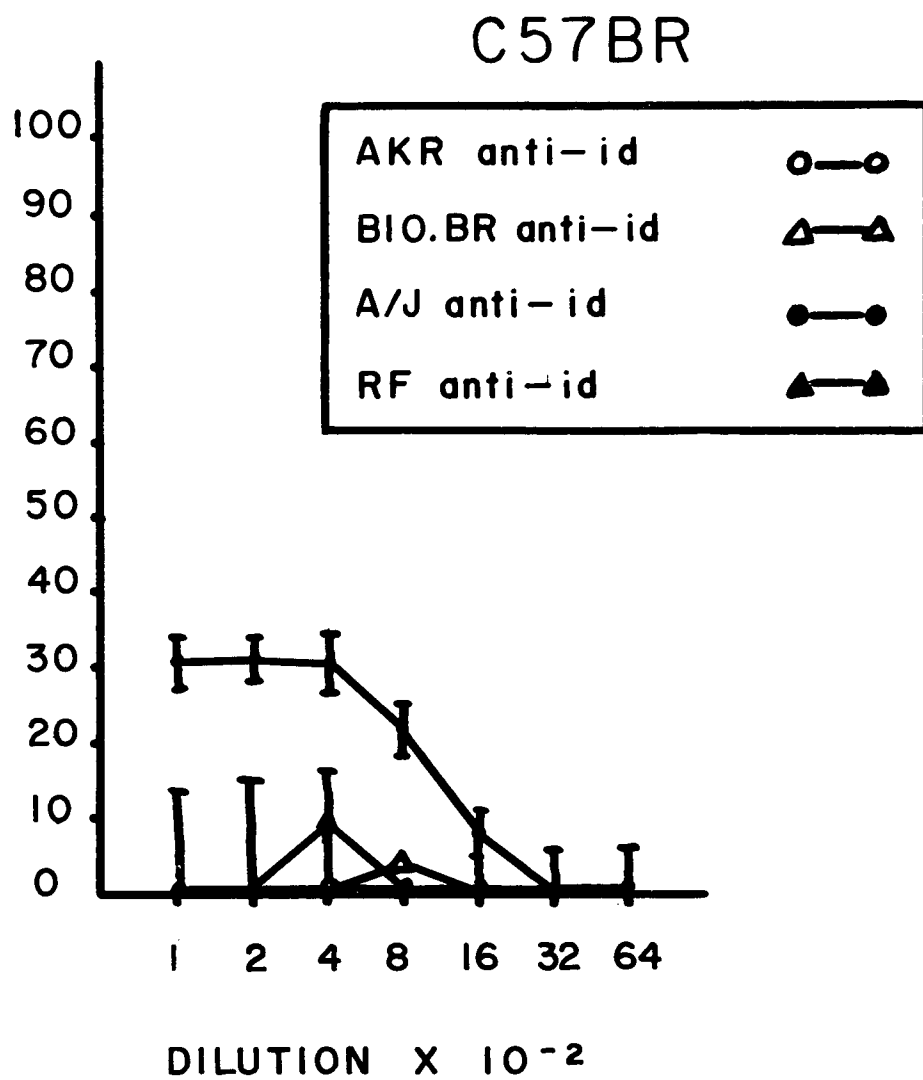
B)   Interstrain Cross-reactive Idiotypes in Individual Anti-Fd Sera

The sera of mice immunized with the original Fd preparation were tested for inhibition at various times during the response with the anti-idiotypic reagents discussed in Chapter 3. The results are shown in Figures 9 - 16. The responses of AKR, RF, and B10.BR to their own anti-idiotypes, are also illustrated for comparative purposes only, and have already been discussed in Chapter 3. The standard errors were very rarely above 10% and thus individual errors are not marked. The data are, as expected, quite complex and will be discussed sequentially.

Figure 9 shows the idiotypic response of AKR mice. As previously discussed, and expected, most of the mice are inhibited with the anti-AKR idiotype. Multiple examples exist, however, of strong cross-reactivity with the anti-idiotypes of other strains. In fact, all anti-idiotypes used were capable of inhibiting some individual sera. With AKR mice the frequency of cross-reactivity appears the same throughout the response, although this cannot be individually proven with the unmarked mice used in this experiment. In this experiment (and in all other experiments) one observes some sera that cross-react with more than one anti-idiotype, as well as some sera that are not inhibitable with any anti-idiotype. Some sera inhibitions add up to greater than 100% cumulative inhibition with different anti-idiotypes, implying the presence of the same idiotypic determinants recognized by different anti-idiotypes. The uninhibitable sera show that the anti-idiotypes are specific for idiotypic determinants, but that the four reagents do not define the total anti-Fd response in mice. Statistical analyses are seen in Table III. The results with anti-AKR and anti-RF inhibition frequencies are statistically significant, while it is quite obvious that some mice are making A/J and B10.BR

Figure 9

Inhibition of Individual Anti-Fd Sera

from AKR/J Mice

Individual anti-Fd antisera from AKR
mice obtained at various times in
their anti-Fd responses were tested
in triplicate for inhibition of
binding of Fd by the various
anti-idiotypes. Percent inhibition
is represented by the vertical axis.
Data for a single serum can be
followed vertically. Standard errors
were less than 10 percent inhibition.

| | 1°, DAY 15 | 1°, DAY 21 | 2°, DAY 7 | 2°, DAY 15 | 3°, DAY 7 | 3°, DAY 15 |

anti-AKR

anti-RF/J

anti-A/J

anti-BIO.BR
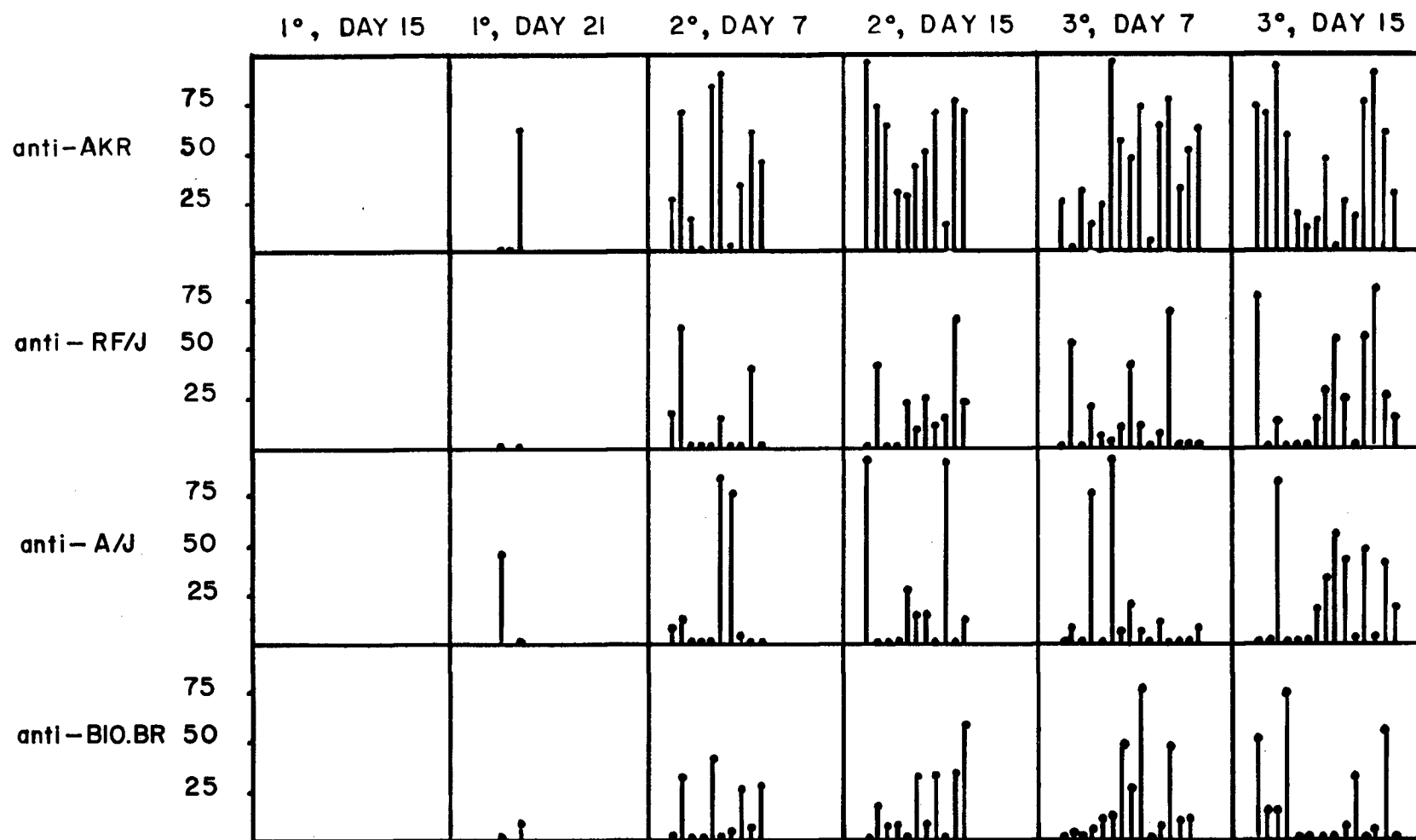
INDIVIDUAL AKR anti-Fd SERA

Figure 10

Inhibition of Individual Anti-Fd Sera

From RF/J Mice

Individual anti-Fd antisera from RF/J
mice obtained at various times in
their anti-Fd responses were tested
in triplicate for inhibition of
binding of Fd by the various
anti-idiotypes. Percent inhibition
is represented by the vertical axis.
Data for a single serum can be
followed vertically. Standard errors
were less than 10 percent.

INDIVIDUAL RF/J anti — Fd SERA
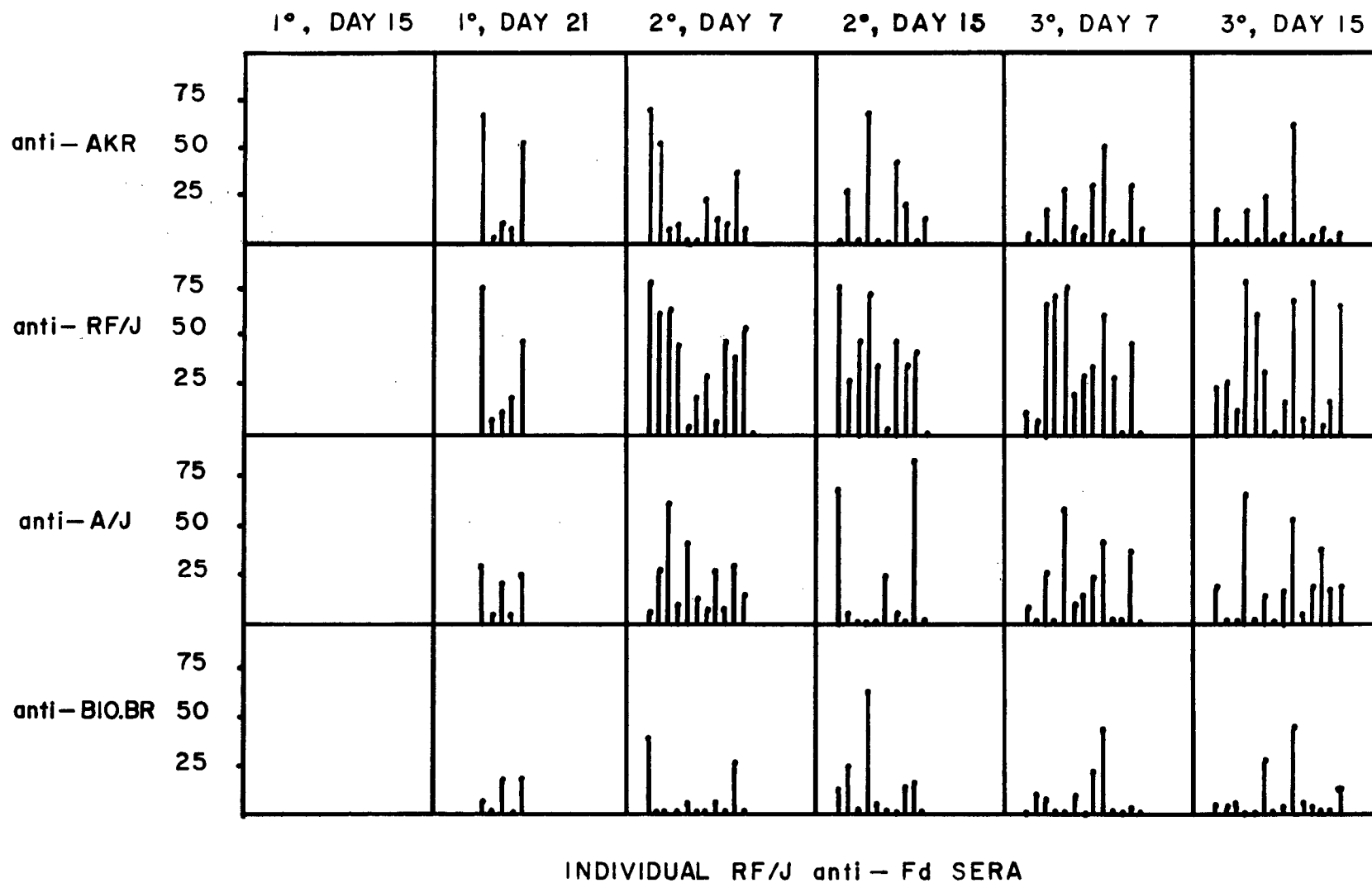
Figure 11

Inhibition of Individual Anti-Fd Sera

from A/J Mice

Individual anti-Fd antisera from A/J mice obtained at various times in their anti-Fd responses were tested in triplicate for inhibition of binding of Fd by the various anti-idiotypes. Percent inhibition is represented by the vertical axis. Data for a single serum can be followed vertically. Standard errors were less than 10 percent.

93
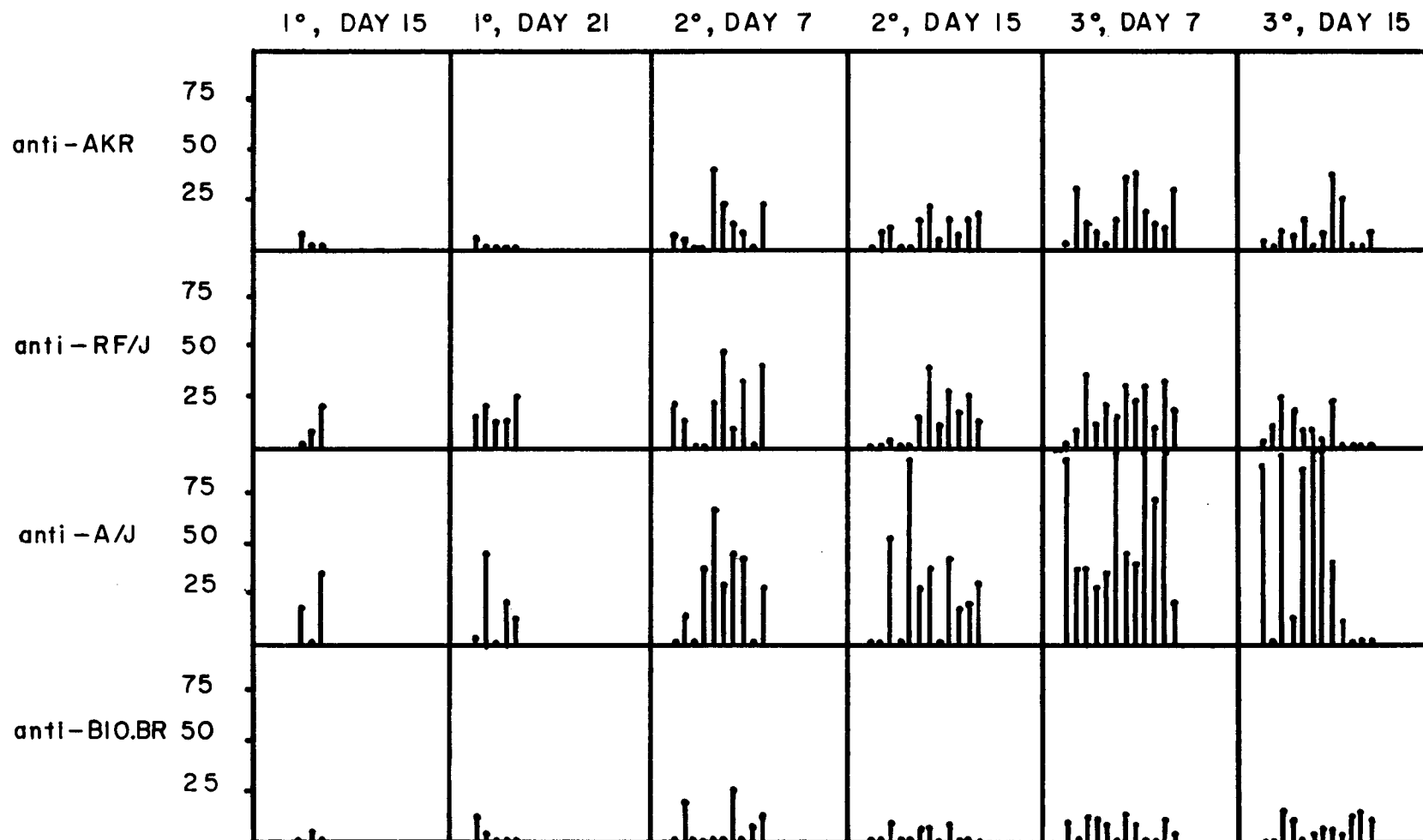
INDIVIDUAL A/J anti-Fd SERA

Figure 12

Inhibition of Individual Anti-Fd Sera

from B10.BR/SnJ Mice

Individual anti-Fd antisera from B10.BR mice obtained at various times in their anti-Fd responses were tested in triplicate for inhibition of binding of Fd by the various anti-idiotypes. Percent inhibition is represented by the vertical axis. Data for a single serum can be followed vertically. Standard errors were less than 10 percent.

INDIVIDUAL BIO.BR anti- Fd SERA
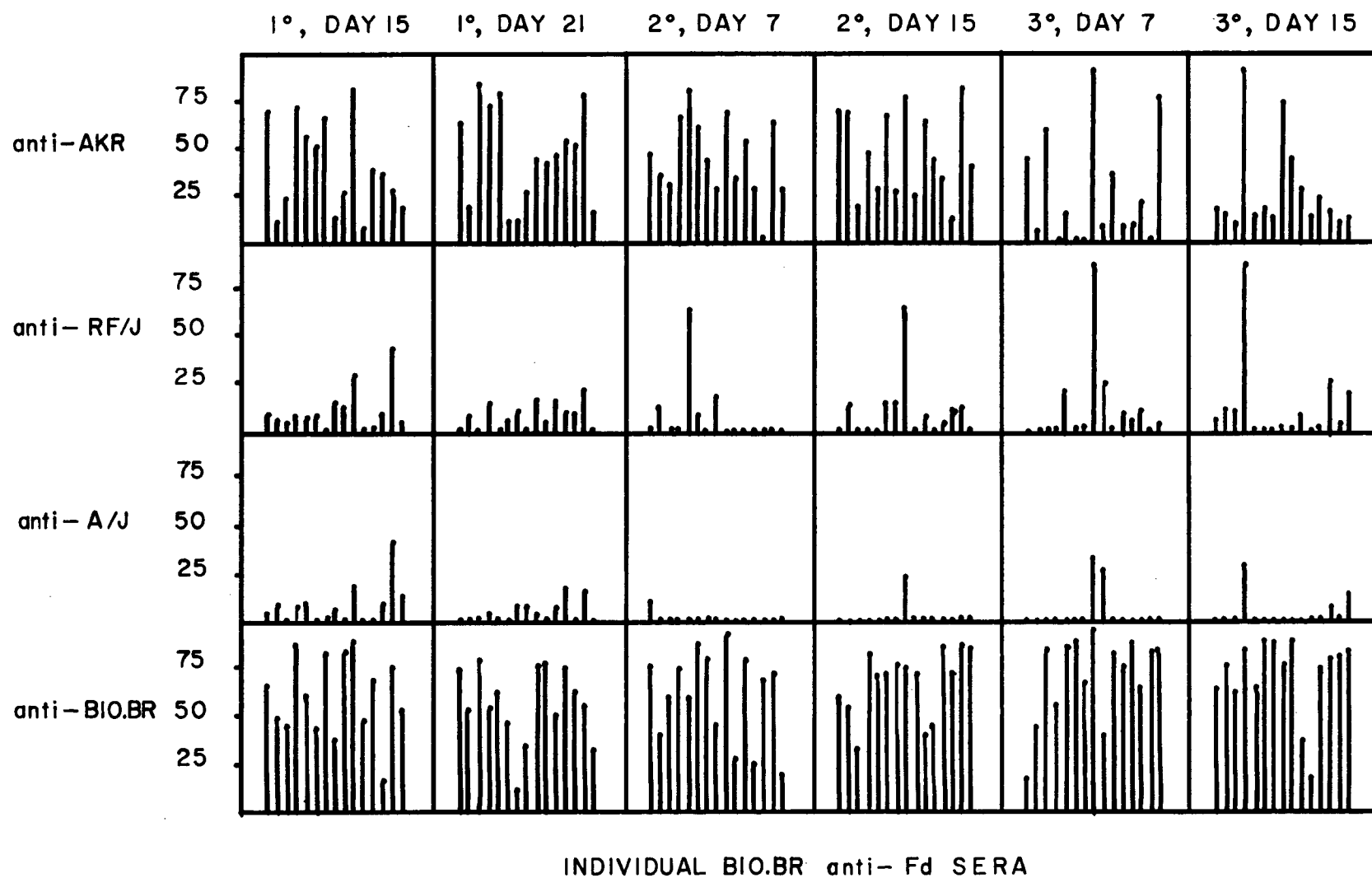
Figure 13

Inhibition of Individual Anti-Fd Sera

from C58/J Mice

Individual   anti-Fd   antisera   from
C58/J mice obtained at various times
in   their   anti-Fd   responses   were
tested in triplicate for inhibition
of binding of Fd by the various
anti-idiotypes.   Percent   inhibition
is represented by the vertical axis.
Data for a single serum can be
followed vertically. Standard errors
were less than 10 percent.
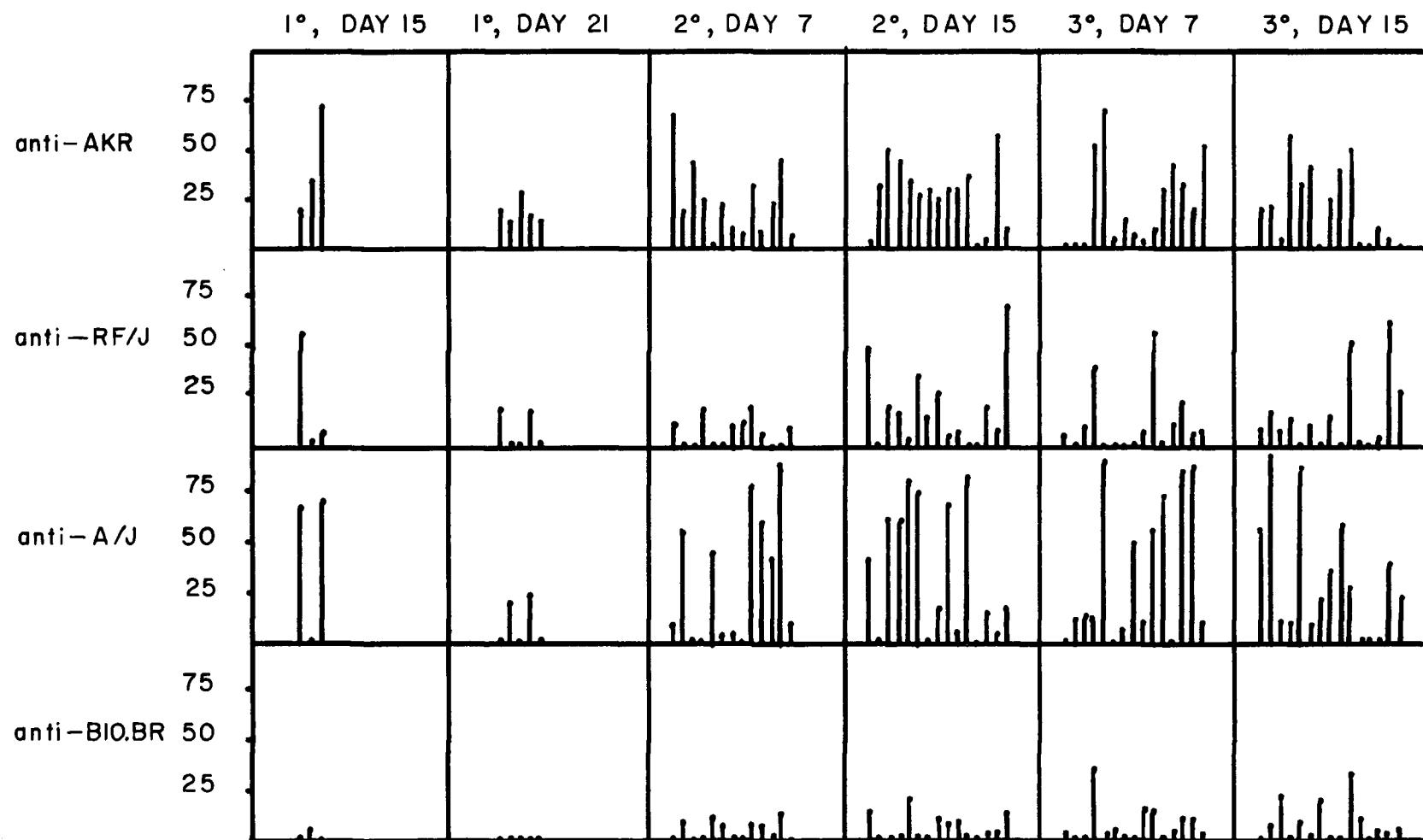
INDIVIDUAL C58 anti—Fd SERA

Figure 14

Inhibition of Individual Anti-Fd Sera

from C57BR/cdJ Mice

Individual anti-Fd antisera from C57BR/cdJ mice obtained at various times in their anti-Fd responses were tested in triplicate for inhibition of binding of Fd by the various anti-idiotypes. Percent inhibition is represented by the vertical axis. Data for a single serum can be followed vertically. Standard errors were less than 10 percent.

INDIVIDUAL C57BR anti — Fd SERA

Figure 15

Inhibition of Individual Anti-Fd Sera

from C3H Mice

Individual anti-Fd antisera from
C3H mice obtained at various times in
their anti-Fd responses were tested
in triplicate for inhibition of
binding of Fd by the various
anti-idiotypes. Percent inhibition
is represented by the vertical axis.
Data for a single serum can be
followed vertically. Standard errors
were less than 10 percent.

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INDICATED C3H anti — Fd SERA
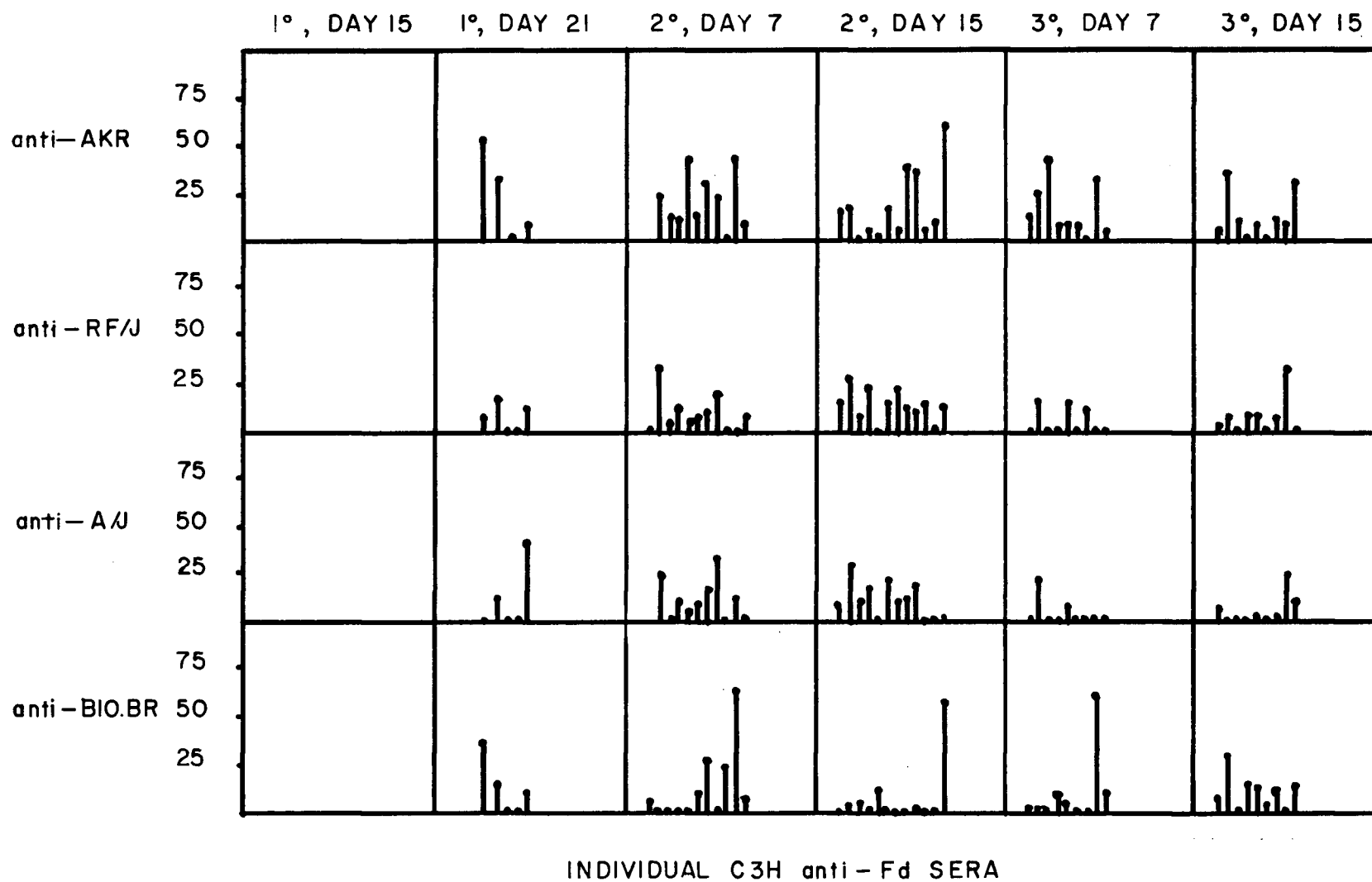
Figure 16

Inhibition of Individual Anti-Fd Sera

from CBA/J   Mice

Individual anti-Fd antisera from
CBA/J mice obtained at various times
in their anti-Fd responses were
tested in triplicate for inhibition
of binding of Fd by the various
anti-idiotypes. Percent inhibition
is represented by the vertical axis.
Data for a single serum can be
followed vertically. Standard errors
were less than 10 percent.

2° day 8   **2° day 15**   **3° day 8**

anti — AKR

anti — RF

anti — A/J

anti — BIO.BR

INDIVIDUAL CBA anti — Fd SERA
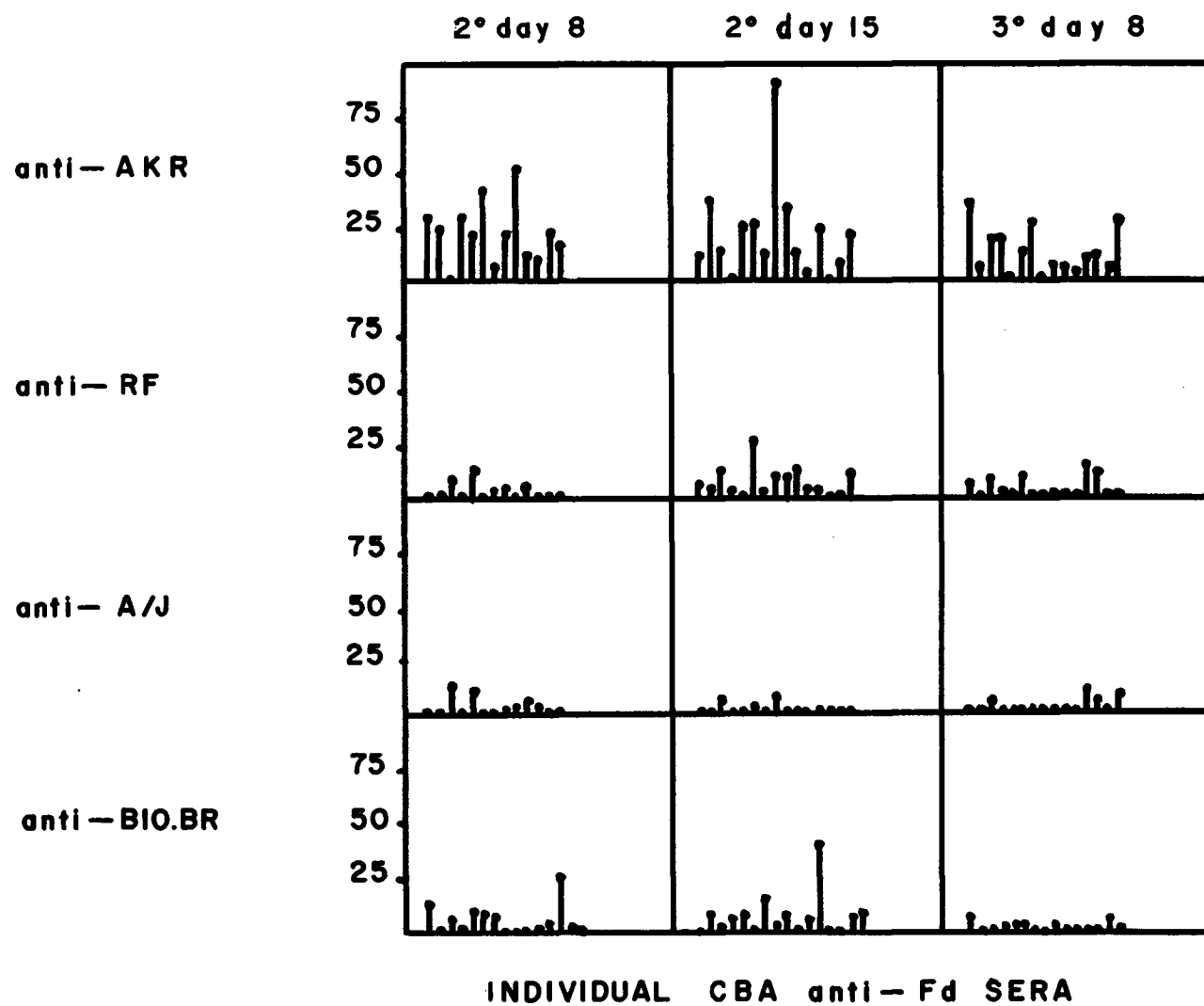
idiotypes, although the frequency of inhibition of these mice with those two anti-idiotypes is not statistically different from the KLH controls. Thus, some AKR mice produce idiotypic determinants that are seen in other strains by rabbits, though, as well some AKR sera are not inhibitable with any of the four reagents. There seems to be no highly specific cross-reactivity with any single strain, and no obvious change in the frequency of inhibition over time.

The results for RF/J mice are shown in Figure 10. There is a significant frequency of cross-reactivity with AKR and A/J idiotypes but not with those of B10.BR (see Table III). At least one RF/J serum reacts far above background with the anti-B10.BR idiotype. Again, uninhibitable sera and cumulative inhibitions greater than 100% are seen with RF/J sera, as was the case with AKR sera, with up to four different anti-idiotypes. This strongly implies the presence of multi-strain determinants on the same molecule. No obvious changes over time are seen.

A/J individual sera were tested with the results shown in Figure 11. A significant frequency of A/J sera are inhibitable by anti-RF/J idiotype, but not by anti-AKR or anti-B10.BR. Generally, A/J mice showed poor cross-reactivity with other idiotypes. This is in agreement with the results discussed in Chapter 3, in which it is likely that A/J mice express many idiotypes in the response to Fd.

Figure 12 shows the results with B10.BR sera. A very striking cross-reactivity is seen between B10.BR and AKR idiotypic repertoires. Almost

Table III

A Summary of the Reactivities of Various Mouse Strains

to Four Anti-Fd Anti-idiotypes

This table shows the statistical comparisons of the various strains of mice with different: anti-idiotypes. The percent frequency of response, time averaged to secondary Day 15 is given in a column for each anti-idiotype, followed by the "P" value of a statistical comparison with the KLH control (as carefully explained in Appendix 1). These data merely summarize the graphical results of Figures 9 to 16 and 17 to 20.

Table III - Summary of Statistical Comparisons

for Chapters 3 and 4

Test	Strain	% AKR	P	% RF	P	% A/J	P	% B10.BR	P
1	AKR	73.5	≤.005	34.0	≤.025	32.1	NS	30.2	NS
1	RF	30.0	≤.05	66.0	≤.005	45.3	≤.025	17.0	NS
1	A	18.5	NS	35.2	≤.025	64.8	≤.005	3.8	NS
1	B10.BR	58.9	≤.005	11.1	NS	4.5	NS	94.5	≤.005
1	C58	58.2	≤.005	20.9	NS	52.3	≤.005	7.6	NS
1	C57BR	10.0	NS	16.7	NS	80.0	≤.005	26.7	NS
1	C3H	33.3	≤.025	13.3	NS	20.0	NS	15.6	NS
1	CBA	46.5	≤.005	0.0	NS	0	NS	4.5	NS
1	Mix (KLH)	6.4	---	6.4	---	9.7	---	9.7	--
2	AKR	55.8	≤.005	0.0	NS	0	NS	26.9	NS
2	RF	50.0	≤.005	83.3	≤.005	39.6	≤.025	27.1	NS
2	A/J	26.7	NS	18.3	NS	16.7	NS	21.7	NS
2	B10.BR	58.3	≤.005	35.0	≤.025	23.3	NS	58.3	≤.005
	B10.BR(KLH)	4.8	NS	16.6	NS	9.5	NS	4.1	NS

NS = P > .05 (Not Significant)

60% of B10.BR mice are significantly inhibited by anti-AKR idiotype (see Table III). Anti-B10.BR is a fair reflection of the repertoire in the B10.BR animals, as almost 95% make a significant amount of recognized idiotype. Many antisera express both AKR and B10.BR idiotypes with cumulative inhibitions approaching 200%. One serum is 90% inhibitable with anti-AKR, 90% inhibitable with anti-RF/J and 95% inhibitable with anti-B10.BR. This is very strong circumstantial evidence that the three strains express the same idiotypic determinants, on the same molecule.

Why is it that B10.BR is inhibitable with anti-AKR, whereas the reverse is not the case? The easiest explanation is that the repertoire of B10.BR animals is quite restricted, and overlaps with AKR. AKR repertoire may be larger, thus less likely to be inhibited with anti-B10.BR reagents.

Another interesting observation is the change in frequency of AKR idiotype expression in the B10.BR response, over time. This change is highly significant (p $\leq$ .005 , Df. =5) and occurs during the 50 day gap between the 2° and 3° responses. Strangely enough, there is no change in the frequency of anti-B10.BR inhibition, and no change in the mean anti-B10.BR inhibition. Thus the anti-AKR and anti-B10.BR idiotypic reagents do not always recognize the same determinants. As the anti-B10.BR anti-idiotype was made to the late 30 sera, it understandably would not recognize the lost AKR idiotypes and would thus not greatly inhibit AKR anti-Fd sera. The concept of idiotypic maturation will be discussed later.

In conclusion, Figure 12 shows that a high proportion of B10.BR mice express AKR idiotype, but this frequency changes over time. These results may be due to an overlap between unequal size repertoires, or to a loss of AKR determinants of B10. BR antibodies before the late tertiary response.

The results of experiments with C58 mice are shown in Figure 13. A high frequency of these mice express idiotypic determinants recognized by both anti-A/J and anti-AKR/J idiotypes. Not all C58 idiotypes can be defined with the four reagents, though sera inhibited with anti-A/J are almost completely inhibited, possibly implying a restricted idiotypic response in these mice. No obvious changes in inhibition frequency over time are observed.

C57BR/cdJ mice have an interesting pattern of inhibition, as shown in Figure 14. Almost all sera were highly inhibited by anti-A/J idiotype. Almost no reactivity is seen with any other anti-idiotype. Thus, the data from Figures 13 and 14 show that though the anti-A/J idiotype will not inhibit different groups of A/J mice, C58 and C57BR antisera are highly inhibitable at high frequency with this reagent.

C3H and CBA mice may be discussed together as they display almost identical patterns of expression, shown in Figures 15 and 16. Both groups are significantly inhibited by anti-AKR idiotype, and are uninhibitable with any other reagent. The amount of inhibition with anti-AKR is quite low. Plainly, the majority of the idiotypic response of these two strains cannot be defined by the four anti-idiotypic reagents.

Although the individual inhibition data is quite complex, several generalities can be made concerning the presence of cross-reactive idiotypic determinants in the various high responder strains. All of the strains tested cross-react with at least one of the four anti-idiotypes, though not all with the same reagent. This pattern is almost always constant over the duration of the response. Some sera cannot be defined by any of the four anti-idiotypes, and some sera probably contain antibodies

with determinants shared by many strains, though this cannot be definitively proven by these experiments.

C)   Kinetics of Individual Interstrain Cross-Reactive Idiotype Expression

Mice were individually marked and immunized by the protocol discussed in Chapter 3. The results from AKR mice are shown in Figure 17. As can be seen, not much cross-reactivity is observed with other idiotypes. Thus, the slight degree of cross-reactivity seen with the anti-RF/J idiotype in Figure 9 cannot be reproduced. There does appear to be some cross-reactivity with B10.BR, of the same order as observed earlier (see Table III). This is important, since earlier experiments showed strong cross-reactivity between B10.BR and AKR idiotypes. The kinetics show changes in only the frequency of AKR idiotype, which will be discussed in the next chapter.

The data from RF/J animals fully corroborate earlier experiments. Figure 18 shows an even higher frequency of inhibition with anti-RF/J idiotype and with anti-AKR idiotype than that found in results presented in Figure 10. The frequency of inhibition with anti-A/J idiotype is almost unchanged (see Table III). Also, no significant frequency of inhibition with anti-B10.BR idiotype is observed. A statistical analysis of the change in inhibition frequency over time (Table IV) shows no significant differences between sample periods.

A/J sera tested for inhibition exhibit the pattern shown in Figure 19. Although the time averaged data showed no significant inhibition frequencies (Table III) above the KLH controls, inspection of the data shows that most primary sera are somewhat inhibited, especially with anti-RF/J idiotype. When these data are tested for changes over time

Figure 17

Inhibition of Anti-Fd Sera from

Individually Marked AKR/J Mice:

Kinetics of the Response

Anti-Fd antisera from marked AKR/J mice
obtained at various times in their anti-Fd
responses were tested in triplicate for
inhibition of binding to Fd by the various
anti-idiotypes. Percent inhibition at
various times in the response is represented
by the vertical axis. Data from a single
animal can be followed vertically. Standard
errors were less than 10 percent inhibition.
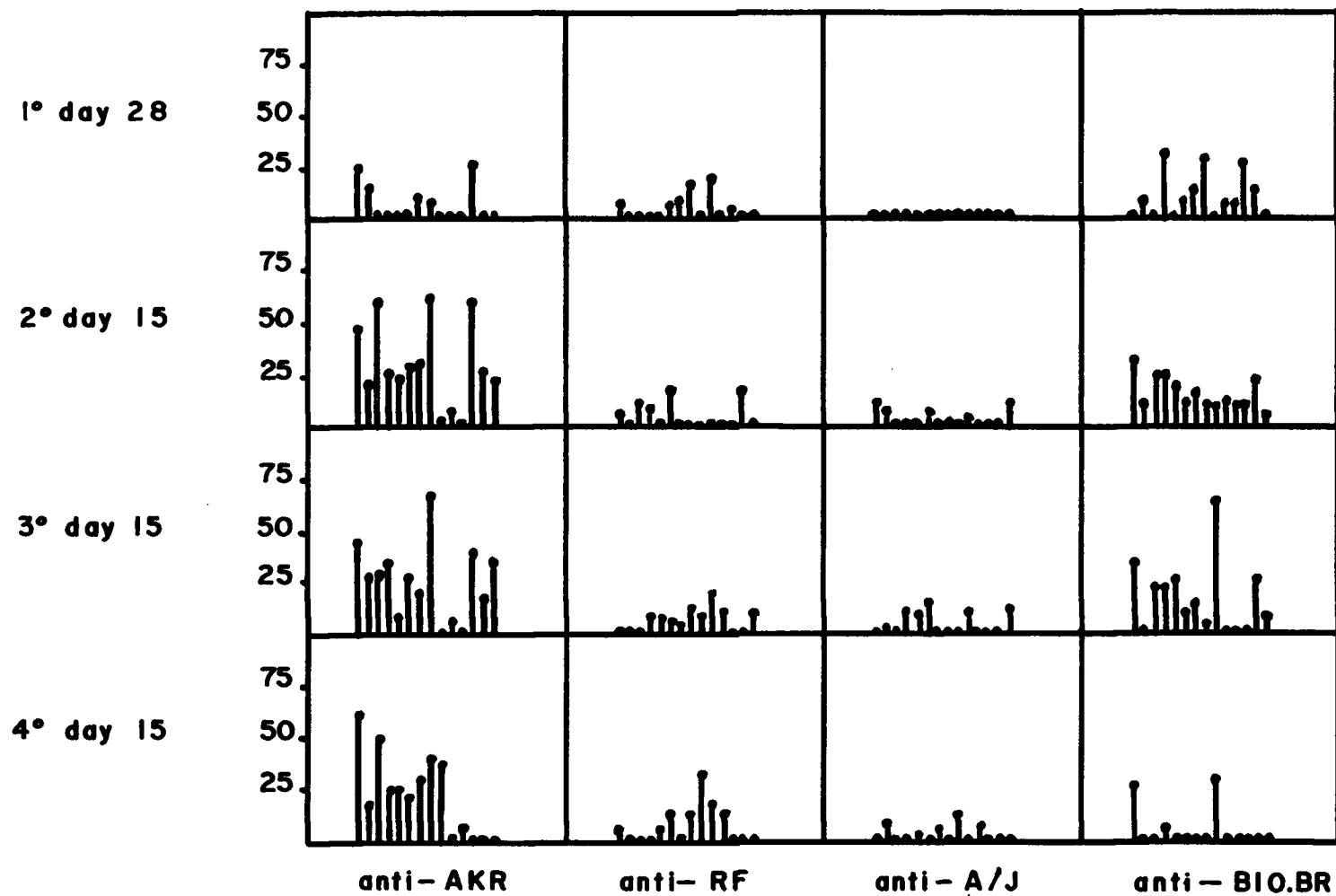
INDIVIDUAL AKR anti-Fd SERA

Figure 18

Inhibition of Anti-Fd Sera from

Individually Marked RF/J Mice:

Kinetics of the Response

Anti-Fd antisera from marked RF/J mice
obtained at various times in their anti-Fd
responses were tested in triplicate for
inhibition of binding to Fd by the various
anti-idiotypes. Percent inhibition at
various times in the response is represented
by the vertical axis. Data from a single
animal can be followed vertically. Standard
errors were less than 10 percent inhibition.

113

anti – AKR   anti – RF   anti – A/J   anti – BIO.BR

INDIVIDUAL RF anti – Fd SERA
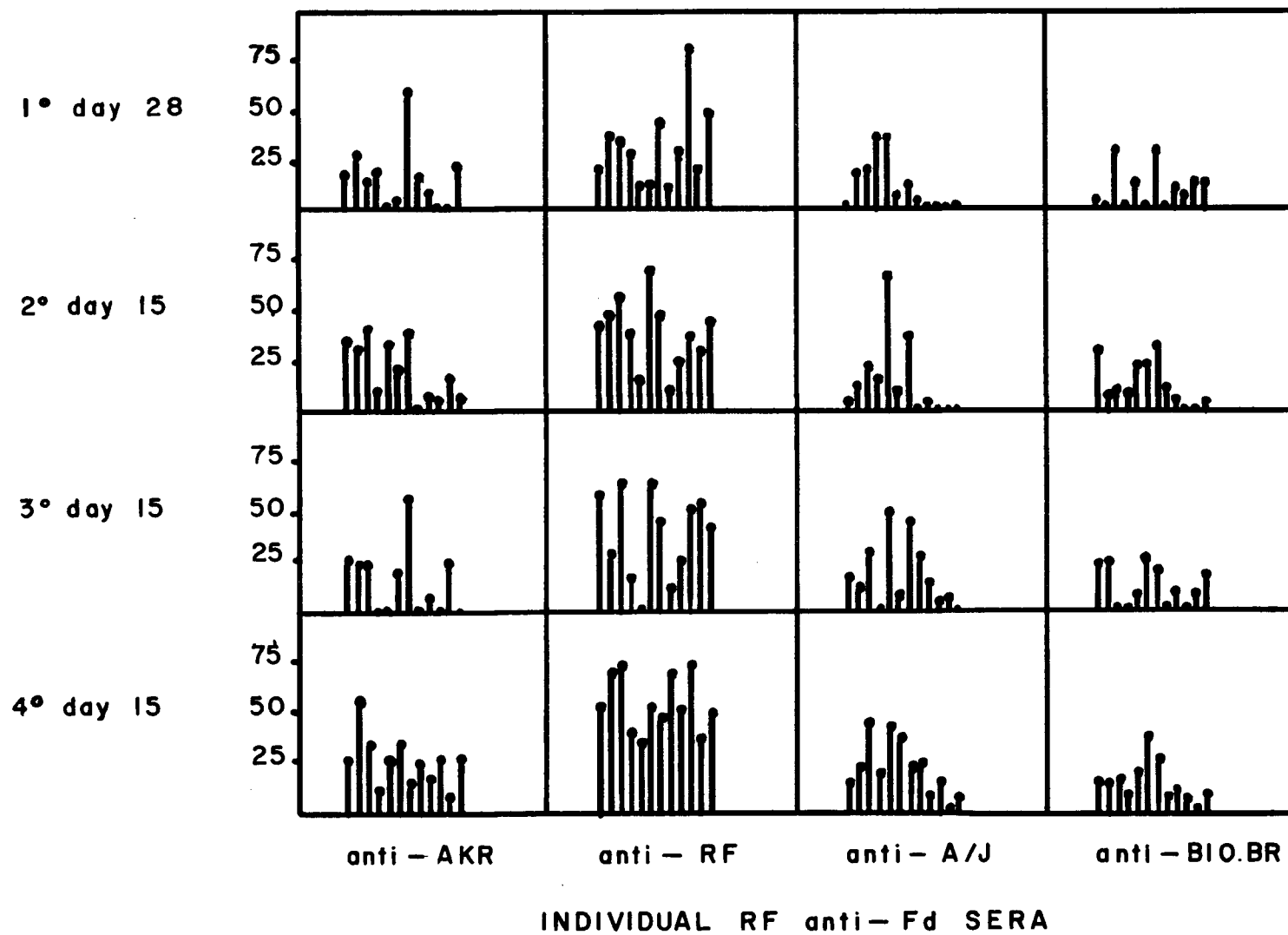
Table IV

A Summary of the Change in Idiotype Expression over

Time of 4 Strains of Mice

This table is a composition of the data of Figures 3, 17, 18, 19 and

20. For each strain and anti-idiotype indicated, the fraction of sera

inhibited above threshold are listed. The value of the Chi-square testing

for no change in inhibition frequency over time, and the corresponding "P"

value are given. The tests were performed as described in Appendix 1.

Table IV - Summary of Kinetics of Idiotype Expression

Strain	Anti-idiotype	1°	2°	3°	x^2	P
AKR/J	AKR	2/13	11/14	9/14	11.8	≤.005
"	RF	0	0	0	ND	NS
"	A	0	0	0	ND	NS
"	B10.BR	3/13	4/14	5/14	0.52	NS
RF/J	AKR	4/12	6/12	6/12	0.90	NS
"	RF	9/12	10/12	9/12	0.32	NS
"	A	4/12	3/12	5/12	0.75	NS
"	B10.BR	2/12	4/12	4/12	1.11	NS
A/J	AKR	7/15	2/15	3/15	4.77	NS
"	RF	9/15	1/15	1/15	15.40	≤.005
"	A	6/15	1/15	2/15	5.84	NS
"	B10.BR	6/15	4/15	2/15	2.73	NS
B10.BR	AKR	8/15	9/15	9/15	0.19	NS
"	RF	2/15	5/15	10/15	9.26	≤ .01
"	A	1/15	2/15	6/15	5.84	NS
"	B10.BR	8/15	11/15	9/15	1.32	NS
B10.BR(KLH)	AKR	1/14	0/14	1/14	1.05	NS
"	RF	3/14	0/14	3/14	3.50	NS
"	A	2/14	1/14	1/14	0.55	NS
"	B10.BR	1/14	1/14	0/14	1.05	NS

NS = P ≤ .05

ND = Not Done

Figure 19

Inhibition of Anti-Fd Sera from

Individually Marked A/J Mice:

Kinetics of the Response

Anti-Fd antisera from marked A/J mice
obtained at various times in their anti-Fd
responses were tested in triplicate for
inhibition of binding to Fd by the various
anti-idiotypes. Percent inhibition at
various times in the response is represented
by the vertical axis. Data from a single
animal can be followed vertically. Standard
errors were less than 10 percent inhibition.

INDIVIDUAL A/J anti—Fd SERA
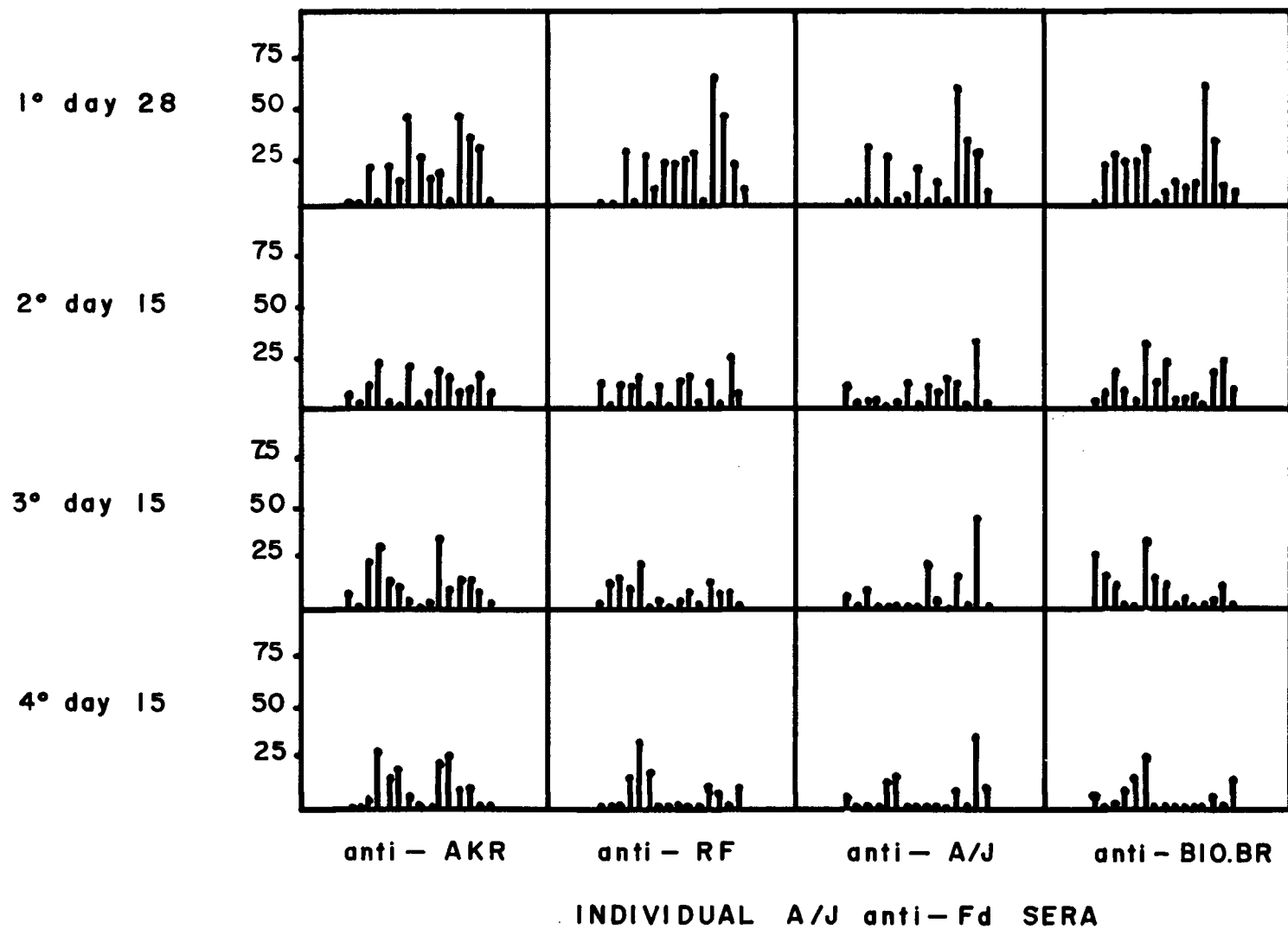
(Table IV), there is significant dropoff of the frequency of inhibition with anti-RF/J idiotype. Although the frequency data is not statistically significant for other anti-idiotypes, one can see that the cumulative amount of inhibition decreases after the primary bleed for all anti-idiotypes. These observations seem to indicate that a change in idiotype expression occurs during the maturation of the immune response of A/J mice to Fd. This change is idiotypic profile over time will be referred to as idiotypic maturation for simplicity, and will be discussed in the next chapter.

The data from B10.BR antisera are shown in Figure 20. These data completely reproduce the findings of earlier experiments (Figure 12) although the frequency of expression of the B10.BR idiotype is somewhat lower. Exactly the same frequency of inhibition with anti-AKR idiotype is seen as before, and, in addition, a significant degree of cross-reactivity with RF/J idiotypes is present (Table III). This cross-reactivity changes significantly over time, though, in contrast to the earlier experiments, the frequency of AKR idiotype expression appears constant. Though not statistically significant ($P \geq .1$) some evidence may also exist for an increase in A/J expression over time.

Thus, several interesting points emerge from the data of the second set of experiments. Generally, with a few exceptions, once an animal is making a cross-reactive idiotype, it continues to make it throughout the response. The exceptions are with some B10.BR and A/J animals, which exhibit examples of idiotypic maturation, causing noticeable changes in the patterns of cross-reactivity over time. The cross reactivities observed in the first sets of experiments are also observed in the second set, with the exception of the A/J data. Individual sera may express more than one cross-reactive idiotypic family, possibly with the determinants

Figure 20

Inhibition of Anti-Fd Sera from

Individually Marked B10.BR/SnJ Mice:

Kinetics of the Response

Anti-Fd antisera from marked B10.BR/SnJ mice obtained at various times in their anti-Fd responses were tested in triplicate for inhibition of binding to Fd by the various anti-idiotypes. Percent inhibition at various times in the response is represented by the vertical axis. Data from a single animal can be followed vertically. Standard errors were less than 10 percent inhibition.

INDIVIDUAL BIO.BR anti-Fd SERA
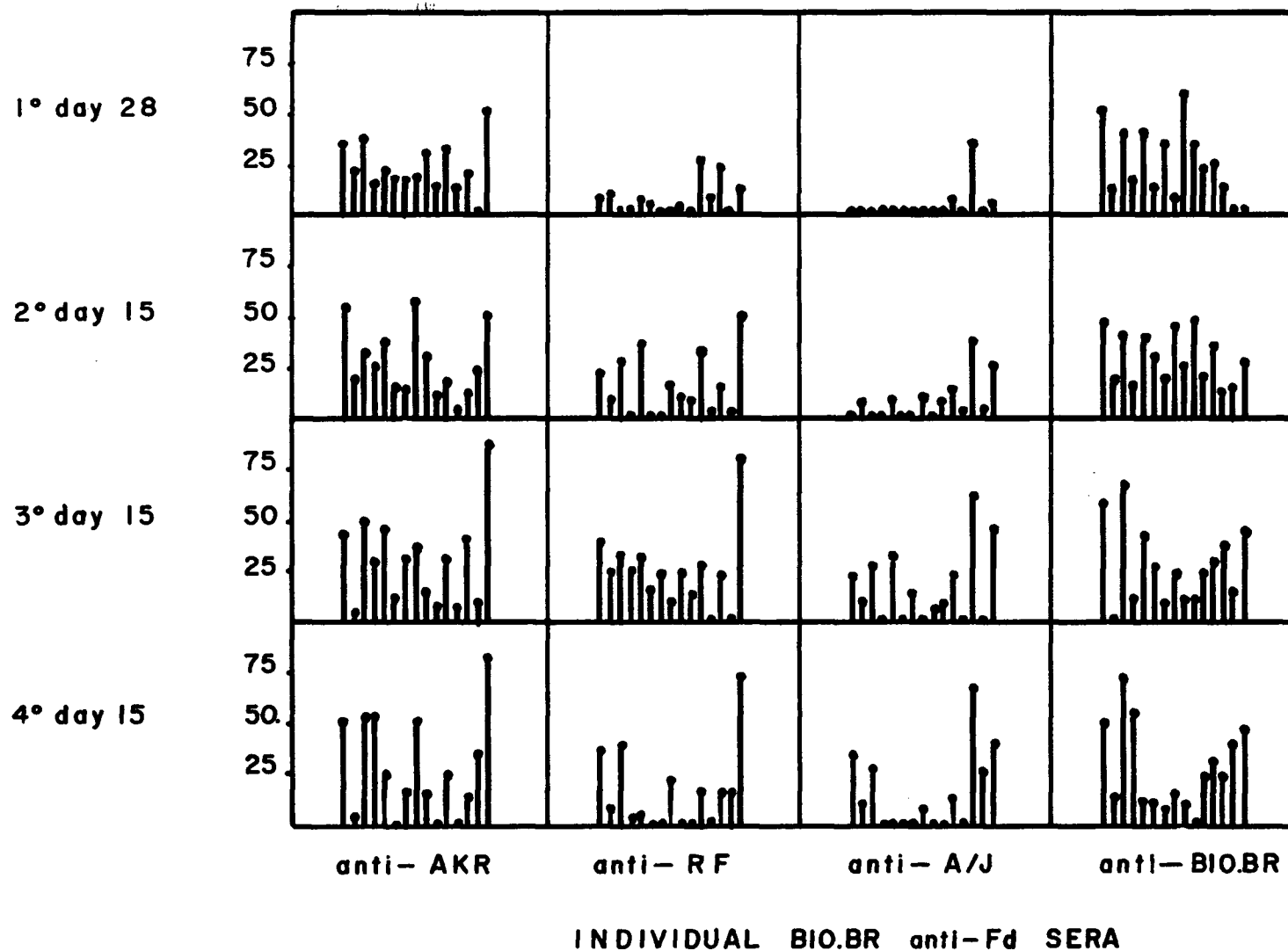
on the same molecule conserved across all of the strains. As well, some sera cannot be inhibited by any of the anti-idiotypes.

D)   Effect of H-2 Genes on Idiotype Expression

The sera of mice used in earlier experiments mapping total and determinant specific responses to Fd in recombinant strains of mice were assayed for idiotypic specificity. These mice had been immunized by Dr. L. Sikora with her preparation of Fd (251). Figure 21 shows the effect of H-2 linked genes on idiotype expression. Sera from B10.BR mice show the classic pattern of inhibition with both anti-AKR and B10.BR anti-idiotypes. B10.S and SJL share the same H-2 region, but only B10.S is inhibited with anti-A/J anti-idiotype. Obviously more genes than H-2 are involved in that particular idiotypic expression. B10, B10.A(3R) and B10.S(9R) do not have a high frequency of response with the expression of any of the four idiotypes, whereas B10.A and B10.A(4R) have a reasonably high expression of B10.BR idiotypic determinants. Surprisingly, B10.A express B10.BR idiotypes but not those of AKR. Some B10.A(2R) and B10.A(5R) are also able to express B10.BR idiotypes.

Though these data are from small sample sizes, they are very interesting. H-2 linked genes do affect idiotype expression, as all of these B10 responding congenics would be expected to produce at least B10.BR idiotypic determinants. This is not seen, instead a variety of patterns of idiotype expression are seen. Generally, mice with H-2K/IA^k loci (251) seemed to produce the best expression of the B10.BR idiotype, though the data of B10.A(5R) and B10.A(3R) are not conclusive due to the small sample size. Strains with H-2K/IA^b have members that are still able to express B10.BR idiotypes. No explanation can be found for the B10.S expression of

Figure 21

Inhibition of Secondary Anti-Fd Sera from

Various Strains of

Mice Differing at H-2 Loci

Individual anti-Fd sera from the secondary
responses of mice differing at H-2 loci were
tested for inhibition of binding to Fd by
various anti-idiotypes. The vertical axis
represents percent inhibition using the
indicated anti-idiotypes. Data from the same
serum can be followed vertically. Standard
errors were less than 10 percent inhibition.

anti-AKR

anti-RF

anti-A/J

anti-BIO.BR

BIO.BR   BIO.S   SJL   C57BL/IO   BIO.A   BIO.A(2R)   BIO.A(3R)   BIO.A(4R)   BIO.A(5R)   BIO.S(9R)

INDIVIDUAL ANTI — Fd SERA
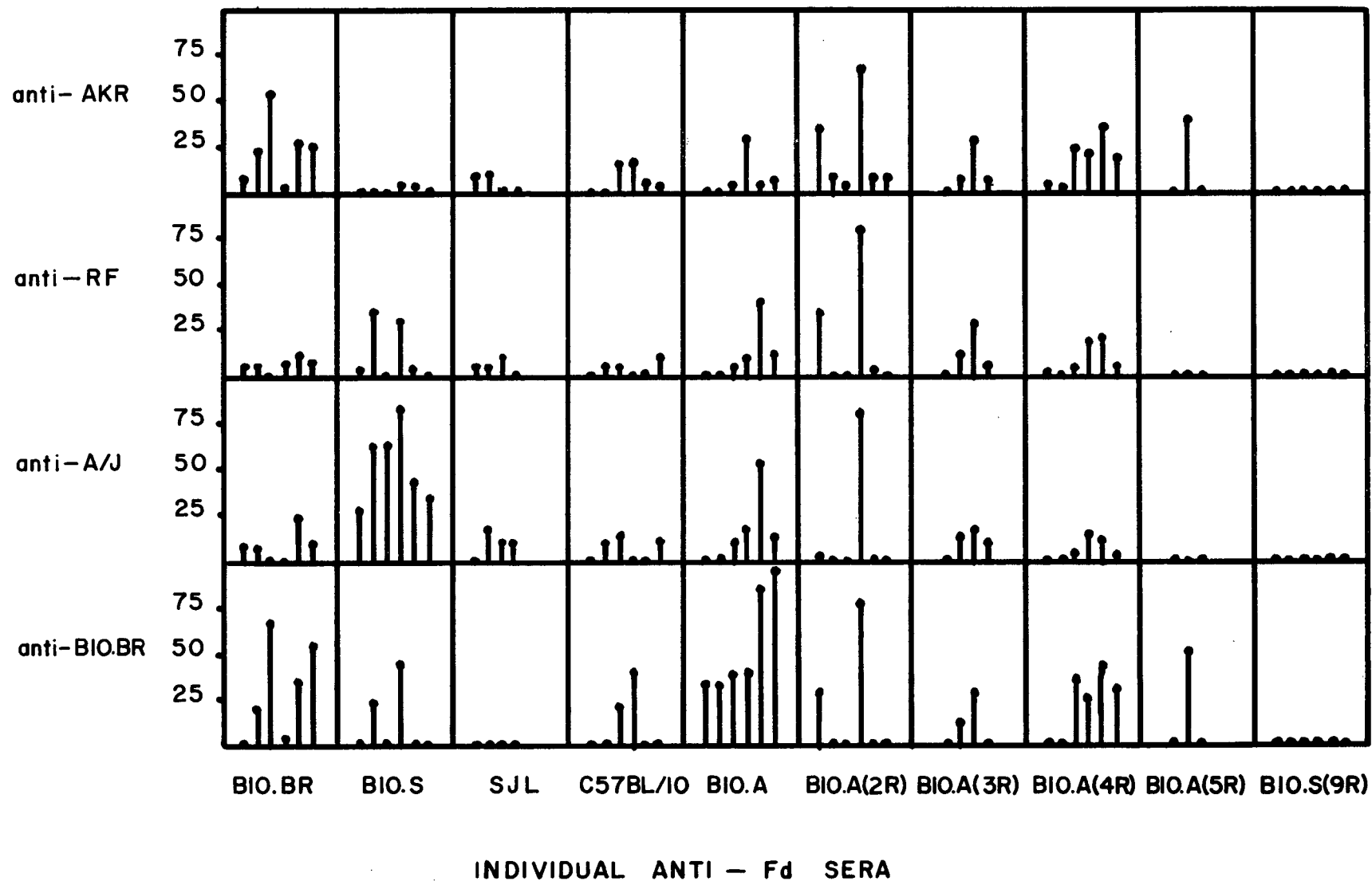
A/J idiotype, as this result is not seen in B10.S(9R) or SJL mice. Probably other non H-2 linked genes and possibly other H-2 linked genes are capable of influencing the idiotypic response.

In summary, the phenomenon of idiotypic cross-reactivity is both widespread and reproducible in the anti-Fd antiserum from high responder mice. The total idiotypic repertoire of the anti-Fd response in mice is not defined by the four anti-idiotypic reagents used, implying a large amount of diversity. B10.BR mice appear to be the most restricted of the strains investigated. The expression of this idiotype appears to be influenced by both H-2 and non-H-2 linked genes.

II. Discussion

Sera from mice immunized with Fd were tested for the presence of four different idiotypes. Two separate experiments were done with AKR/J, RF/J, A/J, and B10.BR/SnJ antisera. One experiment was done with C58/J, CBA/J, C57BR/cdJ and C3H/HeJ mice. These experiments yielded several interesting results. All strains tested contained members whose individual antisera reacted with at least one "foreign" anti-idiotype, and usually representative inhibition was observed with all anti-idiotypes. Some strains had a very significant frequency of cross-reactivity such as B10.BR with AKR, C58 with AKR and A/J, RF with AKR and A/J, C57BR with A/J, and C3H and CBA with AKR. An interesting trend can be observed from Table III. Antisera from most strains could be inhibited with anti AKR idiotype, few strains cross-reacted extensively with A/J or RF/J and only B10.BR were inhibitable with anti-B10.BR anti-idiotype. It would be desirable to find some common ground to explain these patterns of cross-reactivity. Table V

has been assembled to show some additional information between the strains concerning some genes of immunological interest and Figure 22 establishes the relationship between the heavy chain loci by virtue of their Ig2a cross-reactivity (56,277). The cross-reactive anti-Fd idiotype data has been summarized in the relationship diagram shown in Figure 23. No correlation between allotype identity and expression of interstrain cross-reactive idiotypes can be seen. As well, no correlation between idiotype sharing and breeding history (278) can be discerned, with one exception. CBA/J and C3H/HeJ are identical at the loci shown in Table V and are related genetically by virtue of their common breeding histories (278). A computer search of all known mouse gene allotypes, cross-matched with the idiotype cross-reactivity patterns may show an eventual pattern, but such a monumental undertaking is beyond the scope of this thesis. Instead, a brief discussion comparing this work to findings reported in other protein systems is in order.

As previously discussed, the idiotypic response to proteins is much more complex than the response to haptens. Idiotypes to HEL were shown to be present in the anti-HEL response of all mouse strains tested, with no correlation to H-2 or Igh alleles (206) and such idiotypes were also present on antibody directed to different determinants of HEL (208). Diverse strain or species idiotypic cross-reactivity is seen in the anti-GAT response (153, 154) in which antibodies to different determinants can also share the same idiotypes (165). Idiotypes produced in the anti-insulin response are shared by diverse strains of mice, although one public idiotype may occur in greater amounts in mice of Igh^a allotype

Table V

A Summary of Allotypes of Mice at Loci of

Immunological Interest

This table represents the allotypes of mice at heavy chain and light chain loci compiled from the indicated references.

Table V - Allotypes of Mice at Loci of Immunological Interest

Reference Strain	1g2a	1g2b	1g1	1gM	Ef1	Ef2	1gA
AKR/J	d	d	a	NT	a	a	d
RF/J	c	a	a	c	b	a	d
A/J	e	e	a	c	b	a	d
B10.BR/SnJ	b	b	b	b	b	a	b
C58/J	a	a	a	a	a	b	a
C3H/HeJ	j	a	a	NT	b	a	a
CBA/J	j	a	a	NT	b	a	a
CE/J	f	f	a	NT	b	b	f
St/bJ	a	a	a	a	b	a	a
C57BR/cdJ	a	a	a	a	b	a	a

Data from references 56, 271 and 277

NT = Not Tested

Figure 22

<u>Simple Relatedness Tree of Immunoglobulin</u>

<u>Heavy Chain Allotypes in Mice</u>

This diagram is a representation of the relatedness of the heavy chain loci of mice compiled from a serological investigation of their heavy chain allotype immunological specificities as detailed in references 56 and 277.

THEORETICAL ANCESTRAL

Ig 2a ALLOTYPE

BIO.BR   CE   ST   CBA   A/J   AKR   RF

C57BR   C3H

C58
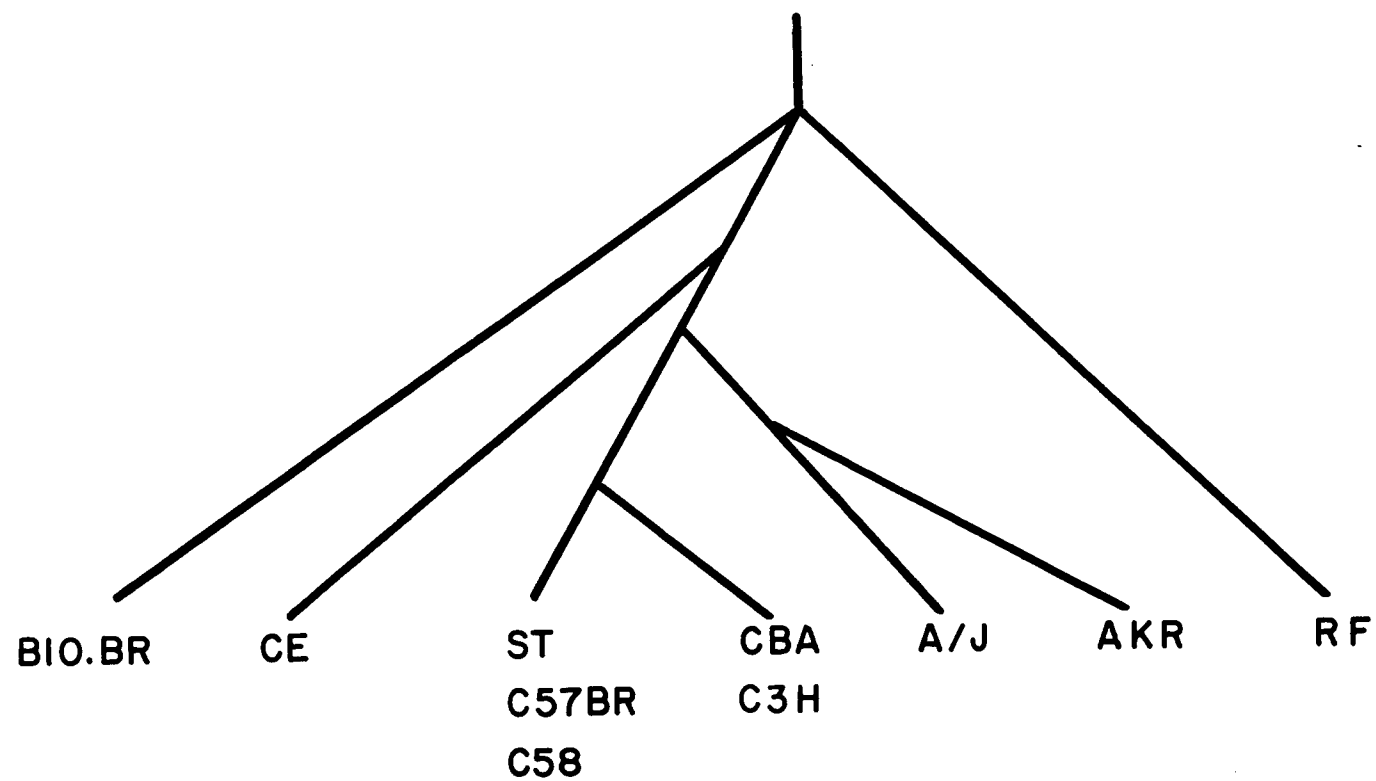
Figure 23

Relationship of Various Anti-Fd Idiotypic

Families

This is a simple relationship diagram illustrating the distribution of idiotypes among the strains of mice used in this thesis. The data are a compilation of the results of Chapter 4.
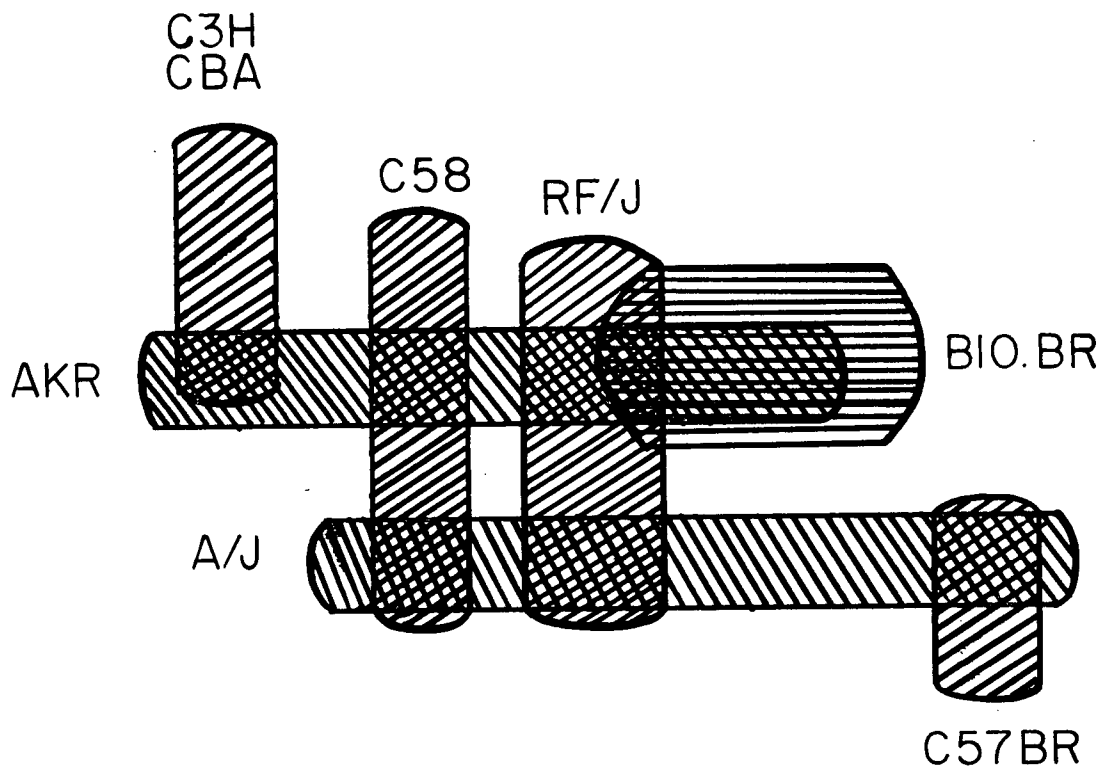
RELATIONSHIP OF IDIOTYPIC FAMILIES

(244). This public idiotype can be shared by antibody directed to different specificities (244) as can public idiotypes in the anti-H-2K^k system (279), the anti-SWM system (194), the rabbit anti-hemoglobin system (280), and even the ARS hapten system (45). Another example of an interstrain cross-reactive idiotype is seen in the response to TGAL. C57BL/10 idiotypic determinants are seen on a wide variety of anti-TGAL antibodies (238). Interestingly, a similar type of one way cross-reactivity seen in this work with the B10.BR and AKR responses is also seen in the TGAL system. C3H.SW mice produce anti-TGAL antibodies inhibitable with anti-B10 idiotype, yet the C3H.SW idiotype is only found in C3H.SW sera (240). This is explained if the B10 idiotypic repertoire is much larger than and includes the C3H.SW repertoire. Alternatively, the B10 mice may not be making the C3H.SW idiotype at the stage of the response at which they were tested, but are capable of expressing those determinants at an earlier or later stage.

Some examples of idiotypic maturation are evident in the data presented in Chapters 3 and 4. Other systems where this phenomenon may occur are NP (88), ARS (281), and HEL (208). The HEL system (208) shows an increase in the frequency of IdX over time, whereas the ARS system (281) and the NP system (88) show that the predominant idiotype is expressed more poorly as the response progresses. An interesting correlation between IgM and CRI expression at the level of the precursor B cell has been shown. IgM PFC remain associated with the CRI but IgG PFC undergo a substantial decrease in the frequency of CRI expression. This disappearance of CRI correlates with an up to 10 fold increase in ARS affinity, showing that the idiotype maturation of IgG PFC may be affinity driven (281). If idiotype

maturation exists, one would expect to see an increase in detectable idiotype frequency over time since the anti-idiotype is directed to tertiary determinants. Such examples are seen in the AKR idiotypic and in the B10.BR cross-reactive RF idiotypic responses (Table IV). The A/J idiotype appears to change with respect to all reagents. Differences in this maturation between the two experiments may be due to differences in presentation of the two different Fd preparations, promoting different regulatory circuits, or to a large A/J anti-Fd repertoire and many regulatory methods so that no two groups of A/J mice respond alike. The last possibility appears unlikely based on observations with other antigenic systems.

The best explanation for idiotypic maturation is that it is affinity driven (62, 281). This explanation assumes that idiotypic determinants are very closely related to paratopic determinants. Many publications show that this assumption is not always correct (165, 208, 244, 279, 280). No satisfactory explanation accounts for all cases of idiotypic maturation, including those observed in this work. Probably many mechanisms including affinity, ease of genetic rearrangement and mutation, and T cell regulation, and auto-anti-idiotypic regulation may account for these phenomena.

In summary, the data of this chapter show that extensive interstrain idiotypic cross-reactivity exists in the murine anti-Fd response. No correlation between known genetic markers and idiotypic pattern is obvious, but H-2 and non-H-2 genes seem to play a role in influencing the expression of at least the B10.BR idiotype. Several cases of change in idiotypes expressed over time have been observed. Not all of the possible anti-Fd idiotypes can be defined by the four anti-idiotypes used. A thorough

discussion of the concepts of idiotypy and cross-reactivity is needed. Such topics will be discussed in a later chapter.

Chapter 5. Idiotypic Characterization of Monoclonal

Anti-Ferredoxin Antibodies

1.) Results

A)   Specificity of Monoclonal Anti-Fd Antibodies

Monoclonal antibodies were produced from hybridomas resulting from a fusion of anti-Fd hyperimmune splenocytes from C57BR mice, and NS-1 as described in Methods. The splenocytes of the mice were pooled for the fusions. One of the mice was a high producer of A/J idiotype, whereas the other was a low producer (data not shown). It was hoped that the monoclonal antibodies produced would reflect the immune response of the donor mice.

Six cell lines were isolated from 42 anti-Fd positive fusion products by virtue of their specific reactivity for Fd over KLH. Since 11 Fd-binding cell lines previously isolated showed poor specifity of antigen binding, it was necessary to establish the specificity of these monoclonals. A comparison between Fd and KLH binding in the ELISA assay is shown in Figure 24. One can see the very high specificity of the monoclonal antibodies for Fd, although the activity of H21 is quite low. Since this hybridoma proved difficult to clone, it was not used for further experiments.

B)   Determinant Characterization of Monoclonal Anti-Fd Antibodies

The % N-determinant and % C-determinant characterizations were performed as previously described (250). The inhibition percentages were calculated as follows, where 0% control is the uninhibited serum binding:

Figure 24 : <u>Specificity of C57BR Anti-Fd</u>

<u>Monoclonal Antibodies</u>

Monoclonal antibody supernatants were tested for binding to Fd (▲) or KLH (●). Standard errors were less than 10 percent. Hybridomas are designated by numbers 1 to 34, and utilized at the indicated dilutions.
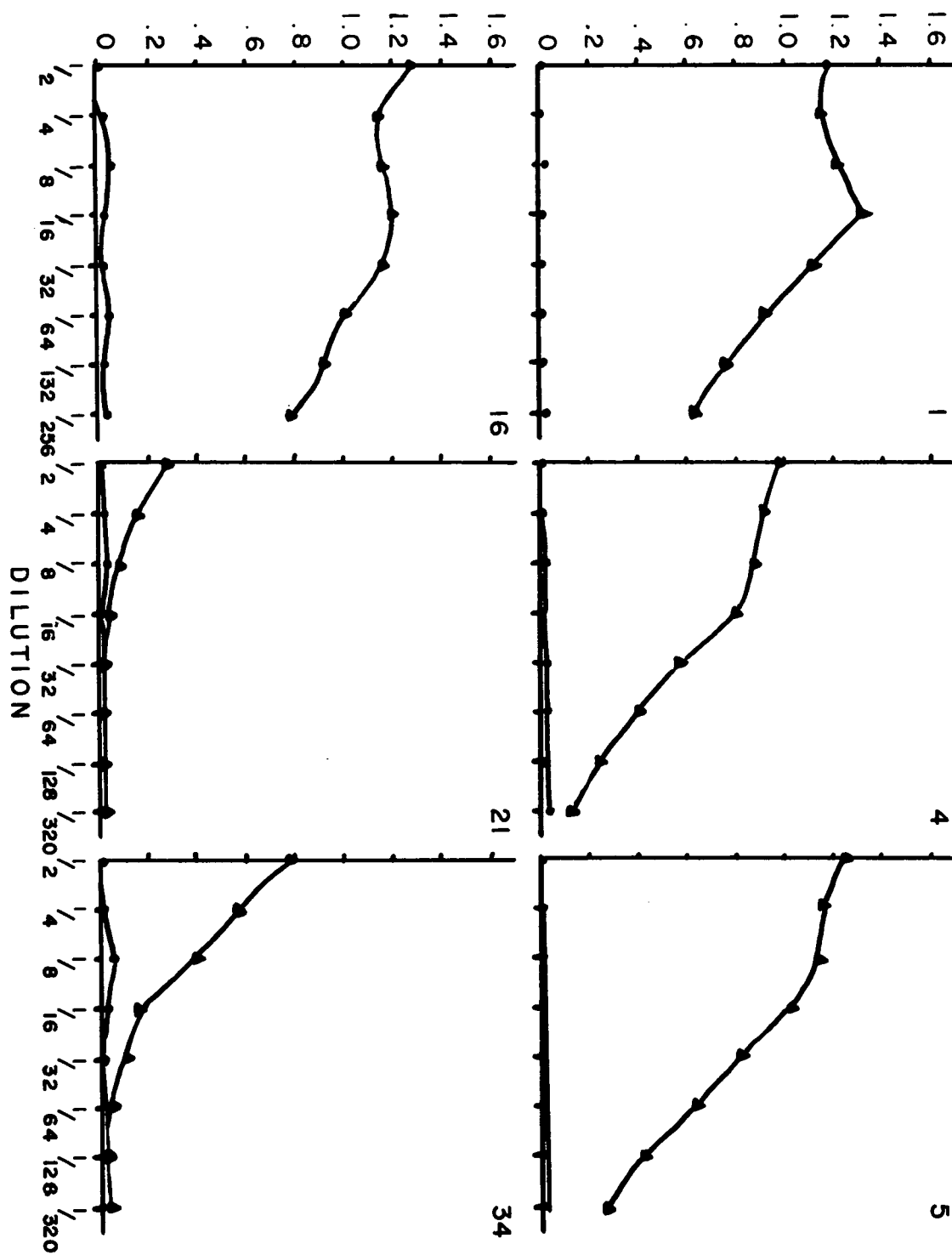
$$\% \text{ I} = 1 - \frac{(N \text{ or } C) \text{ inhib}^n - Fd \text{ inhib}^n}{0\% \text{ control} - Fd \text{ inhib}^n} \times 100\%$$

The results of these experiments are shown in Figure 25. One can see that all of the C57BR monoclonals are inhibited much more with the C-determinant fragment, except for Fd-1, which is known to be N-specific (250), and H4. This antibody probably recognizes a conformational determinant that is destroyed in the enzymatic digestion of Fd required to produce the N or C- determinants. The C57BR monoclonals isolated follow the characteristics of H-2^k mouse sera in that most of them are directed to the C-determinant. Consequently, one may expect their idiotypic profile to represent some of the serum patterns.

C)   Idiotypic Characterization of Monoclonal Anti-Ferredoxin Antibodies

Five C57BR monoclonal antibodies, Fd-1 (from cells donated by Dr. M. Weaver) and R10 and R32 (donated by R. Singhai) were tested in the inhibition assay. The results are shown in Figure 26. Note that Fd-1, H1, and H5 do not react with any of the anti-idiotypes. Though Fd-1 has been proposed as a major idiotype in the anti-Fd response of B10.BR mice (252) and is present in over 30% of the anti-N response, its net amount would be at best only 5% of the total anti-Fd response. Thus, it is probably present in such low amounts in B10.BR anti-Fd sera that it would fail to immunize a rabbit. This observation is in agreement with the results of Chapter 4 in which congenic mice which produced a high anti-C directed response appeared to be more inhibitable with anti-B10.BR idiotype. The H1 and H5 monoclonals, though C-determinant specific, bear idiotypic determinants that do not cross-react with any of the four test strains.

The interesting results can be observed from the data of H16 and H34. These two monoclonals are very strongly inhibited by A/J anti-idiotype.

Figure 25:

Determinant Specificity of

C57BR Anti-Fd Monoclonal Antibodies

A 1:10 dilution of hybridoma supernatant was incubated with a fragment of Fd bearing either the C or N determinant. The percent inhibition of Fd binding is represented by the vertical axis. The standard errors are indicated by the vertical bars.
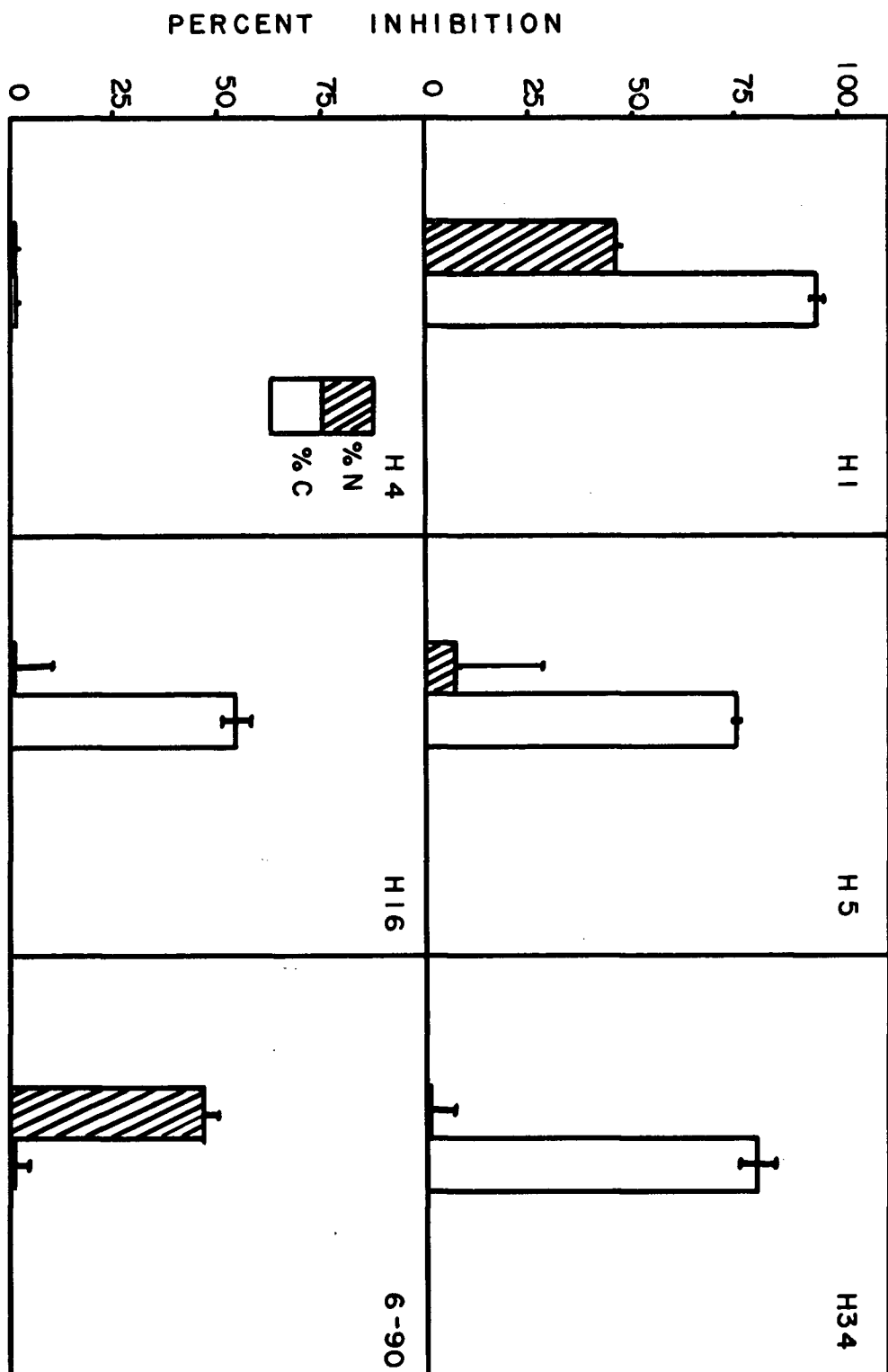
Figure 26: Inhibition of Monoclonal Anti-Fd

Antibodies with Various Anti-idiotypes

A dilution of monoclonal antibody corresponding to 0.5 A_{450}/hour was incubated with anti-idiotype and tested for inhibition of binding to Fd. Each panel indicates a separate monoclonal antibody and four anti-idiotypes, with the vertical axis representing percent inhibition. The standard errors are as indicated by the vertical bars.
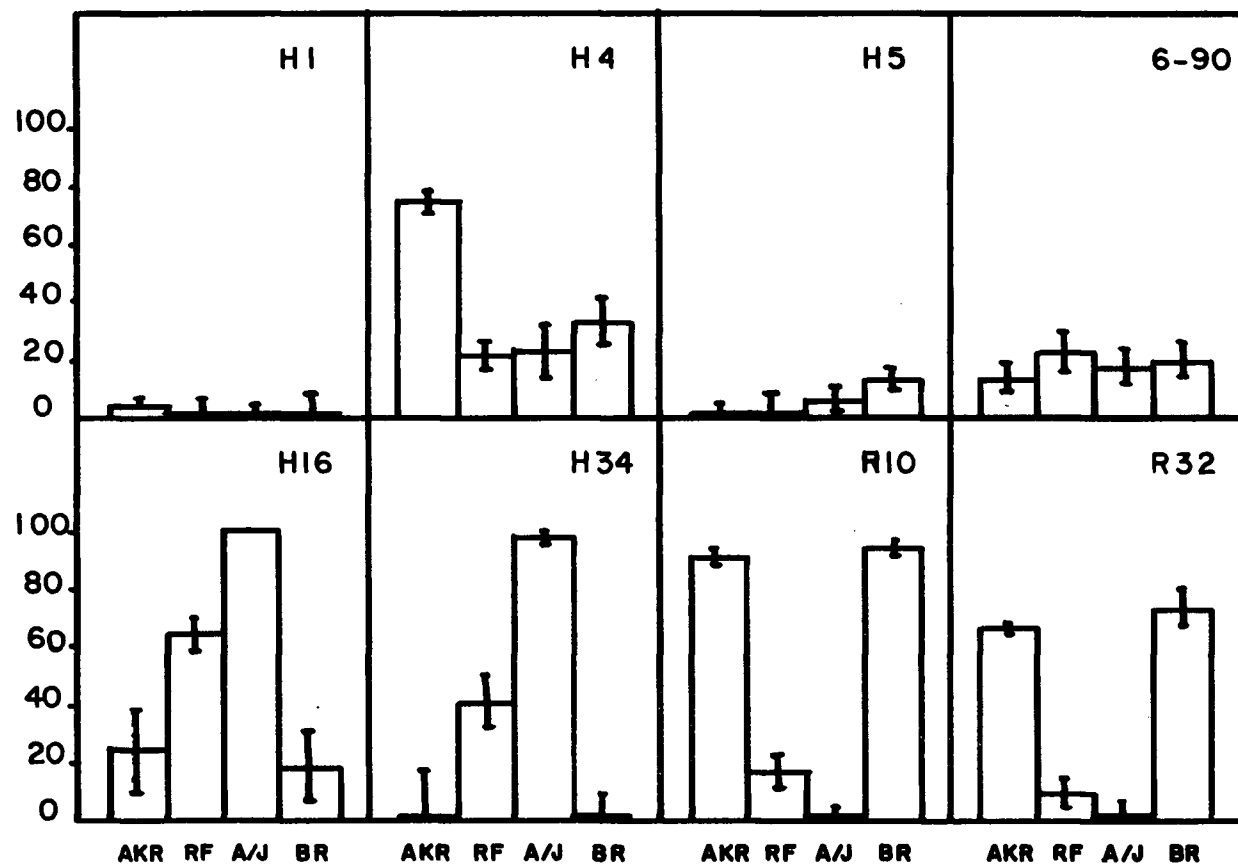
Such a situation is observed with individual C57BR anti-Fd sera (Chapter 4). In addition, R10 and R32 are strongly inhibited with both anti-AKR and anti-B10.BR anti-idiotypes, again reflecting characteristics seen commonly in individual B10.BR anti-Fd sera. H4 is inhibited by anti-AKR anti-idiotype, again, an idiotype occasionally expressed in the C57BR serum response. Thus, three of five monoclonals bear idiotypic determinants observed in the C57BR serum anti-Fd response. Two B10.BR monoclonals also show characteristics of individual parental sera.

Another very important conclusion can be gained from H16, H34, R10 and R32. These monoclonals are inhibited by more than one anti-idiotype. This observation confirms the data from Chapter 4, in which it was postulated that major cross-reactive idiotypic determinants of different strains were present on the same molecule.

Therefore, the use of monoclonal antibodies has strongly coroborrated earlier observations. The same phenomena seen with the serum response are observed at the cellular, indeed molecular, level. Major cross-reactive idiotypic determinants are shared between different strains and several of these determinants can be present on the same molecule.

II. Discussion

The preceding two chapters have dealt with the expression of idiotypes on serum immunoglobulins. These observations may be quite different from these at the level of the B cell (41). If the B cells producing a certain idiotype are relatively few in number, compared to a large suppressed population, one would not expect to see serum patterns produced from a small number of antigen binding B cells. In fact, 3 of 5 C57BR, and 2 of 3

B10.BR hybridomas exhibit the same cross-reactive patterns as serum antibodies. These data indicate that a few dominant clones may determine the serum idiotype pattern in these strains. The data from the monoclonals also indicates the universality of some idiotypes. Since both A/J and RF/J determinants are found on one C57BR hybrid, and B10.BR and AKR determinants are found on B10.BR hybrids, there must be shared major idiotypes in these strains. As well, the monoclonals serve as good controls for the specificity of the anti-idiotypic reagents. Figure 26 shows almost no inhibition of H1 and H5 by any anti-idiotype. Since monoclonal antibodies consist of only one specific immunoglobulin (by definition) one would expect any non-specific RaMIg effect to seriously impair the monoclonal binding.

Several other systems have first been defined by serum idiotypes and then have been further characterized with monoclonals. This approach has been taken in at least the ARS (15), HEL (207), NP (282), and TGAL (240) systems. These monoclonal antibodies have been very useful probes in determining the structure of idiotypic determinants, and the regulation of idiotype expression.

Chapter 6. Two-Dimensional Electrophoretic Analysis

of Anti-Ferredoxin Antibodies

1.) <u>Results</u>

Although the serological data strongly indicated the presence of multiple idiotypes in the mouse anti-Fd response, some biochemical evidence was desireable. Several ways to examine this problem were available. Since immunoglobulin heavy and light chains have relatively constant molecular weights, the only way to resolve the different chains would be to use isoelectric focussing (IEF). Standard flatbed IEF has been used in many situations to examine immuloglobulin heterogeneity (52, 115, 128). Such a situation usually provides quite complex data because the IEF pattern is influenced by variation in the heavy, light and carbohydrate chain residues, producing many bands, even with monoclonal antibodies (252). Analysis of immunoglobulins by two dimensional gel electrophoresis (2-D) represents the best resolution and sensitivity. The advantage of this method would be the ability to compare the light or heavy chains of different samples to see if any identity existed, possibly indicating a structural basis for idiotypic cross-reactivity. The original technique was to purify small amounts of individual sera by immunoabsorption, digest the charged carbohydrate with neuraminidase and reduce, denature, electrophorese and detect the proteins with a sensitive, silver based protein stain.

Though neuraminidase digestion improved the charge smear of the heavy chains (data not shown), satisfactory resolution was still not achieved. Therefore, light chains, though probably not representing major idiotypic

specificities, were the only realistic biochemical reflection of diversity. Possible improvements to this technique will be discussed later.

Figure 27 shows the 2-D pattern of 1.0 ul of normal mouse serum. Obviously, some kind of purification is vital to analyze the immunoglobulins. In Figure 27, the vertical dark streaks represent staining artifacts.

Figure 28 shows the 2-D staining pattern of minicolumn purified Fd-1 monoclonal anti-Fd antibody. The charge heterogeneity of the heavy chain is observed at the top label. As well, multiple spots can be seen representing light chain heterogeneity. Multiple spots can also be observed in the 2-D analysis of other anti-Fd monoclonals (data not shown). Although deamidation or carbamylation could account for the different charges, no completely consistent explanation has yet been offered (265). The extra heterogeneity of such monoclonal light chains may make interpretation of the actual number of different light chains difficult. Figures 29, 30 and 31 show the patterns of immunopurified immunoglobulin chains from 50 ul of pooled anti-Fd antisera. The label indicates the heavy and light chains. Other spots indicate contaminants or standard protein carbamylation trains. The results for the RF/J serum pool (data not shown) is very similar to that of AKR/J. These results indicate that many light chains are expressed in the anti-Fd responses of all four strains, even accounting for charge heterogeneity of single molecules. No obvious spots were shared between strains, though quantitative analysis would be better performed on an ISO-DALT system (265).

Analysis of individual sera generally yielded poor detection of light chains, possibly due to a diverse variety of light chains with poor protein representation of each. Figure 32 shows the best example of light chain

Figure 27   Two Dimensional Gel

   Electrophoresis of Normal Mouse Serum

1.0 ul of normal mouse serum was analyzed by two-dimensional electrophoresis and silver staining for proteins. The ampholines were from pH 3-10 and the slab gel was 12% acrylamide.

10 ————————————————▶ 3 pH
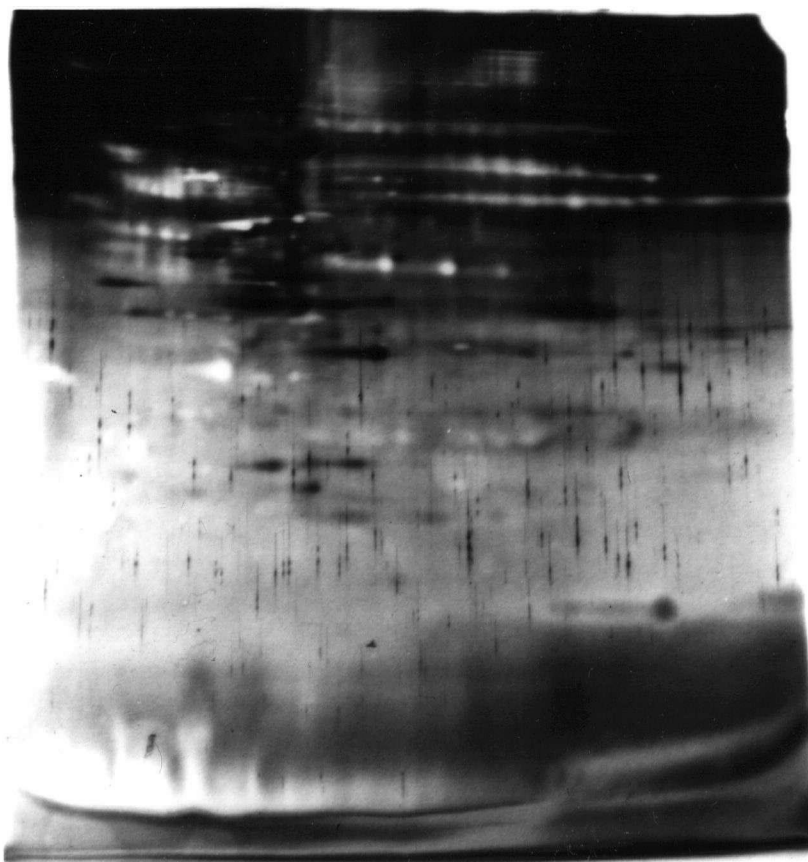
normal mouse serum

Figure 28. Two-dimensional Gel Electro-
phoresis of Monoclonal Anti-Fd Antibody
(Fd-1)

10 ul of Fd-1 was purified on a
Fd-Sepharose minicolumn, denatured and
reduced, resolved by IEF with pH 3-10
ampholines, separated into components on a
12% acrylamide slab gel and the proteins were
detected by silver staining.

3   10   pH

H chain

L chain
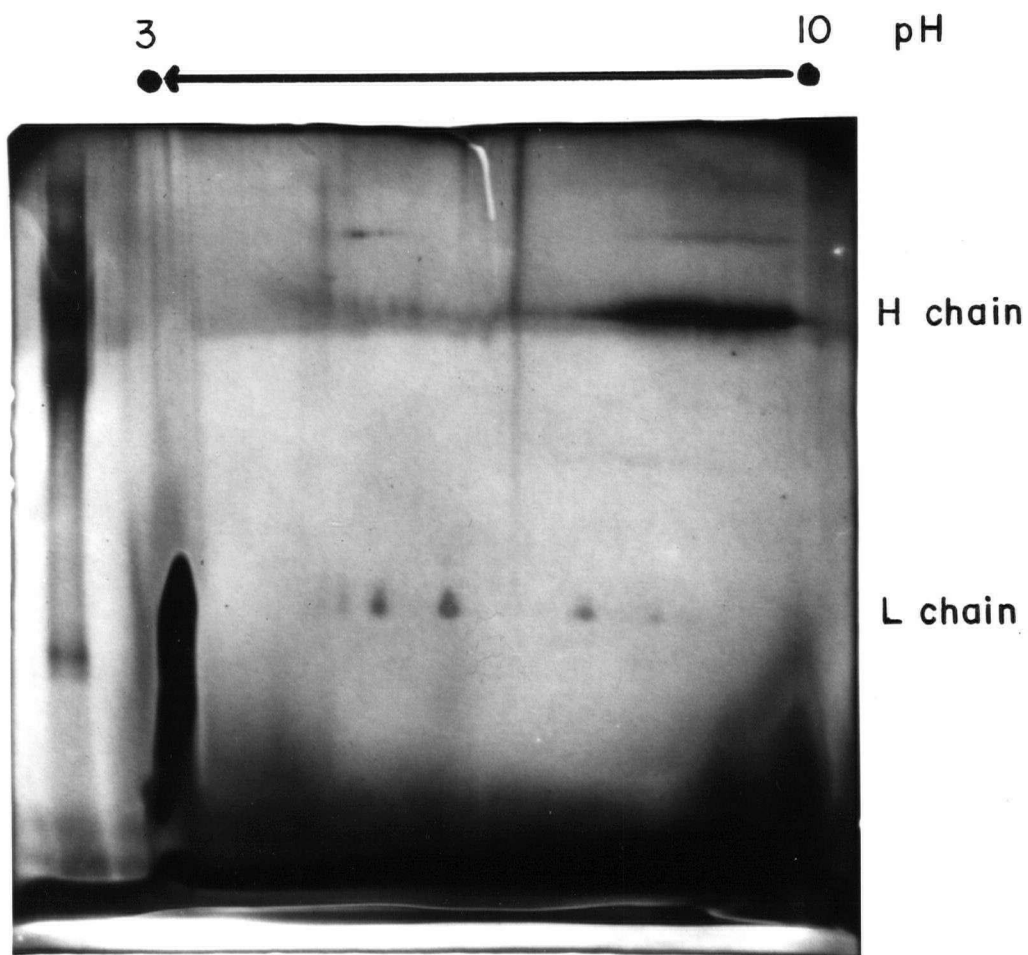
monoclonal anti - Fd (Fd-1)

Figure 29.   Two-Dimensional   Gel
Electrophoresis of Pooled Anti-Fd Antibodies
from AKR/J Mice

50 ul of anti-Fd antisera pooled from 15 AKR
mice   was   purified   on   a   Fd-Sepharose
minicolumn, denatured and reduced, resolved
by IEF with pH 3-10 ampholines, separated
into components on a 12% acrylamide slab gel
and the proteins were detected by silver
staining.
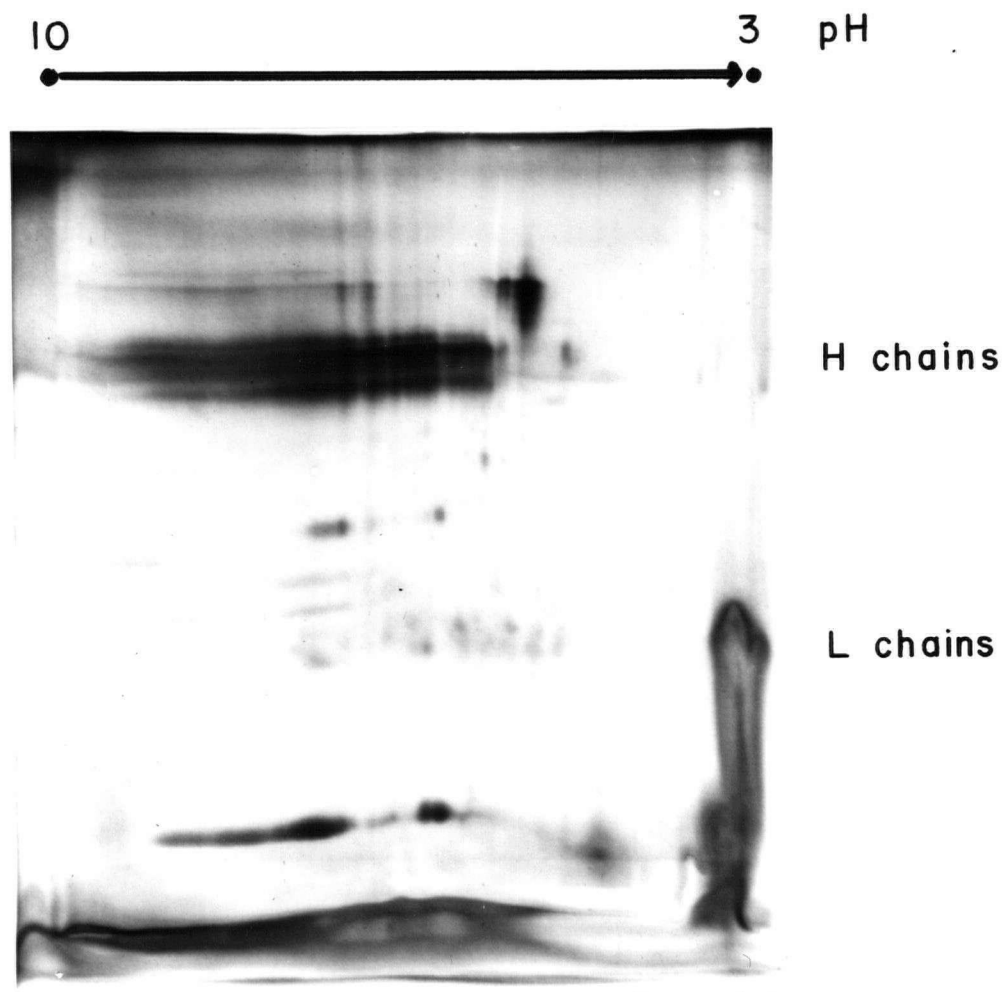
AKR anti — Fd pool

Figure 30.   Two   Dimensional   Gel
Electrophoresis of Pooled Anti-Fd Antibodies
from A/J Mice

50 ul of anti-Fd antisera pooled from 15 A/J
mice   was   purified   on   a   Fd-Sepharose
minicolumn, denatured and reduced, resolved
by IEF with pH3-10 ampholines, separated into
components on a 12% acrylamide slab gel and
the   proteins   were   detected   by   silver
staining.

3   10 pH

H chains

L chains
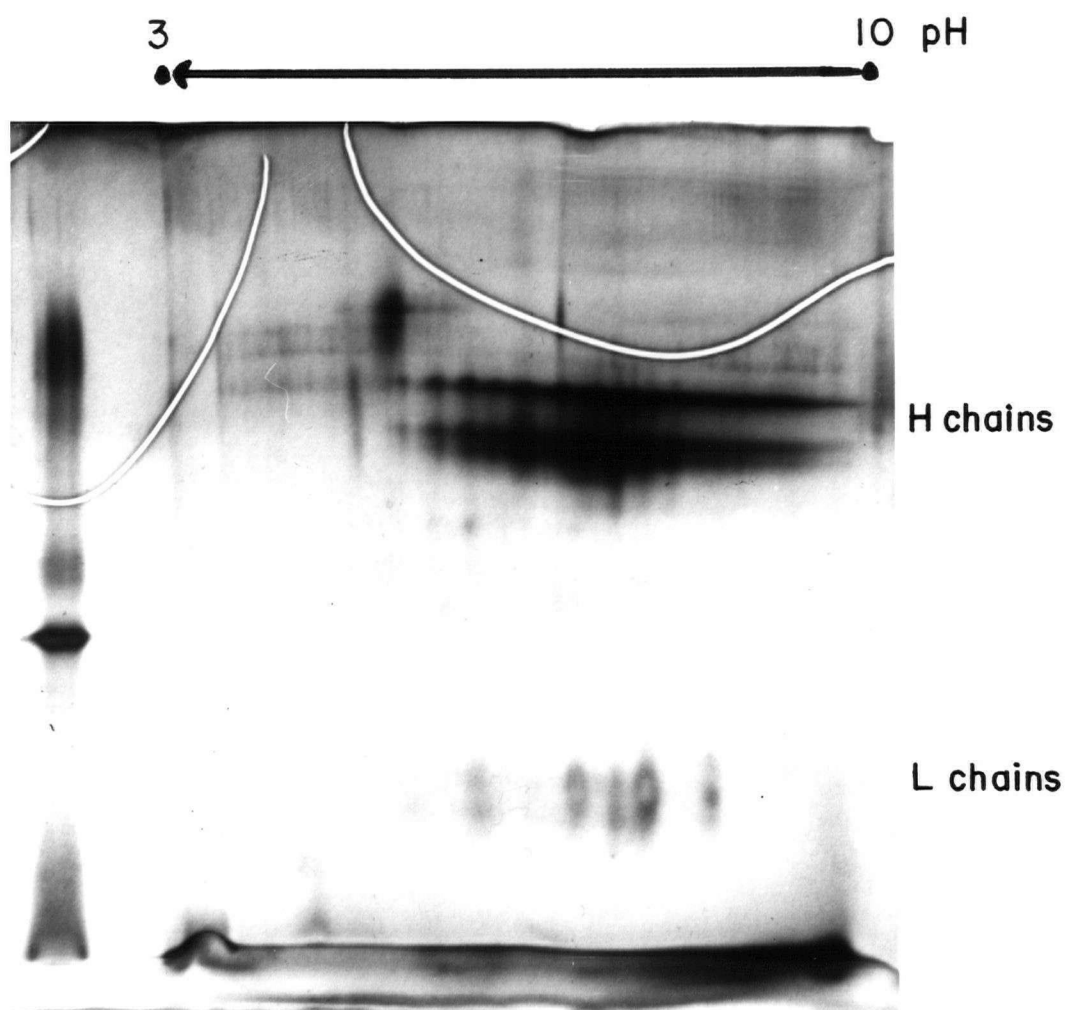
A/J anti-Fd pool

Figure 31.   Two   Dimensional   Gel
Electrophoresis of Pooled Anti-Fd Antibodies
from B10.BR/SnJ Mice

50 ul of anti-Fd antisera pooled from 15
B10.BR mice was purified on a Fd-Sepharose
minicolumn, reduced and denatured, resolved
by IEF with pH 3-10 ampholines, separated
into components on a 12% acrylamide slab gel
and the proteins were detected by silver
staining.

3   10   pH

H chains

L chains
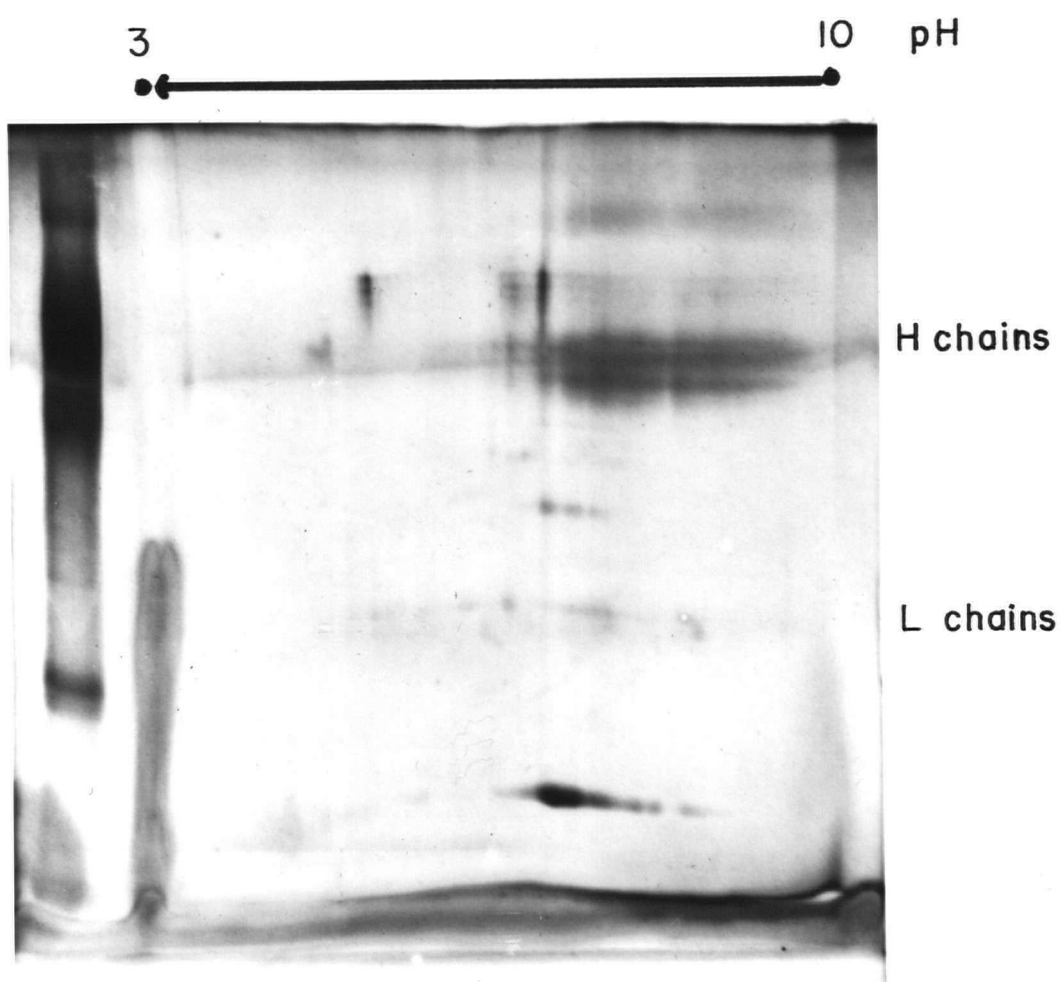
BIO.BR anti — Fd pool

Figure 32. Representative Two-Dimensional Gel
Electrophoresis of an Individual B10.BR
Anti-Fd Serum

20 ul of anti-Fd antiserum from a single
B10.BR animal producing a high level of both
B10.BR and AKR idiotypes was purified on
Fd-Sepharose. The eluted antibody was
denatured and reduced, resolved by IEF with
pH 3-10 ampholines, separated into components
on a 12% acrylamide slab gel and the proteins
were detected by silver staining.
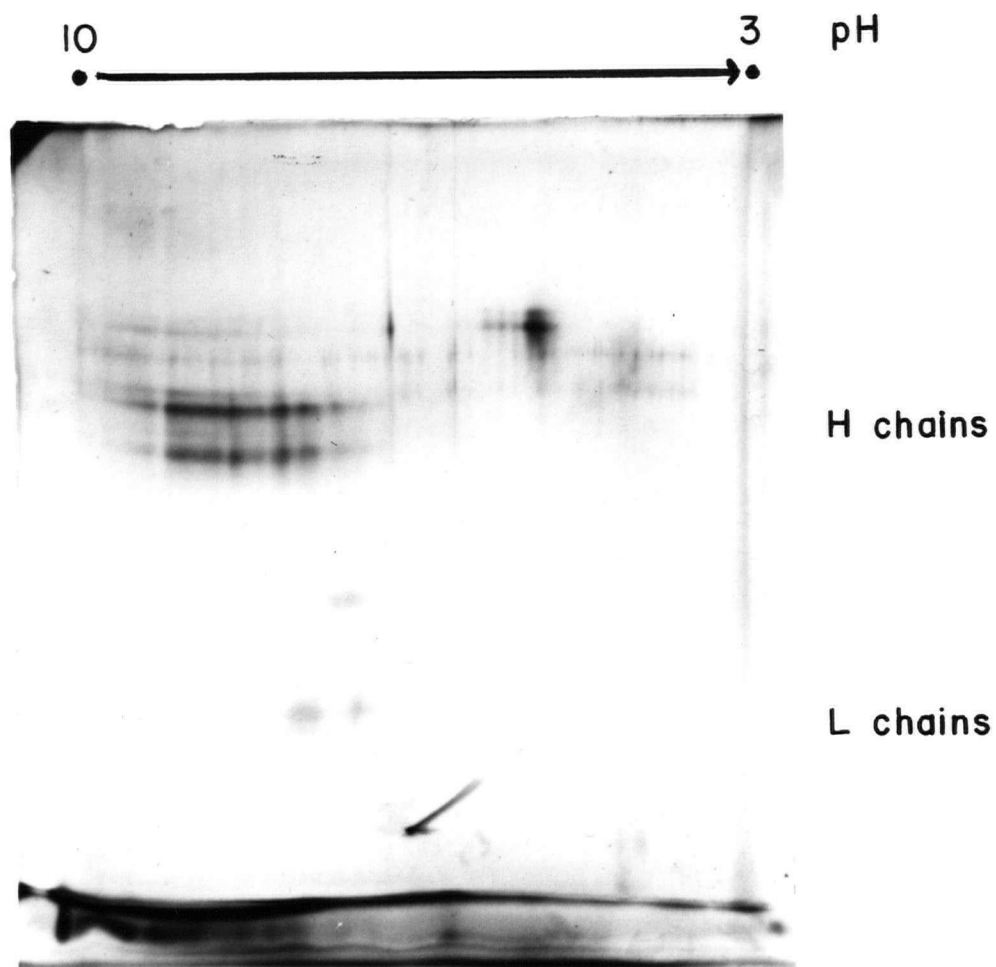
BIO. BR anti—Fd individual serum

restriction in an individual B10.BR serum, strongly cross-reactive with AKR. Again, several light chain spots can be observed.

Thus, the results show that several dominant light chains exist in the anti-Fd response of all inbred strains tested. No obvious identity between light chains of different strains could be observed.

II. Discussion

Since these mice produce such small amounts of specific antibodies in their serum, detection of all light chains being manufactured is impossible by these procedures. The results show that several immunodominant light chains are produced, averaging to at least about 2 to 3 light chains per mouse, as observed. This number is only a fraction of the number of light chains involved in Fd binding, and certainly does not reflect all of the variability observed in the serological data, and as such must only be considered an indication that more than one light chains are involved in the anti-Fd response in these strains of mice.

The 2-D method of analysis is very useful but further work must go into it to properly investigate the cross-reactivity of individual sera. It would be very hard to improve the sensitivity of this system by direct staining. If 20 ug/ml of anti-Fd antibody is produced by high responder mice (251) and 50 ul of serum is absorbed, then about 1 ug of immunoglobulin is applied to the gel, of which stainable light chain represents about one-third (by molecular weight). If 15 spots are visualized, then the stain is staining about 20 ng! This number represents a conservative level of sensitivity. Aside from using large samples (which defeats the purpose of the microassay), very few options are available.

Direct iodination of the antibody on the Fd immunoadsorbent was done

and worked satisfactorily (data not shown) but the 2-D pattern differed from the uniodinated pattern. This may be due to Chloramine T oxidation or the pKa change of the tyrosine phenolic group upon iodination (283). Thus, this method will not be useful. As well, the light chains label poorly since they appear to be protected by the immunoadsorbent (data not shown).

Unfortunately, the best method of increasing sensitivity is by blotting onto nitrocellulose and detecting with immunoperoxidase (284) or iodinated antibody (285). This would be extremely time consuming. Possibly it would be less complex to directly analyze the light chains by IEF (286), followed by blotting and detection (284, 285). Though detection of small amounts of protein on flatbed IEF by Sammon's silver staining is almost non-existent (data not shown), blotting of the light chains onto nitrocellulose would allow very sensitive detection (284).

Neuraminidase was not found to be consistently effective at removing charged carbohydrate from immunoglobulin (data not shown). As well, most neuraminidase preparations contain proteases. A better method may be incubation with Endoglycosidase H (per. comm. Dr. O. Witte, UCLA).

This enzyme cleaves the carbohydrate side chain distal to the first N-acetyl-glucosamine residue (287). Theoretically, this enzyme should totally eliminate the sialic acid charge heterogeneity (288) however, problems have been encountered at cleaving charged side chains (287). Perhaps a change in reaction conditions may circumvent this problem.

In conclusion, the results of Chapter 6 show that at least 3 different light chains are expressed in individual mice responding to Fd (assuming the usual 2-3 spots per light chain, and not the worse case scenario observed from Figure 28).

These light chains vary from mouse to mouse and do not appear to determine idiotypic expression, though the superior reproducibility of an ISO-DALT system would be essential for truly quantitative comparisons. Better detection and the resolution afforded by pure IEF separations are necessary for accurate quantitation of total anti-Fd light chain expression. Nonetheless, the results of chapter 6 are valuable in demonstrating that many light chains are produced in the mouse anti-Fd response, and this biochemical evidence correlates quite nicely with the serological data.

Chapter 7. Implications of Results and Further Experiments

1. Thesis Discussion

The work discussed in the preceding chapters was carried out to define a new protein idiotypic system. Several important questions must be addressed before this work is complete. In this chapter four important topics will be discussed, followed by a brief summary of the work of this thesis, and several suggested experiments.

A)   The T Cell Receptor

Even though it is not a major theme of this thesis, it would be appropriate at this time to briefly mention the T cell receptor.

The relationship between T and B cell idiotypes has been extensively studied (28, 75, 134, 222) and much evidence exists for serological cross-reactivity. However, much data exists to demonstrate that T cells do not express immunoglobulin genes (80, 180, 181). How then do anti-B cell idiotypes recognize T cells? At least two possibilities exist. T and B cell receptors may be different, but if they recognize the same epitope, they may bind the anti-idiotype if it has residues that are structurally similar to the recognized epitope. This hypothesis will be discussed in more detail later. Another possibility is that the T cell receptor and immunoglobulin (the B cell receptor) are separate but similar proteins. Thus they may share idiotypes by virtue of similar protein structure. Some experimental evidence may support this hypothesis. Monoclonal antibodies specific for certain T cell lines or hybridomas can be used to purify molecules consisting of two disulphide-linked glycoproteins of 37,000 - 50,000 molecular weight (289, 290) which may consist of variable and constant regions (319, 320). Molecular geneticists have isolated cDNA

from mouse mRNA that hybridizes to genomic regions that are rearranged in T cells and not B cells, and vary for different antigen specificities, suggesting that the nucleic acid sequence coding for one chain of the T cell receptor has been isolated (321). Examination of the nucleic acid sequences of this putative receptor chain gene indicates that it shows significant homology to immunoglobulin heavy chains with a distinct arrangement into constant and variable regions (322). Furthermore, a Canadian group has cloned and sequenced a human mRNA specific for mammalian T cells. The protein from this cDNA is estimated to have a molecular weight of about 35,000 and shows extensive homology to immunoglobulin light chains, including the positions of the relevant cysteine residues (323). These results are very supportive of a close structural similarity of B and T cell receptors, which may explain data indicating such idiotypic cross-reactivity.

B)   Who Constitutes Idiotype?

As mentioned before (1, 2), idiotype has been defined as the antigenic specificities of antibodies produced by an individual or group of individuals in response to a given antigen. This description was modified to define idiotype as the set of epitopes (or antigenic determinants) displayed by the variable regions of a set of antibody molecules, in a paper proposing the formal Network Theory of the regulation of the immune response (3). In this theory, individual antigenic determinants on the variable region of an antibody were classified as paratopes, when they were associated with the antigen binding site and idiotopes when they were outside it. Regulation occurred by events associated with the recognition of idiotopes by paratopes of other antibody molecules. These interactions could result in changes in the recognizing and recognized populations.

Importantly, distinction was made between the paratopes and the idiotopes. Since a paratope from the antibody specific for an antigen could recognize idiotopes of other antibodies, an "internal image" of the antigens was available within the immune system itself. The presence of exogenous antigen thus changed the dynamic equilibrium of the network, resulting in the phenomena of the immune response to that antigen. Unfortunately, no real description of the physical properties of either idiotypes, or paratopes was offered. Only one paratope was proposed per Fab arm. The theory was very elegant, and some very supportive evidence existed. Idiotypes were serologically defineable (1, 2) but were not always associated with an antigen binding specificity (291). Unfortunately, the distinction between paratopic and idiotopic determinants became somewhat blurred by the finding of multi-specificity of antibodies. Hybrids of antibodies to two different haptens could bind both at the same time but in different locations (292). True multi-specific antibodies were found that could bind two separate haptens (293, 294). When fluorescence quenching techniques were used by some investigators, as much as 1.4 nm existed between the binding sites (294). When rabbits were immunized with an antigen X, a small percentage bound an unrelated hapten Y. When the animals were boosted with Y, the frequency of distinct antibodies binding both X and Y rose by 80 times (295). As well, crystallization data from hapten binding myeloma proteins showed a relatively huge cleft binding a tiny hapten (296). It is not conceptually difficult to imagine a large protein being able to bind two different ligands -- this is observed frequently with enzymes. A rational modification for interpreting antibody interactions was proposed. The entire variable region of the antibody could serve as a "sticky end" with no distinction between recognizing and

and being recognized (297). Thus, no real distinction would be made concerning paratopes and idiotopes, which would exist only as defined by the particular system of interest. Such a definition would explain why paratopes and idiotopes need not be associated (165, 194, 208, 244, 279, 280, 298) as well as why they could be associated (15, 92, 299, this work). Accordingly, the formal Network Theory was modified to define the paratactic epitope as a binding region, and the paratactic idiotope as a bound region of antibody, which would be only defined in a particular system as a convenient reference (300). As well, a possible idiotope could consist of a cleft or protuberance conformationally, or an amino acid configuration chemically (300).

Much work has been done attempting to define the structure of the idiotopes for idiotypes of different antibodies. Such attempts have been reviewed here earlier, and elsewhere (64, 98, 301). Only the amino acid correlates of very simple idiotopes, from very simple systems have been defined (112, 116, 302). As well, direct observation has localized idiotopes to the amino terminus of the Fab fragment (303). By definition, idiotopes are as complex as protein epitopes, the structures of which are currently being revealed. As new, high power, computer assisted X-ray diffraction techniques are used, and data accumulated, idiotypic determinants will be defined chemically.

C) What Constitutes Cross-reactivity and Specificity?

In order for bonding to take place between an epitope and an antibody an appropriate complementarity must exist. As with enzyme binding, ionic, hydrophobic, and polar bonding forces can all be utilized to produce a high

binding constant for the epitope (304). The spatial arrangement of the hapten, as well as the appropriate complementary functional groups of the hapten and antibody determine binding. These groups may be continuous or may be brought together by the conformation of the molecules (305). Thus, molecules of quite different primary structure may cross react by virtue of conformational determinants, as observed for morphine and enkephalins (306). As cross-reactivity of antibodies to different haptens by virtue of recognition at the same site or multi-hapten binding at different sites is well recognized (292, 293, 294), one would postulate that multi-idiotypic cross-reactivity would be possible. An example of this is seen in the T15 system (86).

The concept of cross-reactivity has been elegantly explored (307). "True" cross-reactivity may be defined as competition of two determinants at the same site, with different binding constants. Thus, one hapten may completely inhibit the other from binding. The second type, or "shared reactivity" is a reactivity to different parts of a protein. Thus, with heterologous sera, the different populations could not all be inhibited with a fragment of the protein or another protein with some shared determinants (307). True cross-reactivity would be observed with monoclonals; shared with heterologous sera.

If one postulated that all antibodies are multi-specific, but that only a very few will cross-react with any other antigen (307) at one time, then even though all of the antibodies are individually multi-specific, the serum appears specific. Such a situation is probably common with an anti-protein response.

Cross-reactivity will be discussed with respect to several mechanisms. A small amount of cross-reactivity between some idiotypes may exist by virtue of the multi-specificity of antibodies (307), since some antibody in the system will bind any antigen. This will be compounded by assuming a "sticky end" model of antibody binding (297) but should still only account for a small percentage of the cross-reactivity with an anti-idiotype representing idiotype of another strain.

An alternative explanation of idiotypic cross-reactivity invokes the "internal image" model (300). In this model, cross-reactivity exists because the anti-idiotype binds to the paratope of the idiotype bearing immunoglobulin, thus simulating the antigen epitope. One may be inclined to reject this hypothesis at first glance, expecting all specific antigen binding specificities to be inhibited by an anti-idiotype, and, indeed, this would be expected in simple hapten systems. The response to proteins, however, is usually complex, with many determinants involved (150, 195). If the response is directed to many epitopes, and the anti-idiotype is the "internal image" of only a few, dominant epitopes, one would expect that the individual sera would be inhibited in a manner reflecting their anti-epitopic content. Thus, varying degrees of cross-reactivity would be expected universally among all sera directed against the antigen. This result would be consistent with observations from the GAT (153, 154), insulin (244), lysozyme (206) and Fd (this work) systems. If such a result is not seen with all strains (or species) one may postulate that though the anti-idiotypes reflect the "internal images" of the immunizing strain, the other strains respond to different epitopes. Indeed, this result has often been observed (reviewed in 147) but is usually correlated to the H-2

haplotype. Nonetheless, much more epitopic heterogeneity via topical determinants may exist, but would be very difficult to detect. Thus, the "internal image" theory has quite plausible grounds for support in a protein system, backed with some evidence (300). Such a theory explains very nicely the presence of B cell cross-reactive idiotypic determinants on T cells, in light of the previous lack of structural data.

This theory does not explain cross-reactive idiotypes that do not correlate with antigen specificity (194, 207, 244, 280) or genetically mapped idiotypes in the hapten systems (24, 66). In fact, it has been observed that the "internal image" model of idiotypic cross-reactivity is rarely valid, since, quite often immunization with anti-idiotype does not produce much antibody capable of binding antigen (308). This is thought to be as a result of the frequency of B cells bearing the original idiotypic determinants. If the original idiotype is determined by one or very few germline genes, immunization with anti-idiotype is likely to produce antigen binding, idiotype positive antibody (309), whereas if the idiotype is determined by many germline genes, or V_H V_L pairing, as in many protein systems, then anti-idiotype will cause the production of many antibodies that bear idiotypic determinants, but do not bind antigen (309).

Another mechanism to explain cross-reactive idiotypes may be by virtue of shared germline V genes. This mechanism has been shown to account for the major idiotypes in PC (66), ARS (24), dextran (113), NP (101), GAC (130) and Nase (220) systems. The Nase and GAT systems have shown the presence of multiple germline genes. The wide interstrain idiotypic sharing seen in other systems (153, 154, 206), and in this protein system may be due to the conservation of certain V genes across all strains of

mice, certainly not an unreasonable possibility considering that all inbred strains may have been derived from a more limited gene pool than originally thought (310, 311).

It is interesting to try to interpret the work of this thesis in the context of the various explanations for idiotypic cross-reactivity previously discussed. Predominant idiotypes exist in the AKR, RF, and B10.BR strains, but not all anti-Fd producing mice bear cross-reactive idiotypic antibodies in their antisera. Thus, if there is a genetic reason for the intra and interstrain cross-reactivities observed, its effect is not tremendously important in the response. It must be noted that the anti-idiotypes were prepared using tertiary antisera from 10-15 mice. One would expect most possible epitope specificities to be present. Genetic reasons for the idiotypic cross-reactivities observed would very nicely explain the results if many genes, or gene combinations, were capable of yielding anti-Fd antibodies. Thus, the resulting anti-idiotypes would recognize mainly a few of the dominant idiotypes. If each individual mouse expressed only a few members of the total set of possible idiotypes, and overlaps existed between strains, then the variability of idiotype expression and the intra and interstrain cross-reactivities, represented in Figure 27 would be explained. Some biochemical data exists to support the hypothesis of many possible idiotypes (see Chapter 6).

Interpretation of these results with the "internal image" model is more difficult. These cross-reactivities could be explained if the anti-idiotypic antibodies were functionally similar to antigen, and thus represented some of the dominant epitopes of Fd. The variation in cross-reactivity could be explained by microheterogeneity in terms of

epitope recognition by the various anti-Fd sera. Traditional evidence argues against this view. Careful molecular genetic evidence supports the genetic inheritance of idiotypy (24, 66, 130). Idiotypes can cross-react when associated with different antigen specificites (194, 207). As well, in this system, a pool of at least 10 mice should represent almost all of the possible epitopes recognized by the antibody response to Fd. In addition, the Fd molecule is very small, with most antibody directed to one determinant (or region location at the carboxy terminus) (251). With these facts one would expect to see greater cross-reactivity, if the anti-idiotype is simply mimicking antigen epitopes. In situations where such mimickry is investigated, very little seems to be found (308). In short, though it would be difficult to disprove entirely, epitope "disguise" by the anti-idiotypic antibodies probably does not play a major role in the observed cross-reactivities presented in these results. The "internal image" theory, however, appears to be a good explanation for the presence of B cell idiotypic determinants on T cells.

Thus, the genetic inheritance of V genes coding for idiotypic determinants seems to be the best explanation for cross-reactive idiotypes in the Fd system. Somatic mutation during the course of antibody maturation may give rise to high titre antibody recognized by the anti-tertiary (anti-Fd) idiotypes. Presumably, different regulatory mechanisms and many V genes could explain the variability in idiotype expression, though the "internal image" model cannot be ruled out, and may explain a proportion of the cross-reactivity observed.

D)   Idiotypic Regulation

The mechanisms by which antibody levels in vivo are regulated are largely unknown. The most accepted explanation at present was proposed by Jerne (3). Much data exists to support the formal Network Theory and has been reviewed extensively elsewhere (98, 297, 309, 312, 313). Obviously, some regulatory phenomena are occuring in mice injected with Fd. It is assumed that regulation occurs in a similar manner in other animals -- probably by a network of idiotypic interactions. These interactions are usually thought to involve T cells (133, 179), although B cells are capable of regulating antibody expression, perhaps by virture of the recognition of different D_H segments (314). It is not the aim of this thesis to explore the regulatory aspects of the Fd system, which is the subject of ongoing work by others. Instead, an exploratory investigation of expressed idiotypes in the Fd system was undertaken with the hope that a relatively restricted response existed. Such an idea was considered possible in light of the small size, and the determinant uniformity of the response in $H-2^k$ strains of mice. However, this was not the case. As has been shown, the idiotypic response to Fd appears complex, with some relatedness noticed between different strains of mice. This relatedness is probably due to conservation of germ line idiotypic markers, although the "internal image" model of cross-reactivity (300) cannot be ruled out. The reagents exhaustively purified, the many anti-Fd antisera collected, and the hybridomas produced for these experiments can be put to use, however, in the continued investigation of anti-Fd idiotypes in inbred mice.

II. Further Experiments

Many experiments need to be done to fully define the expression of idiotypes in the Fd system. These will be listed in order of priority or simplicity.

1) Although the data of this thesis indicate that many idiotypes are expressed in the Fd response, not much is known about their dominance. To try to determine this, columns of monoclonal antibodies bearing reactive idiotypic determinants to one or more anti-idiotypes should be used to absorb the anti-idiotypic reactivity. Very good evidence exists for the dominance of that idiotype if most of the anti-idiotype is absorbed.

2) If a monoclonal antibody possessing dominant idiotypic determinants is found, anti-idiotypic reagents to that monoclonal antibody should be prepared. This anti-idiotype could be used to re-screen the individual sera to estimate the importance of that idiotype.

3) With the anti-monoclonal idiotypes, cell mixing studies after idiotype or anti-idiotype treatment and reconstitution may define the importance of these idiotypes in regulation.

4) As well, _in vivo_ studies with idiotype or anti-idiotypic reagents should be done to characterize important regulatory characteristics.

5) As not all anti-Fd idiotypes can be defined with the four anti-idiotypes, perhaps another anti-idiotype to CBA or C3H anti-Fd antibodies should be made.

6) The four anti-idiotypic reagents could be injected into mice. If the mice began to produce Ab capable of binding Fd, good evidence for the role of the "internal image" model in idiotypic cross-reactivity may be obtained, as well as evidence for V gene restriction (309).

7) Western blots of IEF or 2-D separated antibodies and detection with labelled subclass specific antibodies would be useful to associate idiotypic cross-reactivity with antibody isotype.

8) As well, the use of more sensitive Western blotting, the ISO-DALT system (269) and modifications discussed in Chapter 6 may permit the identification of common H or L chains in individual sera displaying interstrain cross-reactive idiotypes.

9) Since the carboxyl terminus of Fd may exhibit some sequence homology to cytochrome c (315), and $H-2^k$ mice are high responders to cytochrome c it would be interesting to determine if anti-cytochrome c idiotypes cross-react with anti-Fd idiotype in, for example, the B10.BR strain. Such a result may imply the expression of similar germ line V genes.

10) If a very useful monoclonal idiotype probe can be found, then cV_H and cV_L probes can be readily prepared from the mRNA. These could be used to screen non-idiotype producing responders or non-responder strains to determine if the capability to react to Fd is there. For example, although evidence exists that non-responders in the Fd system can be induced to respond (255), the results of the ARS system show that the absence of the CRI germline gene determines the lack of CRI expression in mice (24).

11) More work is needed to define the idiotypic response of B10.D2 or DBA non-responders that, through regulatory manipulation, are capable of responding to ferredoxin.

12) A mixture of the four anti-idiotypes should be made, and used to inhibit sera that are not inhibitable over background with any single idiotype. The cumulative effect may inhibit well over background, showing that at least some of the idiotypes are cross-reactive.

The important question that was the motivation for all of the work of this thesis is still unanswered. Are idiotypes that are conserved in the anti-Fd response of different strains of mice conserved because they are important regulatory idiotypes? The background work has been done, now is the time to finally address this question.

III. Summary

The work of this thesis demonstrates the production of at least 3 major cross-reactive idiotypic families among the response of 3 strains of mice to ferredoxin. Whether these idiotypes are restricted or are repeated "individual" determinants cannot be completely defined at present, however, the evidence indicates that many idiotypes are expressed. Mice of 8 strains can express idiotypic determinants that cross-react outside their own strain. Both H-2 and non-H-2 genes appear to influence idiotypic expression, but no pattern is correlated with Igh or Igl allotype. Monoclonal antibodies specific for ferredoxin express idiotypic determinants capable of reacting with "foreign anti-idiotypes." Possible mechanisms for the intra and interstrain cross-reactivities have been discussed, and further experiments have been proposed. This work represents a study of idiotype expression in the Fd system, and was intended to provide a platform from which investigations into immune regulation at the cellular level could be launched.

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Appendix 1

I) Statistical Methods

Essentially, quite simple statistical tests were used to compare populations of idiotype producing mice in this thesis. Since the distributions of idiotype producing mice are not normal with respect to percent inhibition, the students t test must not be used (316). Consequently, a threshold level of inhibition was arbitrarily designated, and responder frequency was calculated. The X^2 test was used to compare these frequencies (317).

II) Threshold of Response

KLH immunized mice, representing 7 strains, were tested with the different anti-idiotypic reagents at the 2° day 7 period in their response. Since the inhibitions were quite low, and the mice were responding to a ferredoxin unrelated protein, these results were designated as background. The values for all mice with each anti-idiotype were averaged and the threshold point was set as

$$T = \bar{x}_{KLH} + 2 (s_{KLH})$$

where: T = threshold

$\bar{x}_{KLH}$ = Mean

s_{KLH} = Standard deviation

$\therefore T_{AKR}$ = 19.3%

T_{RF} = 19.7%

T_{A} = 17.1%

$T_{B10.BR}$ = 18.8%

Since these values were so similar they were assumed to represent the background level of inhibition. Thus, values of inhibition higher than threshold were regarded as positive for a specific inhibition.

III) Statistical Comparisons

X^2 contingency tables were prepared (per. comm. Dr. Siu Dept. of Community Health Sciences, University of Calgary) to test strain specificities. These X^2 values were calculated by establishing the total strain frequency over all sample times, and standardizing them with the 2° day 15 population for that strain. For example:

Observed frequency of AKR inhibition was 73.5% over all times. Standardized to the 2° day 15 population (11 mice), 8.09 mice were inhibited, 2.91 mice were not inhibited. This was compared to 2 of 31 positively inhibited anti-KLH mice. Thus, $X^2 = 20.0$ with $p \leq .005$ where:

$H_o : F_{AKR} = F_{KLH}$.

By this method the significance of interstrain cross-reactivity could be established.

The kinetics of the idiotypic response could be analyzed statistically to determine if the frequency of inhibition varied over time. Contingency tables were prepared, for example:

AKR Marked Individual Mice

	1°	2°	3°	Total
Positive	2	11	9	22
Negative	11	3	5	19
Total	13	14	14	41

df = 2 $X^2 = 11.8$ $p \leq .005$

Ho: $f_1 o = f_2 o = f_3 0$

Appendix 2.

Summary of Idiotypic Systems

Antigen	Idiotype	Igh	IgI	References
ARS	CRI$_c$	a	k	318
ARS	CRI	e	k	6, 21
PC	T15	a	k	51, 60
PC	C3	b,e,j	k	55
NP	NP-a	a	λ	90
NP	NP-b	b	λ	88
NP	B1-8	b	λ	97
Dextran	J558	a	λ	113
Dextran	M104E	a	λ	114
GAC	A5A	e	k	120
GAC	S117	a	k	126
GAC	V_{kGAC}	various	k	127
LPS	IdX	all	-	146
GAT	CGAT	various	k	153
GAT	pGAT	various	k	154
GAT	srGAT-1	a,c,e	-	162
GAT	Gte	b	-	163
HEL	IdX	various	k	206
Nase	5 idiotypes	a	k	220
phOx	Ox-1	various	k	206
DNP	M-460	a	k	227, 228
BI	not named	a,b,c	-	244

Appendix 3

The Structure of Ferredoxin

TRYPSIN

ALA-TYR-LYS-ILE-ALA-ASP-SER-CYS-VAL-SER-CYS-GLY-ALA-CYS-ALA-SER-ALA-CYS-PRO-VAL-ASN-ALA-ILE

THE N - DETERMINANT

SER
|
GLN
|
GLY
|
ASP
|
SER
|
ILE
|
PHE
|
VAL
|
ILE
|

THE C- DETERMINANT

GLU—GLN-VAL-PRO-ALA-GLY-VAL-PRO-CYS-VAL-ASN-ALA-CYS-ASN-GLY-CYS-ASP-ILE-CYS-THR-ASP-ALA-ASP

CARBOXYPEPTIDASE A
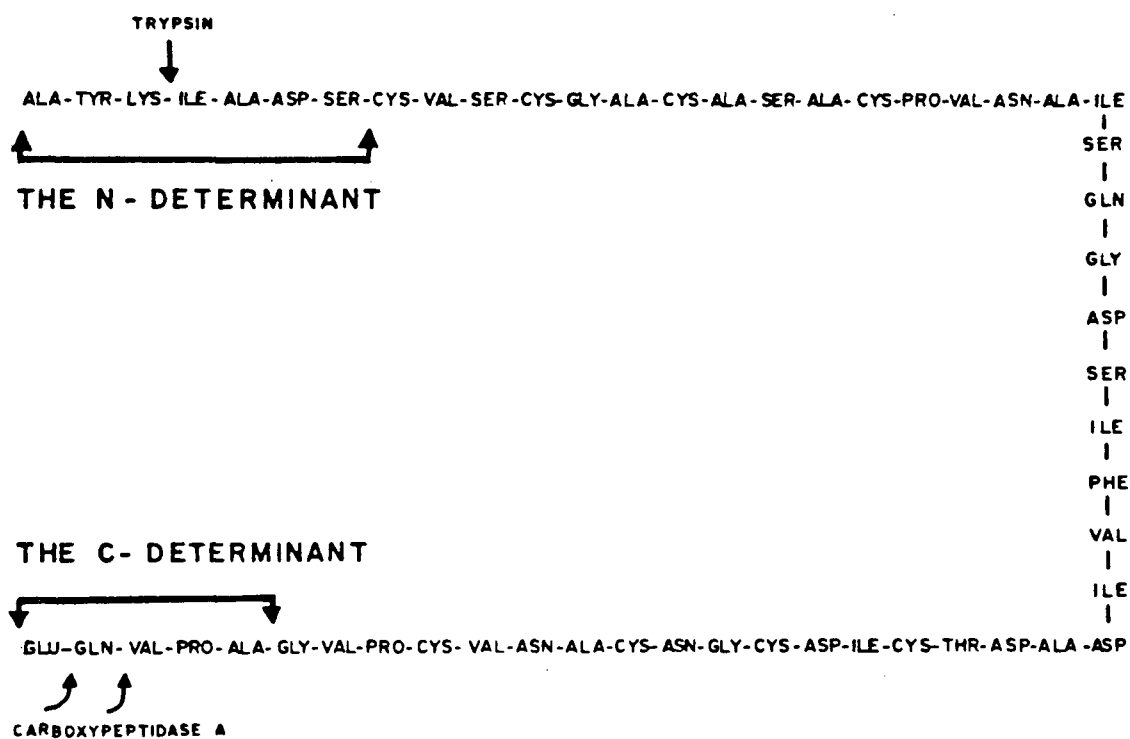
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