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Immunochemical and molecular analysis of $\beta_{2\rightarrow6}$ fructosan binding monoclonal antibodies bearing the A48 regulatory idiotype

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City University of New York, 1988

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IMMUNOCHEMICAL AND MOLECULAR ANALYSIS OF $\beta 2 \rightarrow 6$ FRUCTOSAN BINDING MONOCLONAL ANTIBODIES BEARING THE A48 REGULATORY IDIOTYPE.

by

ZAHAVA T. BARAK

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosopy, The City University of New York

1988

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Abstract

IMMUNOCHEMICAL AND MOLECULAR ANALYSIS OF $\beta_{2\rightarrow6}$ FRUCTOSAN BINDING MONOCLONAL ANTIBODIES BEARING THE A48 REGULATORY IDIOTYPE

by

Zahava Tova Barak

Advisor: Professor Constantin A. Bona

The overall goal of this study was to investigate the immunochemical and molecular properties of monoclonal antibodies expressing A48 regulatory idiotype. The experimental strategy devised was to activate A48-UPC10 regulatory idiotype bearing clones subsequent to administration of syngeneic antiidiotypic reagents and then immortalize those clones as hybridomas. Hybridomas secreting antibodies were selected for the expression of A48-idiotype by sensitive radioimmunoassay.

Regulatory idiotypes are autoimmunogenic, shared by antibodies exhibiting different antigen specificities, and recognized by regulatory T cells which could contribute to their dominant expression.

In order to fully appreciate the implications of immunoregulation through regulatory idiotypes, I performed a complete immunochemical and molecular characterization of antibody molecules bearing the A48-UPC10 regulatory idiotype.

The V_H region expressed by A48, UPC10 and the hybridomas in this work derives from the V_H441-1 germline gene which belongs to the small X-24 V_H gene family. Northern-blot analysis of mRNA extracted from the

hybridomas revealed that in addition there is a preferential usage of the V_L gene belonging to the V_K10 family located on chromosome 16 in the mouse, like the V_L used by UPC10 and MOPC-173 A-48RI bearing myelomas.

Surprisingly, the antigen binding specificities manifested by those hybridomas are different from that of A48 and UPC10, which exhibit $\beta_{2\rightarrow6}$ fructosan binding activity. They include specificities to $\beta_{2\rightarrow6}$ fructosan, $\beta_{2\rightarrow6}$ and $\beta_{2\rightarrow1}$ fructosan, branch point determinant in bacterial levan, and non-fructosan determinants. These data which show heterogeneous antigen binding specificities and yet preferential pairing of heavy and light chains, suggest differences in the sequence of genes derived from the same germline gene.

The sequencing data presents a random usage of J_H and J_L segments, and the variety of D segments used by the heavy-chain genes, suggest no possible contribution of J_H, J_L or D segments to the regulatory idiotype expression or antigen binding specificity. Differences are found in the sequences of the hypervariable regions of the V_H and the V_L genes, but nevertheless they still derive from a unique V_L germline gene, the V_K10 and a unique V_H germline gene belonging to the X-24 germline gene family.

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To Dr. Carol Victor-Kobrin, you are very important to me, and I thank you for your guidance and support, and your true friendship, it was truly a pleasure to work with you and I am grateful for our successful collaborative efforts.

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I would like to thank my family, my husband, Menachem, and my children, Merav, Erez and Alon; because it was their love, support, confidence, and encouragement during the difficult times that truly inspired me to achieve this goal.

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Introduction

1. Antibody Structure

The antibody molecule is a heterodimer protein composed of two identical light chains, and two identical heavy chains. The immunoglobulin proteins have many disulfide bonds per molecule, mostly within chains, and a few between chains.

The chain structure was established in 1959 by Edelman who discovered that after cleavage of the disulfide bonds the molecular weight of an immunoglobulin molecule dropped and electrophoresis revealed two components. By gel filtration after exposure to an organic acid (acetic, propionic), which breaks noncovalent hydrophobic bonds and imposes many positive charges on the chains, leading to their mutual repulsion, two components are recovered, with essentially 100% yield; the molecular weight of the heavier component is about 50,000 and the lighter one is about 25,000 d.

On the assumption that each fraction is made up of a single kind of chain, the original molecular weight of an immunoglobulin 150,000 daltons consists of two heavy chains (2x50,000) plus two light chains (2x25,000). Though analysis of antigenic differences and of amino acid sequences have revealed a great variety of heavy chains and of light chains, any particular immunoglobulin molecule has identical heavy chains and identical light chains.

Mouse immunoglobulins are encoded by three unlinked gene families. λ light chain genes, κ light chain genes, and heavy chain genes located on chromosomes 16, 6 and 12 respectively. In mice λ light chain genes are less

heterogeneous than the κ light chains or the heavy chains, and form only about 5% of the total serum immunoglobulin light chains.

Extensive sequence analysis of many immunoglobulin genes manifested highly variable amino termini regions while the carboxy-terminal regions are one of several types within a given species. The hypervariable region is primarily responsible for antigen recognition and contains particularly hypervariable subregions whose residues have been implicated in actual antigen contact. These subregions, the hypervariable regions which are referred to as complementarity determining regions CDR1, CDR2 and CDR3, are flanked by less hypervariable subregions termed framework regions FR1, FR2, FR3 and FR4. The constant regions of the heavy chain (C_H) are responsible for a variety of effector functions such as complement fixation and binding to the Fc receptor, and define the chain isotype, classes or subclasses (Heinrich, G. et al. 1984; Tonegawa, S. et al. 1983).

The variable region of the heavy chain is encoded by three germline DNA segments: V_H, D and J_H. V_H = variable region D = the third hypervariable region or diversity segment, J_H = joining segment. The three types of segments are encoded in three separate clusters in the mouse genome. There are 4 J_H segments, approximately 12 D segments which can be divided into 3 families based on nucleic acid sequence homology. At an unknown distance upstream from the D segments lie approximately 200 to 1000 V_H gene segments which can be grouped into families based on nucleotide sequence homology (Alt, F.W. et al. 1986).

The joining between two segments is mediated by a consensus recognition sequence which consists of a conserved heptamer and nonamer separated by a spacer of either 12 or 23 base pairs. Joining normally occurs only between segments flanked by recognition sequences of the different

spacer length, double-stranded cleavage occurs at the heptamer-segment border, and the re-ligation of the two segment ends which have frequently been modified by the deletion and insertion of nucleotides takes place (Alt, F.W. et al. 1986; Sakano, H. et al. 1980; Seidman, J.G., and Leder, P. 1978).

Following assembly of the V_HDJ_H, variable region gene transcription is initiated from a promoter which lies upstream of each germline variable region gene. The primary transcript extends downstream through the exons which encode the first constant region gene expressed during development: the C_μ gene. There are four major C_μ exons as well as the secretion μ(S) and the membrane specific exons μ(M). Transcription is terminated at 1 of 2 downstream sites. The latter feature allows the cell to generate the mRNAs encoding the membrane-bound and the secreted forms of immunoglobulin protein. Following appropriate RNA splicing events, the V_HDJ_H sequences are fused to the C_H encoding sequences to form a complete heavy chain mRNA (Alt, F.W. et al. 1984).

Although expression of rearranged V_HDJ_H segments is initiated from a promoter that is carried upstream of all germline V_H segments, only the promoter of the rearranged V_H is active in immunoglobulin-producing cells, whereas those flanking unrearranged V_H segments are inactive.

Although many germline V_H gene promoters are active early in development, the exclusive activity of the rearranged V_H gene in more mature cells is thought to be provided by an enhancer element which lies in the J_H to Cμ intron, and which maintains activity of the rearranged V_H gene promoters at a time when the germline V_H promoter has been turned off (Yancopoulos, D.G. and Alt, F.W. 1985).

The flexibility of the joint increases the V_HDJ_H diversity (Seidman, J.G. et al. 1978). However, it also results in many pre-B cells which do not

produce immunoglobulin heavy chains and have non-functional V_HDJ_H complexes carrying the V_H and J_H coding sequences in different reading frames (Hagiya, M. et al. 1986). In the work published by Reth, M.G. et al. (1986) it has been shown that such "null cells" are not dead end products of the B-cell developmental pathway but can preform a novel V'_H to V_HDJ_H joining using a 5' V'_H segment to replace the V_H sequence of non-producing immunoglobulin sequence of V_HDJ_H complex. In that work they studied the order and control of immunoglobulin gene arrangements in the Abelson transformed pre-B-cell line 300-19 derived from the bone marrow of an outbred NIH/Swiss mouse, which originally carry a DJ_H3 complex. Approximately 30% of the V_H to DJ_H joinings place the V_H and J_H coding sequence in the same reading frame thus resulting in the production of a μ chain. Many of the 300-19 subclones, however, contain $V_HDJ_H^-$ complexes and fail to produce a μ chain. Two such complexes, the P17 and Pa2-5, which were $V_HDJ_H^-$ were analyzed. The P17 became more and more-μ positive over a 3-4 week culture period, and so did the Pa2-3 subclone. A process which can result in generation of a V'_HDJ_H immunoglobulin producing complex and the subsequent expression of an immunoglobulin heavy chain.

Developing B-lymphoid cells which express cytoplasmic μ heavy chains but which have not yet assembled or expressed light chain genes are referred to as pre-B cells. Subsequent to and perhaps signalled by the production of a μ heavy chain, pre-B cells begin to assemble light chain genes.

κ light chain is encoded by a single copy of κ constant region C_k located at the 3' end of the gene, a cluster of five homogeneous but differing joint segments J_K are located 5' to the C_K segment between 4.0 to 2.5 kilobases

(Kb) (Sakano, H. et al. 1979). Only four out of the five J_K genes are being used during V_KJ_K rearrangement, the J_{K3} gene is referred to as a pseudo-gene. In embryonic DNA the V_K genes are separated by a large intron from the J gene cluster. The V_K DNA cluster is located 5' to the J_K cluster and composed of 90-300 different V_K DNA segments (Tonegawa, S. 1983).

Immunoglobulin κ light chain gene formation involves site specific somatic recombination between one of the several hundred variable regions of germline genes and one of the J segments localized 5' to the constant region gene. The structural homology shared among the five J segments suggests a common ancestral gene. In all the different J segments excluding the J3 sequence, 10 out of 13 codons are unchanged except in a few base positions. It is likely that the preserved coding sequence is of significance for the configuration of the immunoglobulin molecule (Sakano, H. et al. 1979).

Two other regions in the κ light chain gene of preserved homology are of interest. These are the G and T-rich sequence on the 5' side of each J segment, and the short palindrome [CAC(T/A)GTG] present with some minor variations adjacent to each J-region combination site. The palindromic sequence is present and adjacent to each germline V-region recombination site. These palindromes are complementary inverted repeat sequences and therefore can be visualized as forming an inversion loop or stem structure drawing the V and J sequences together (Max, G. et al. 1974). The rearrangement affects only one member of an allelic pair of light chain genes.

Translation of the immunoglobulin κ light chain genes depends on the presence of a TATA box upstream of the leader gene segment, and is regulated by an enhancer sequence in the large intron (Falkner, F.G. et al.

1984; Kelley, D.E. et al. 1985; Hamlyn, H.P. et al. 1978). By using internal deletions within a rearranged immunoglobulin κ light chain gene, the presence of the intron regulatory sequence the enhancer has been confirmed, by internal deletions within the J_K-C_K intron the intron regulatory element was identifiable. It occurs concidentally with a region of sequence conserved between mouse, man, and rabbit κ chain genes and a region of hypersensitivity to deoxyribonuclease digestion. The intron regulatory sequence behaves as an enhancer by conventional criteria: its activity is evident whether it is upstream of the gene or within it and in both orientations. It also stimulates, weakly, transcription of a β-globin gene and a μ heavy chain gene.

By using 5' deletions, a second regulatory element was located upstream of the TATA box. In studying the region 5' to the mouse V_K region, using 5' deletion mutants, sequences essential for κ chain transcription were identified. They are located upstream of the TATA box between position -69 and -104. Deletion of sequences 5' of position -104 had no effect on the efficiency of κ chain transcription, but deletion of sequences between -69 and -104, which left intact the TATA box around position 30 upstream of the initiation site, showed no accumulation of transcripts. These two elements both are required for efficient κ chain gene expression (Bergman., Y. et al. 1984; Morrison, S.L. et al. 1984).

Seventy-nine V_K sequences were grouped into seven size groups based on the length of the sequence to the first invariant tryptophan (Trp 35, Kabat number) (Potter, M. et al. 1982). Then all of the sequences within a size group were sorted and matched with the parent sequence, i.e., the sequence from which all others could be derived with the least number of nucleotide changes. In this sorting process, each sequence was given a distance value

from the parent sequence, based on the number of amino acid interchanges necessary to convert the sequence to the parent sequence. It has been shown that the V_K region sequences can be divided into two very large families, one containing the 33, 34, 35 and 36 amino acids size groups, and the 35 amino acids group to which the V_K10 group belongs. The second family contains the 39, 40 and 41 amino acids size groups. There are also large subdivisions in this family: 39 amino acids group and 41 amino acids group each has two subdivisions. Some sequences of different sizes show closer sequence relationships than do some of the sequences of the same size, e.g., the 35 and 39 amino acid subdivisions present great homology. These relationships suggest that mutations causing either deletions or insertions of one or two amino acids have occurred during the evolution of the mouse immunoglobulin κ complex.

Mouse λ light chain genes which are located on chromosome 16 are organized in a similar manner to that of κ light chain gene, but unlike the latter has only two V genes, four J_λ genes and four C genes, of which only three are functional.

Due to diversity in the mechanisms of assembly and expression of the immunoglobulin genes, each B cell has the capability of somatically creating an almost unlimited number of variable regions at each immunoglobulin locus. Furthermore, since a B cell can and often does rearrange both "alleles" of a given locus, the theoretical potential exists for a given B cell clone to assemble and express totally different variable regions at both of its heavy chain alleles and at each of its several light chain alleles. However, various studies have demonstrated that each B lymphocyte produces a mono-specific immunoglobulin receptor due to: allelic exclusion i.e., a B-cell clone expresses only one of its two heavy chain

alleles and only one of its several light chain alleles as immunoglobulin chains which associate to form a mono-specific antibody receptor. The unique regulatory processes which mediate allelic exclusion appear to have evolved to ensure the generation of the mono-specific B lymphocytes that are a prerequisite to an immune system whose specificity is based upon clonal selection.

2. Antibody idiotype

When an antibody or cellular receptor is regarded as an immunogen, it possesses numerous determinants toward which specific antibodies can be produced. Among such determinants, those which are found in the variable region of a receptor, and which make that receptor unique, these can be defined collectively as the "idiotype" (Id). Therefore, an immune receptor has two unique properties related to its specificity: antigen-binding activity and idiotype. Any monoclonal anti-idiotypic antibody can only recognize a determinant of limited size, much smaller than the entire variable region of another immune receptor. Therefore, one could envision the existence of shared idiotypic determinants on two antibodies with different antigen specificities. Likewise, two different antibodies to the same or slightly different determinants on the same antigen might not necessarily share idiotypes.

Idiotypic interactions form the basis by which the immune system can regulate the expression of its diverse resident lymphocyte clones. Numerous investigators have demonstrated that the appearance of auto-antiidiotypic antibodies coincides with a decrease in the titer of idiotype bearing antibodies during the course of an immune response (Bona, C. A. et al. 1978; Kluskens, L. et al. 1974).

One of the postulates of the idiotype network theory was that idiotype-anti-idiotype interactions are vectorial. Thus, a given idiotype (Ab1=P1i1) is diminished by interacting with a complementary paratope (Ab2=p2i2) which by virtue of its own idiotopes (i2) stimulates the clones (Ab3=p3i3) possessing a paratope (P3) specific for idiotope (i2) of Ab2 clones. A

"cascade" of complementary antibodies (Ab1-Ab4) was obtained in an allogeneic rabbit system (Wikler, M. et al. 1979) and in the mouse system,(Bona, C.A. 1979). The immunochemical analysis of the properties of four members of an idiotypic pathway showed that the immunization with Ab1 (anti-epitope antibody) gave rise to a heterogeneous population of Ab2 and that the immunization with Ab2 stimulates clones (Ab3) sharing mainly the idiotopes of Ab1. When the animals were immunized with Ab3, the Ab4 obtained resembled Ab2 since, like Ab2, it bound to Ab1 (Wikler, M. et al. 1979).

Thus the experimental data demonstrated that this cascade immunization led to the activation of clones exhibiting the expected idiotypes, since a particular idiotypic pathway operates through a circular causality, which tends to repeat itself. In order to explain these phenomenon it was proposed that Ab1 molecules express a special set of regulatory idiotopes which differ from conventional idiotopes. (Bona,C.A. et al.1981a).

In general regulatory idiotypes are defined by three criteria: The ability to function as autoimmunogens, the potential to become dominantly expressed because they are recognized by regulatory T cells, and their shared expression by antibodies with different antigenic specificities (Victor-Kobrin, C. et al. 1985a,b, Bluestone, J.A. et al. 1986). The Ab1 bearing regulatory idiotypes (RI) activates Ab2 clones, producing anti-RI antibodies, lacking themselves RI. The immunization with Ab2 leads to the activation of clones bearing RI on their receptors. Finally, immunization with these so-called Ab3 [anti(anti-Id) antibodies], results in the activation of clones recognizing RI. Indeed, the majority of Ab4 [anti-anti(anti-Id)

antibodies] obtained subsequent to the immunization with Ab3 were similar to Ab2 (Bona, C.A. et al. 1981a).

Rubinstein et al. (1983) have shown that exposure to antiidiotypic antibodies or the idiotype bearing antibodies themselves *in utero,* by placental crossing or by parenteral administration to neonatal and adult mice, can dramatically expand the number of B cell precursors bearing that idiotype in those animals. However, it was observed that when working in an autologous system i.e., the antiidiotypic antibodies were elicited from the same mouse strain from which the idiotype bearing antibodies were derived, idiotype driven events were mediated by the special category of idiotopes, the regulatory idiotypes (RI) (Bona, C.A. et al. 1981a).

This regulatory idiotype concept emerged from an immunochemical analysis of 4 members of an idiotypic pathway initiated by A48, an IgAk, $\beta_{2\rightarrow6}$ fructosan binding myeloma protein which prototypically bears the A48 idiotype. Examination of the idiotype of polyclonal populations of syngeneic Ab2 (anti-A48-Id), Ab3 (anti-[anti-A48Id]) and Ab4 [anti-anti(anti-A48Id)] type antibodies, showed that the Ab3 antibodies expressed the A48-Id since the Ab3 and Ab4 antibodies, respectively bound to both the A48 and Ab3 antibodies. Thus, it was proposed that those particular idiotopes of Ab1 (A48) which are immunogenic in a syngeneic system will elicit the production of Ab2 antibodies. Parenteral administration of these antibodies will activate Ab3 clones which for the most part will express the A48 regulatory idiotype. Therefore, immunization with these Ab3 antibodies will lead to the production of Ab4 antibodies which will primarily be anti-A48 regulatory idiotype antibodies.

Working in the anti-tobacco mosaic virus (TMV) Id and anti-Micrococcus lysodeikticus (*M. lysodeikticus*) Id systems of the rabbit,

the Ab1, through Ab4 members of an idiotypic pathway and demonstrated the same phenomena. Namely, the diversity of the idiotype network did not increase along the chain of immunization. The Ab1 and Ab3 populations extensively shared idiotypy since the Ab2 and Ab4 populations, each bound equally well to Ab1 and Ab3. In a similar way, autoimmunogenic anti-idiotypic antibodies have demonstrated a clear immunoregulatory role in determining the idiotypy of the immune response to the peptidoglycan (PG) moiety of the cell wall of *M. leuteus* in the rabbit (Wikler, M. et al. 1980).

The existence of regulatory T cells specific for regulatory idiotypes has been demonstrated in 2 separate systems. First, Rubinstein et al. (1982) showed that helper cells specific for the A48 RI were responsible for the expansion of A48 RI bearing B cell clones in neonatal mice treated with A48 protein. Second, are the observations made by McNamara et al. (1984) on idiotype specific helper T cells which participate in the anti-phosphorylcholine (PC) immune response in BALB/c mice. Such T cells naturally appear during the PC immune response; however, they can be elicited by immunization with the isolated heavy chains of the 2 PC binding myeloma proteins, TEPC15 and MOPC167. TEPC15 bears the T15-Id which is a dominant Id of the PC immune response and does not share any idiotypic determinants with MOPC167, a minor Id component of this immune response. In order to exert their immunoregulatory abilities, these T cells recognize a RI associated with the heavy chains of anti-PC antibodies which is distinct from the dominant serologically detected T15 idiotype.

The demonstration of antibodies bearing different antigen specificities, yet sharing a common regulatory idiotype has also been well substantiated in the literature. The vast majority of Ab3 type antibodies elicited by mice

immunized with anti-A48 RI antibodies (Bona, C.A. et al. 1981b) or rabbits immunized with anti-*M. leuteus* RI antibodies and anti-TMV RI (Wikler, M. et al. 1979) clearly bear the respective A48RI, anti-*M. leuteus* RI ot anti-TMV RI, but they do not bind bacterial levan, *M. leuteus*, or TMV, respectively. According to the classical Jerne network theory (Jerne, N. 1974) these RI bearing, non-antigen binding antibodies are considered to belong to the "parallel set". Only after these Ab3 producing animals are challenged with antigen, are the Id bearing, antigen binding clones sufficiently expanded for us to see an Id^+ antigen specific component of this Ab3 subset.

Idiotypic networks have been proposed to play an important role in lymphocyte receptor repertoire formation and in immune regulation (Elaine, A.D. et al. 1986). The MOPC460-Id has been observed as a minor idiotypic component of antibodies specific for the hapten 2,4 dinitrophenyl (DNP), as well as a major idiotypic component of antibodies specific for the opportunistic murine pathogen Pasteurella pneumotropica (*P. pneumotropica*). Furthermore, high levels of M460Id bearing immunoglobulin is found in the sera of normal mice which can be absorbed by *P. pneumotropica* and not DNP. The interpretation is that the M460Id is functioning as a RI and is utilized as a selective force for maintaining the expression of V_H genes which encode resistance to this pathogen.

The physiological relevance of the role of regulatory idiotypes is best shown in several autoimmune disorders, where the primary lesion is thought to be an aberration in the RI mediated Id-anti-Id regulation of those clones which are reactive with self-components. In patients with the autoimmune disorder SLE (systemic lupus erythematosus), as well as in lupus murine models, antibodies to DNA are responsible for much of the

pathology associated with this disease (Lambert, P.H., and Dixon, F.J. 1968; Koffler, D. et al. 1967; Schur, P., and Sandson, J. 1968).

Data obtained from both systems indicate that extensive idiotypic similarities exist not only between the anti-DNA clones in a single individual but also between genetically unrelated mouse strains and patients. Furthermore, Abdau et al. (1981) showed that remission of the disease could be correlated with the generation of auto-antiidiotypic antibodies. Hahn et al. (1983) demonstrated that auto-anti-Id could be elicited by immunizing (NZB x NZW)F1 females displaying the symptomology of SLE with a syngeneic monoclonal anti-DNA antibody derived from the kidney eluates of mice with glomerulonephritis. The production of auto-anti-Id caused the disappearance of anti-DNA antibodies bearing that Id which resulted in a transient improvement in the SLE symptomology. Similar observations were made by Dwyer et al. (1984) in myasthenia gravis (MG) patients where the pathology is attributed to production of autoantibody against the acetylcholine receptor (AChR). In 40% of MG patients studied, autoantiidiotype to the anti-AChR antibodies were observed during the remission phase from the symptoms of muscle weakness.

The final relevant observation in this section involves the production of autoantiidiotypes by women who, as a result of pregnancy, are alloimmunized with the fetus' paternal HLA alloantigens. Suciu-Foca et al. (1983) observed that low levels of anti-HLA antibodies are found only in 25% of parous women, while 100% of all parous women produce autoantiidiotypes which bind to the immune receptor for HLA on their own T lymphocytes which have been sensitized *in vitro* to their husband's HLA-D/Dr antigens. Furthermore, these autoantiidiotype antibodies will also react with the auto-anti HLA antibody, as well as allo-anti-HLA antibody

which is directed against the same HLA specificity. Therefore, the immune response to HLA encoded antigens seems tightly regulated by a highly conserved regulatory idiotype.

In contrast to all these experimental observations which substantiate the existence of regulatory idiotypes, few attempts have been made to study the population of antibodies which bear a given regulatory idiotype. Without known anything about RI bearing antibodies, we cannot attain a thorough understanding of how clones which bear such determinants are regulated in the immune system and what the implications are to the immune system in utilizing such a mode of immunoregulation.

The monoclonal antibodies bearing the A48RI used in this work are clonal products resulting from immunoregulation through regulatory idiotypes. The experimental strategy devised to get those hybridomas was to activate A48-UPC10 regulatory idiotype-bearing clones using syngeneic antiidiotypic reagents and then immortalize those clones as hybridomas. By using syngeneic reagents to effect this activation it was assured that the activation was achieved only through regulatory processes involving the recognition of regulatory idiotypes.

Previous studies showed that the activation of A48-UPC10 Id^+ clones could be achieved in several ways. They include: administration at birth of 10 ng of the A48 monoclonal protein; *in utero* exposure to UPC10 monoclonal protein; neonatal injection of syngeneic polyclonal anti-A48 Id antibodies (Hiernaux, J. et al. 1981); immunization of adult mice with a syngeneic polyclonal anti-A48 Id-KLH conjugate followed by immunogenic challenge with bacterial levan; or the immunization of adult animals with syngeneic monoclonal anti-A48-UPC10 Id-KLH conjugates followed by an

immunogenic challenge with bacterial levan (Bona, C.A. et al. 1981b; Goldberg, B. et al. 1983).

Based on these studies, hybridomas used in this study were obtained by a fusion of SP2/0 myeloma cells and spleen cells obtained from pre-treated mice (Table I). The hybridomas generated from these animals were selected according to two criteria: a) expression of antibody bearing the A48-UPC10 regulatory idiotypes which is based on the ability of the monoclonal antibody to inhibit the binding of radioactively tagged A48 or UPC10 to syngeneic polyclonal anti-A48-UPC10 Id antibodies in a competitive inhibition radioimmunoassay; b) demonstration of mRNA in cytoplasmic lysates of the hybridomas which can hybridize to a V_H 441-4 germline gene probe using RNA slot blotting techniques and later confirm the positive hybridization by Northern blot analysis (Victor-Kobrin, C. et al. 1985a; Hartman, A.B., and Rudikoff, S. 1984).

To fully appreciate the implications of immunoregulation through regulatory idiotypes, I proceeded to perform a complete immunochemical and molecular characterization of the antibody molecules bearing the A48-UPC10 regulatory idiotypes. My studies were specifically directed at addressing the following three issues:

a) Whether or not expression of the A48-UPC10 regulatory idiotypes could be correlated with expression of a germline V_H gene derived from the V_HX24 germline gene family. Earlier studies (Victor-Kobrin, C. et al. 1985b) suggested that these regulatory idiotypes were phenotypic markers of germline V_H genes. The murine V_H family identified, is located at the 5' terminus of the murine V_H locus and consist only of two highly homologous V_H genes (≥93% nucleotide homology), the V_HX24 and the

Designation		Treatment of BALB/c donor mice
ABPC-48,	UPC10	BALB/c myeloma
1-5-1	(μ,κ)	BALB/c mice injected at birth with 10 μg A48 and challenged 1 mo. later with 20 μg levan
2-1-3	(γ3,κ)	BALB/c mice injected at birth with 10 ng anti-A48-Id antibodies and challenged mo. later with 20 μg levan
2-1-10	(γ 3,κ)	
2-11-1	(μ,κ)	
2-11-3	(μ,κ)	
2-8-2	(μ,κ)	
2-28-9	(γ3,κ	
2-9-17	(μ,κ)	
2-12-10	(μ,κ)	
2-12-19	(μ,κ)	
3-76-4	(μ,κ)	BALB/c mice hyperimmunized with polyclonal anti-A48-Id/KLH and challenged 1 mo. later with 20 μg levan
3-76-42	(μ,κ)	
3-14-9	(μ,κ)	
3-27-6	(γ1,κ)	
3-101-14	(γ1,κ)	
3-9-9	(γ1,κ)	

TABLE I. Origin of monoclonal antibodies
ABPC-48, UPC10, are myelomas secreting antibodies bearing the A48RI and bind to polyfructosan. Series 1 antibody was derived from BALB/c mice injected at birth with 10 μg of A48 monoclonal protein, and followed by an immunogenic challenge of bacterial levan at the age of one month. Series 2 antibodies are from BALB/c mice immunized at birth with minute amounts of syngeneic anti-A48-Id antibodies and challenged 1 mo. later with bacterial levan (BL). Series 3 antibodies were obtained from adult BALB/c mice which were first hyperimmunized with an anti-A48-ID/KLH and then challenged with BL after completion of immunization.

V_H441-4 germline genes (Brodeur, P.H. et al. 1984). The V_H441-4 germline gene is utilized by A48 and UPC10 to encode their respective V_H regions.

b) Whether or not expression of the A48-UPC10 regulatory idiotypes could be correlated with expression of a V_K10 germline gene, since it is known that two myeloma proteins: MOPC-173 and UPC10 which are known to bind to $\beta 2 \rightarrow 6$ fructosan and bear the A48 RI use the V_K10 light chain gene suggesting that these V_K genes are essential for generating this antigen specificity.

c) To establish the degree of heterogeneity, if any of the antigen binding specificities expressing the A48-UPC10 regulatory idiotypes and utilizing germline V_H genes deriving from the V_HX24 germline gene family and V_K10 light chain germline gene.

Materials and Methods

1. Monoclonal Antibodies

(a) Description Hybridomas secreting antibodies bearing the ABPC48 (A48) regulatory idiotype (Id) were generated from BALB/c mice treated at birth or as adults with minute amounts of syngeneic polyclonal anti-A48-Id antibodies (Table 1). Series one antibody was derived from BALB/c mice immunized at birth with 10 μg A48 protein and challenged one month later with 20 μg bacterial-levan. Series two antibodies are from BALB/c mice immunized at birth with 10 μg of syngeneic anti-A48-Id antibodies and challenged one month later with 20 μg bacterial-levan. Series three antibodies were obtained from adult BALB/c mice which were first hyperimmunized with an anti-A48-Id/KLH conjugate and then challenged with 20 μg bacterial-levan after completion of immunization. The monoclonal antibodies generated from each of these series were screened for two qualities. First for their ability to inhibit the binding of ^{3}H A48 to polyclonal anti-A48-Id coated plates, and second for their ability to hybridize to the V_H441-4 probe in Northern blot analysis of cytoplasmic poly(A)$^+$ RNA. Those hybridomas which demonstrated the above two properties were selected for this work (Victor-Kobrin, C. et al. 1985a).

b) Antibody purification. All antibodies were purified by chromatography on Sephacryl G-300 (Pharmacia Inc., Uppsala, Sweden) in 10mM Tris-HCl, pH 8, 0.9% NaCl, and 0.3% sodium azide.

2. Antigen binding specificity

a) Antigens. Bacterial-levan (BL), a $\beta_{2\rightarrow6}$ linked polyfructosan chain with $\beta_{2\rightarrow1}$ branch points from Aerobacter levanicum (ATCC1552) was obtained as previously described (Bona, C.A. et al. 1981b). Grass (rye) levan (GL), a $\beta_{2\rightarrow6}$ linked polyfructosan was the kind gift of Dr. Franco Celada (Genoa University, Milan, Italy). Inulin-bovine serum albumin (BSA) was coupled according to the method of Chien et al. (1979).

b) Radioimmunoassay (RIA). The antigen binding specificity of the monoclonal antibodies was determined in RIA by assaying their binding to microtiter plates coated with rye levan at 30 μg/ml, inulin-BSA conjugated at 3 μg/ml or bacterial levan 30 μg/ml. Plates were coated by incubating the polysaccharide solutions in the plates overnight at 4°C. The plates were washed 3 times with PBS, after which they were incubated with PBS - 3% BSA for 1 hr at room temperature. Following washing with PBS the monoclonal antibodies were incubated on the plates at a concentration of 5 μg/ml overnight at 4°C. Antibodies binding to these antigens were measured by washing out the monoclonal antibody solution from the plate with PBS and replacing it with 50,000 cpm of ^{125}I-rat anti-mouse C_K monoclonal antibodies for three hrs at 37°C. The plates were extensively washed before counting bound radioactivity in a gamma counter.

3. V_K10 Probe

a) Description. The V_K10 probe used in my experiments was generated from a nonproductive V_K10 germline gene, kindly provided to our laboratory by Dr. M. Weigert (Kelley, D.E. et al. 1985). The V_K10 probe is an EcoRI/HindIII DNA piece cloned in a pUC series vector plasmid, designated pC3386. The 5' region of the probe has the variable region gene of the V_K10 group joined to a site 16 base pairs downstream to the J_{K2} element. This V gene has multiple defects and is therefore nonproductive. It contains three termination codons and has only 92 codons instead of the usual 95 codons. Another feature of this probe is its relative number of somatic mutations in the vicinity of the recombination sites.

b) Nick translation. Nick translation is used to label the V_K10 probe. E. coli DNA polymerase I (Amersham, Chicago, ILL), adds nucleotide residues to the 3' hydroxyl terminus that is created when one strand of the double-stranded DNA molecule is nicked by DNAase I (Amersham, Chicago, ILL). In addition, the DNA polymerase I, by virtue of its 5' to 3' exonucleolytic activity, can remove nucleotides from the 5' side to the nick. The elimination of nucleotides from the 5' side and the sequential addition of nucleotides to the 3' side results in movement of the nick along the DNA. By replacing the pre-existing nucleotides with highly radioactive nucleotides. ^{32}P-labeled DNA has been prepared with specific activity well in excess of 10^8 cpm/μg (Amersham, Chicago, ILL, Nick-translation kit). Separation of nick-translated DNA from unincorporated dNTPs was done by centrifugation through a 3.0 ml column of Sephadex G-50 (Weinstock, R. et al. 1978).

4. Localization of V_K10 genes in BALB/c liver DNA

a) Extraction of BALB/c liver DNA. To extract liver DNA, a general method for isolating high molecular weight DNA from eukaryotes has been used (Blin, N. and Stafford, D.W. 1976). This procedure allows for the preparation of DNA from a variety of tissues. The DNA obtained in such a way has an average molecular weight of about $200x10^6$ d and contains few, if any, single strand breaks. Minced tissue is homogenized in a stainless steel Waring blender in the presence of liquid nitrogen (1-5 min, at top speed), until the tissue is ground to a fine powder. After liquid nitrogen has been evaporated 10 vol. of the following lysis solution is added: 0.5M EDTA (pH 8.0), 100 μg/ml proteinase K (New England Biolabs, Beverly, MA), and 0.5% sarcosyl. The suspension of lysed tissue is placed in a 50°C water bath for 3 hrs and swirled periodically. DNA is gently extracted three times with an equal volume of phenol. After centrifugation, the phenol phase and as much of the interface as possible is removed. After the third extraction, DNA is dialyzed agaisnt 4 liters of solution of 50mM Tris-HCl (pH 8.0), 10mM EDTA, and 10mM NaCl with several changes until the OD_{270} of the dialysate is less than 0.05. Extracted DNA is treated with 100 μg/ml of DNAase-free RNAase (Sigma, St. Louis, MO) at 37°C for 3 hrs. Samples are extracted twice with phenol/chloroform, and dialyzed extensively against TE (10mM Tris-HCl pH 7.4, 1mM EDTA, pH 8.0). The size of the DNA was analyzed by electrophoresis of an aliquot through a 0.3% agarose gel. The DNA should be greater than 100 kb in size and should migrate more slowly than intact bacteriophage λ DNA (~ 46 kb). DNA was stored at 4°C.

b) Southern Blotting. Three samples of 10 μg BALB/c mice liver DNA were digested as follows: one with 50 units EcoRI (New England Biolabs, Beverly, MA), the second with 50 units HindIII (New England Biolabs, Beverly, MA), and the third sample, a double digest, with 50 units EcoRI and 50 units HindII. Samples were digested for 3 hrs at 37°C. Digested samples were then applied to a 40 mM Tris-HCl, 20 mM acetate, 2 mM EDTA, 0.8% agarose gel containing 1 μg/ml ethidium bromide. DNA fragments are separated according to size electrophoresis through the gel. For the transfer process DNA is denatured by soaking the gel in several volumes of 1.5M NaCl and 0.5 N NaOH for 1 hr at room temperature with constant stirring and shaking. Neutralization is by soaking the gel in several volumes of 1 M Tris-HCl (pH 8.0) and 1.5 M NaCl for 1 hr at room temperature with constant shaking. Denatured DNA was transferred to BA85 nitrocellulose filter (Schleicher and Shuell, Inc., Keene NH) by 10xSSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) (Southern, E.M. 1975). The relative position of the DNA fragments in the gel are preserved during their transfer to the filter. Nitrocellulose paper is baked for 2 hrs at 80°C under vacuum to immobilize DNA.

c) Hybridization. Hybridization (Wigler, M. et al. 1979) was carried out to localize V_K10 genes within the digested BALB/c liver DNA. To block nitrocellulose filter, incubation for 2 hrs at 52°C with prehybridization solution 50% formamide, 6xSSC, 0.5% SDS, 5X Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA) and 100 μg/ml denatured Salmon sperm DNA took place. 0.2 ml of solution were used per cm^2 of filter. After 2 hrs prehybridization, solution was removed and replaced with the hybridization solution, 50% formamide, 6xSSC, 0.01 M EDTA, ^{32}P-labeled denatured probe DNA (pre-boiled for 5 minutes) and 5X Denhardt's

solution. 10^8 cpm/μg DNA was used to screen single-copy sequences. Hybridization bags were then sealed and incubated overnight at 52°C.

Regarding buffers containing formamide, each increase by 1% in the formamide concentration lowers the T_m of a DNA duplex by 0.7°C. High stringent washes at 68°C each for 15-30 minutes were done to remove any excess or non-specific binding of ^{32}P DNA. Two washes with 0.5xSSC, 0.1% SDS, one wash with 0.2xSSC, 0.1% SDS, and the last wash with 0.1xSSC, 0.1% SDS were carried out (Grunston, M., and Hogness, D. 1978).

5. V_L Identification

a) RNA extraction. Total RNA was prepared from 3-5x10^7 cells obtained from logarithmically growing hybridoma cultures, washed twice in cold PBS. To break protein disulfide bonds and achieve cell lysis, washed cells were added directly to 4M guanidinium thiocyanate plus 0.1 M 2-mercaptoethanol solution and homogenized immediately using a polytron. This homogenate was carefully layered onto a 5.7 M CsCl cushion and tubes were spun in a SW41 Beckman rotor at 32K for 18 hrs at 20°C. Supernatants were quickly suctioned off. RNA pellets were rinsed in 100% ethanol at 25°C, resuspended in 10 mM Tris 1 mM EDTA, extracted once with phenol sevag (chloroform/Isoamyl alcohol, 24:1) solution and precipitated twice with 1/10 volume 4 M NaCl and 2 volumes of ethanol and stored at -20°C (Chirgwin, J.M. et al. 1979).

b) Oligo(dT) selection. Poly(A)$^+$ mRNA was isolated after two cycles of selection over a type 3 oligo(dT)-cellulose column (Collaborative Research Inc., Lexington, MA), according to the method of Aviv and Leder (1972). Briefly, RNA was resuspended in 2-3 ml binding buffer, 0.5 M NaCl, 10 mM

Tris-HCl (pH 7.4-7.5) and 1 mM EDTA, heated for 5 min. at 65°C, loaded on the column, collected and loaded for two more times. The column was washed with 10 ml binding buffer. To elute RNA 10 ml of elution buffer, 10 mM Tris-HCl pH 7.4-7.5 and 1 mM EDTA was used, and samples of 1 ml were collected. The presence of RNA was followed and quantitated by following absorption readings at wavelength 260 nm. All solutions for RNA extraction and oligo-dT selection were prepared using glassware baked overnight at 150°C. Whenever possible, the solutions were treated with 0.1% diethylpyrocarbonate for 12 hrs and autoclaved (not used for solutions containing Tris).

$5x10^8$ cells 10^6 cells/ml yield 3 mg of total RNA. The first cycle of oligo-dT selection gave rise to 50% cytoplasmic RNA and 50% rRNA. A second cycle of oligo-dT selection was used to get rid of rRNA. Selection of 3 mg total RNA yielded 70 μg of poly(A)$^+$ RNA.

c) Northern blot analysis. 200 ng of poly(A)$^+$ mRNA were resuspended in 50% deionized formamide, 2.2 M formaldehyde, 20 mM 3-(4-morpholino) propanesulfonic acid (MOPS), 5 mM sodium acetate buffer, pH 7.0, and denatured by heating at 65°C for 10 min. After quick chilling on ice for 5 min, Ficoll and bromphenol blue were added and the RNA was fractionated on a 0.8% agarose gel containing 2.2 M formaldehyde in a running buffer of 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7. The electrophoresis was carried out in a wick-type apparatus at 4°C, 115 V, while continuously circulating the buffer between the anodic and cathodic buffer compartments. The RNA was blotted onto BA85 nitrocellulose paper (Schleicher and Schuell, Inc., Keen, NH) and hybridized to the nick-

translated V_K10 probe. Hybridization and washing protocol are as described for DNA blots.

6. Cloning of ds-cDNA into pUC-9 plasmid

Cloning of ds-cDNA was devised as a preliminary step for sequencing heavy-chain or light-chain immunoglobulin genes (Figure 1).

a) First strand cDNA synthesis. 2 μg of oligo-dT selected mRNA were washed with 80% ETOH, lightly dried and dissolved in 10 μl of RNAase free 0.1 mM EDTA. RNA was denatured by heating at 65°C for 2 min. Polyadenylated RNA was used as a template for synthesis of the first cDNA strand using 18 units reverse transcriptase (New England Biolabs, Beverly, MA.), 10 μl of dXTP mixture containing the 4 deoxynucleotide triphosphates at 20 mM, 1 μl 0.5 M DTT (dithiotheitol), 1 μl dCTP - α^{32}P-labeled 10 mCi/ml, 3000 Ci/mmole and 40 ng of primer at a total volume of 100 μl. The primer used was a fifteenmer oligonucleotide (OCS Laboratories, Inc., Dallas, TX) complementary to the 5'-end of the heavy-chain constant region, or the 5'-end light chain constant region. The mixture was incubated at 42.5°C for 2 hrs (Retzel, E.F. et al. 1980).

b) Second strand cDNA synthesis. The DNA-RNA hybrids produced as previously described, were denatured by heating in a 105°C 5 M NaCl bath for 2 min, and cooled on ice for 60-90 sec. The first cDNA strand was used as a template to the nascent second cDNA strand, and its hair-pin structure at the 3' end primed the synthesis. The reaction mixture used was 10 μl of 20 mM dXTP 2 μl 1.0 M DTT, 5 μl dCTP - α^{32}P, 10 mCi/ml, 3000 Ci/mmole and 7.5 units of E. coli Klenow fragment of DNA polymerase I (New England Biolabs, Beverly, MA), an enzyme which lacks the 5'→3'

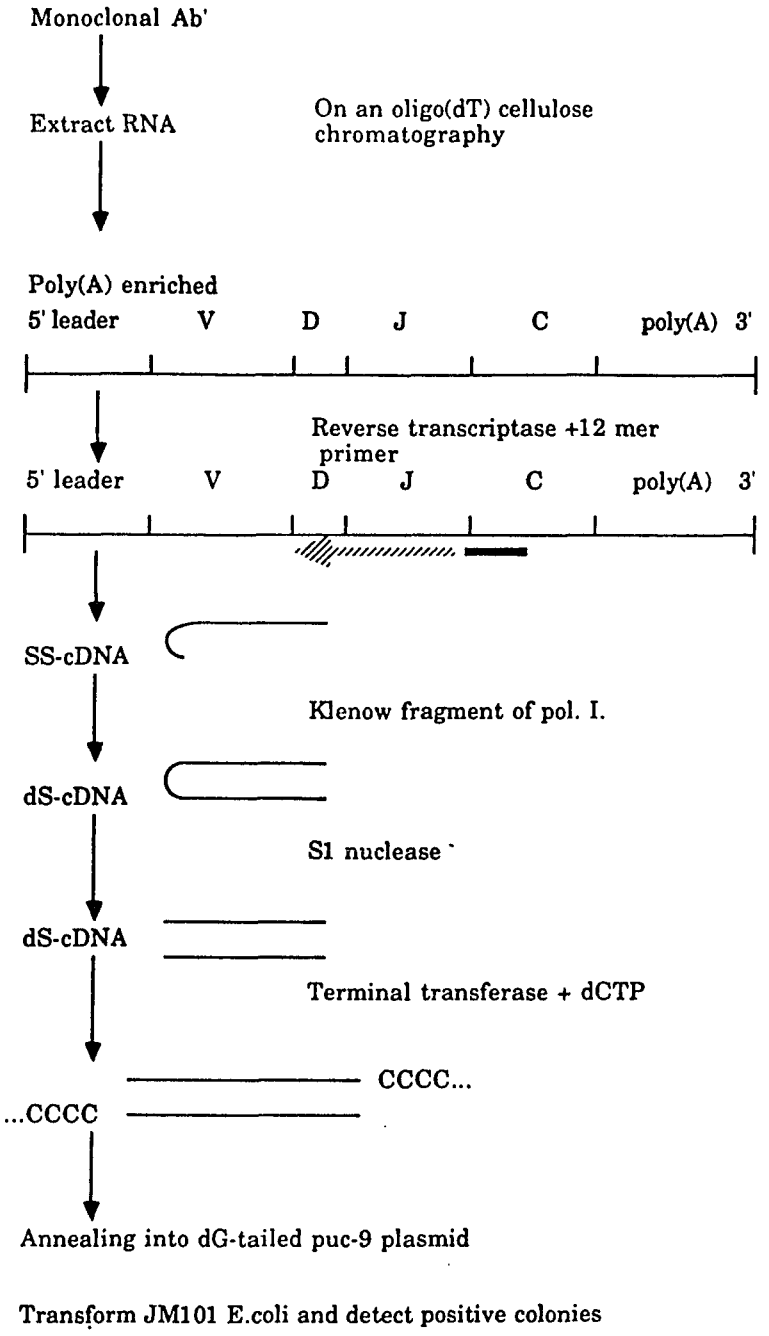


Figure 1. Cloning of ds-cDNA

exonuclease activity. Reaction took place 30 min at room temperature (Efstratiadis, A. et al. 1976; Wickens, M.P. et al. 1978).

c) SI nuclease trimming. The hair-pin loop which covalently joined the first and second cDNA strands, was cleaved by 6000 units of single-strand specific nuclease SI (Pharmacia Inc., Uppsala, Sweden) in 0.28 M NaCl, 0.05 M sodium acetate (pH 4.6), 4.5 mM $ZnSo_4$, and 20 µg/ml carrier ssDNA (Sanger, E. et al. 1977).

d) Double-stranded cDNA separation. To remove unincorporated labeled nucleotides from ds-cDNA, the mixture was phenol extracted. The volume was reduced to 150 µl by sec-butanol extractions. Aqueous phase was extracted with 150 µl chloroform-isoamyl alcohol (24:1). 1 µl of 10% SDS was added to the sample. 150 µl of cDNA were applied on a Sepharose 4B column poured in a 5" siliconized pasteur pipette, and run in 100 µl of column buffer: 0.05 M Tris pH 8, 0.1 M NaCl, 0.02% SDS and 0.001 M EDTA, were used to collect 15 fractions, each of ~100 µl , by repetitive addition of 100 µl of column buffer. The first peak of labeled DNA is the ds-cDNA.

e) Tailing cDNA with dCTP. 10 ng of cDNA were tailed with dCTP, in 0.1 M K Cacodylate pH 6.9, 0.4 mM DTT, 0.2 ng/ml BSA, 2 mM $CoCl_2$ and 50 units of calf thymus terminal deoxynucleotidyl transferase (New England Biolabs, Beverly, MA), which dC-tailed the 3'-hydroxyl ends of the ds-cDNA. The reactions were run at room temperature and stopped at 2, 3 and 5 min, in a dry ice bath with 0.1 M EDTA.

f) Annealing dC-tailed ds-cDNA into dG-tailed pUC-9 plasmid. The homopolymeric dC-tailed cDNA was annealed overnight at room temperature into dG-tailed pUC-9 plasmid (Figure 2) (Pharmacia Inc.,

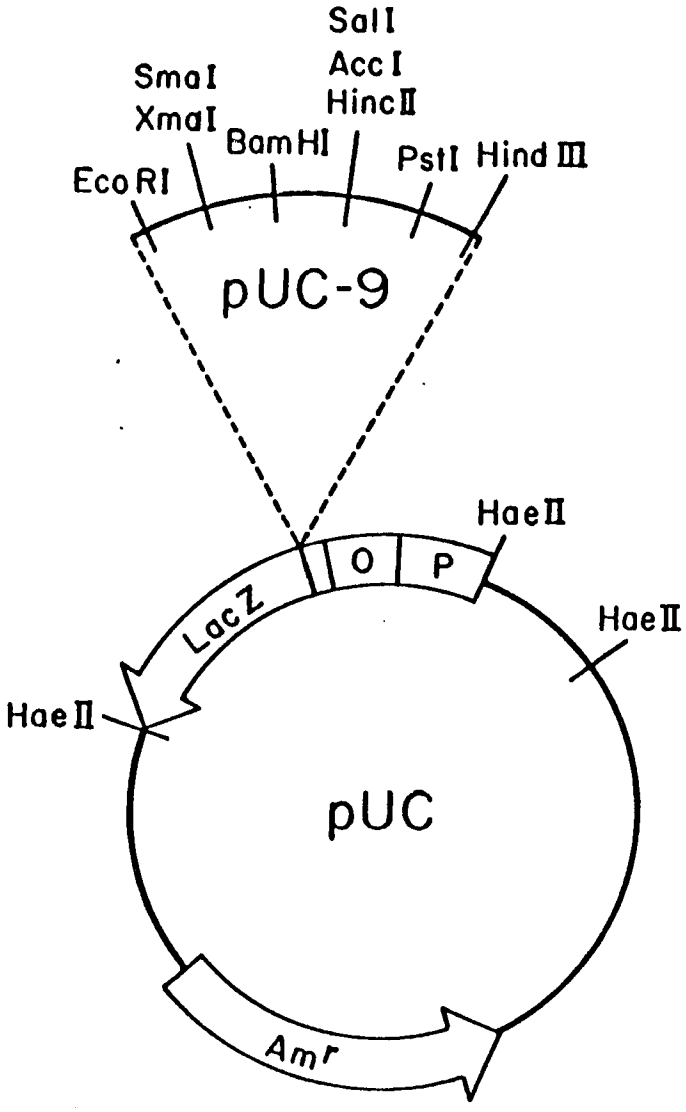


Figure 2. pUC-9 plasmid with the available polylinkers (Pharmacia Inc., Uppsala, Sweden)

Uppsala, Sweden) in the PstI site. 5 ng of the cDNA were mixed with 40 ng of the plasmid in 0.1 M NaCl, 10 mM Tris pH 7.8 and 1 mM EDTA. This mixture was heated to 65°C for 3 min., then incubated at 42°C for 2 hrs, followed by an overnight incubation at room temperature.

The pUC-9 plasmid is ~2 kb in length carrying a dG-tail (tail length is 16 base average) at the PstI site. Two types of selective markers are inserted in the plasmid: One is Ampicillin resistance and the other is the E.coli lac operon containing the regulatory region and some coding information for β-galactosidase lac Z, O and P. Plasmids containing DNA inserts at the polylinkers site give rise to white colonies in the presence of the inducer isopropyl-thiogalactoside (IPTG) and the chromogenic substrate Xgal. When no insert is introduced into the plasmid, blue colonies of transformed bacteria are growing in the presence of IPTG and Xgal

g) Transformation. The method of choice in my experiments to transform bacteria was by the Calcium-chloride/Rubidium-chloride procedure (Kushner, S.R. 1978). 2 ml of logarithmically growing E. coli JM101 bacteria (OD_{550}=0.5) were spun down 10 minutes at 4,000g at 4°C. Pellet was resuspended in 1 ml of sterile 10 mM MOPS (morpholinopropane sulfonic acid, pH 7.0) and 10 mM RbCl 20 min on ice. Cells were recovered by centrifugation at 4000g for 10 min at 4°C, and pellet was resuspended in 1 ml of 0.1 M MOPS (pH 6.5), 50 mM $CaCl_2$ and 10 mM RbCl, placed on ice for 1 hr, spun again, and resuspended in 0.2 ml of the last solution.

h) Plasmid purification. Transformed bacteria were plated on selective media, containing 10 μg/ml Ampicillin , 100 mM IPTG and 2% Xgal. White colonies containing the V_K10 genes were detected by hybridization to the

V_K10 probe (Paterson, B.M. et al. 1977). Plasmid DNA was purified using an equilibrium cesium chloride gradient (Radolff, R et al. 1967).

7. Sequencing

For sequencing the Maxam and Gilbert method was used (Maxam, A.M. and Gilbert, W. 1977, 1980). Both DNA strands of the cloned cDNA insert were sequenced.

To label the 5' terminus of each strand, two separate samples each containing 20 μg of plasmid with the cDNA insert were linearized each with a single restriction enzyme cleaving on either side of the insert. The 5' terminal phosphate residue were removed by phosphatasing with 0.5 units of calf alkaline phosphatase (IBI, New Haven, CT). Those ends were then kinased by 10 units of kinase (BRL Gattersburg, MD) and $\gamma^{32}P$ ATP 10 mCi/ml 5000 Ci/mmole (Amersham, Chicago, ILL). A second restriction enzyme digest cleaved at the 3' terminus of the 5' labeled strand released the 5'-end labeled immunoglobulin cDNA from the plasmid. Digested DNA was run on a 5% polyacrylamide gel to separate the labeled cDNA insert from the plasmid. The band containing the insert was electroeluted 150V 1 hr and used for the sequencing reactions. Following several phenol extractions and ethanol precipitatons to purify the labeled DNA fragment, it was ready for the Maxam and Gilbert chemical modifications. The base specific reactions for sequencing the 5'-end labeled DNA were G, G+A, C+T, C, A>C. DNA nucleotides are modified according to the Maxam and Gilbert method (1977, 1980). The base specific modifications were as followed: G was modified by dimethyl sulfate (DMS), G+A by 50 mM sodium citrate pH 4.5, and 0.1 mM EDTA. C+T was modified by hydrazine. C by hydrazine+5 M NaCl, and A>C by 1.2N NaOH and 1 mM EDTA.

Cleavage of DNA was by 1.0 M piperidine 30 min at 90°C. To read the actual sequence, samples were washed, denatured 5 min 100°C and 3 μl of 2,000 cpm per μl were loaded and electrophoresed at constant voltage 65W through a 40 cm denaturing TBE polyacrylamid gel. This method yielded data with no sequence ambiguities. In addition sequences were confirmed by sequencing the two complementary strands (Figure 3).

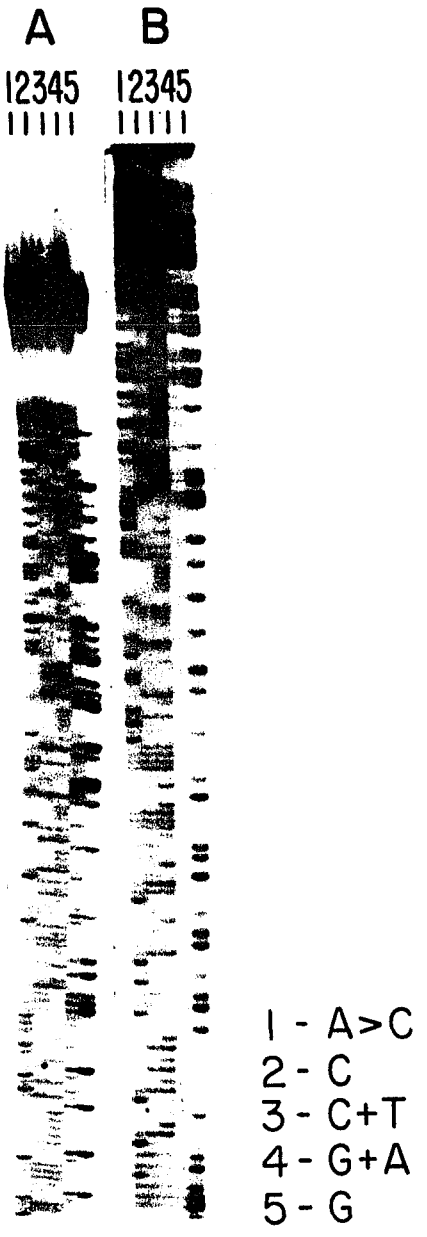


Figure 3: Maxam and Gilbert's sequencing gel.
200,000 cpm of γ^{32}P-ATP are used for each reaction. DNA nucleotides: G + A,G, A > C, C + T,C are modified respectively by sodium citrate, dimethyl sulfate, sodium hydroxide, hydrazine and hydrazine+salt. DNA is cleaved with 100 µl of 1.0M piperidine 30 min at 90°C. Samples are washed, denatured and 3 µl of 2,000 cpm per -µl are loaded and electrophoresed at constant voltage 65W on 8% denaturing polyacrylamide gel.

Results

In this work I present an immunochemical and molecular characterization of hybridomas secreting antibodies exhibiting $\beta 2 \rightarrow 6$ fructosan binding activity and bearing the A48 Id. The experimental strategies employed to carry out this goal were defining the antigen binding specificities by RIA, Northern blot analysis of light chain to define the V_K families of light chain mRNAs of the hybridomas which are known to share the V_H441-4 heavy chain gene and nucleotide sequencing using Maxam and Gilbert method to characterize the immunoglobulin heavy and light chains DNA and pinpoint somatic mutations if any.

1. Antigen binding specificity of the monoclonal antibodies

The antigen binding specificities of the monoclonal antibodies was determined by radioimmunoassay (RIA) using three types of polysaccharides as antigens. Rye levan, a $\beta 2 \rightarrow 6$ linked polyfructosan, bacterial levan, a $\beta 2 \rightarrow 6$ linked polyfructosan chain with $\beta 2 \rightarrow 1$ branch points, and Inulin a $\beta 2 \rightarrow 1$ linked polyfructosan.

MOPC-460, the negative control is an IgAk, with dinitrophenyl binding specificity. A48, and UPC10 are positive controls, which are BALB/c myelomas bearing the A48 Id and which bind strongly to the polyfructosan $\beta 2 \rightarrow 6$ linkage. 1-5-1, a series one antibody manifested strong binding to bacterial levan and weak binding to rye levan and no binding to inulin (Table II).

Of the eight antibodies of series two generated from BALB/c mice injected at birth with 10 ng anti-A48-Id antibodies and challenged one

Antigen binding specificity of monoclonal antibodies

Designation	Rye levan	Bacterial levan	Inulin
MOPC-460	225±79	376±116	44±2
A48	5,659±275	12,819±945	91±2
UPC10	1,353±95	19,233±723	188±30
1-5-1	819±272	12,576±1,867	223±53
2-1-3	4,013±328	12,570±903	8,347±1,024
2-1-10	802±222	8,503±1,516	249±147
2-8-2	1,057±161	16,232±11,418	98±147
2-11-1	1,592±206	13,286±805	186±40
2-11-3	102±3	12,354±697	151±26
2-12-10	113±76	11,230±1,650	40±13
2-9-17	62±15	13,434±2,915	2,228±645
2-28-9	666±294	5,232±1,421	61±21
3-14-9	5,623±947	1,413±263	3,583±733
3-27-6	1,693±293	96±11	177±88
3-9-9	118±30	126±14	105±19
3-101-14	103±19	15±12	1,482±275

Table II.

*Counts per mninute ±S.E. of triplicate determinations

month later with 20 μg levan, four antibodies displayed the $\beta 2 \rightarrow 6$ fructosan binding specificity of A48, i.e., bound to bacterial levan and rye levan as well. Those are the 2-1-10, 2-8-2, 2-11-1 and 2-28-9. It should be noted that the 2-1-10 and the 2-11-1 also displayed very weak binding to inulin. Three antibodies, the 2-11-3, 2-12-10 and 2-9-17, only bound to bacterial levan. The first, the 2-11-3, also bound weakly to inulin. Surprisingly, one antibody, the 2-1-3, bound to all polyfructosans.

Of the four series three antibodies generated from BALB/c mice hyperimmunized with polyclonal anti-A48-Id/KLH and challenged one month later with 20 μg levan, the 3-9-9, did not bind to any of the polysaccharides. The 3-27-6 bound to rye levan only. One bound Inulin the 3-101-14. The 3-14-9 monoclonal antibody bound to all three polyfructosans. Compared to the 2-1-3 of series two antibodies which also bound to all three polyfructosans, the 3-14-9 bound bacterial levan about ten-fold less and about two-fold less than to inulin.

2. Southern blot analysis of BALB/c liver DNA

The molecular configuration of the V_K10 germline gene family has been studied in normal BALB/c liver DNA. Normal BALB/c liver DNA was digested with the appropriate restriction enzymes and analysed by standard Southern blotting protocol. The probe used for hybridization was the non-productive V_K10 germline gene kindly provided by M. Shapiro from the Weigert laboratory.

Since the probe used is an EcoRI/HindIII piece, three sets of 10 μg of DNA were digested with the following DNA restriction enzymes. HindIII only, EcoRI only and a double digest EcoRI/HindIII. In Figure 4 DNA

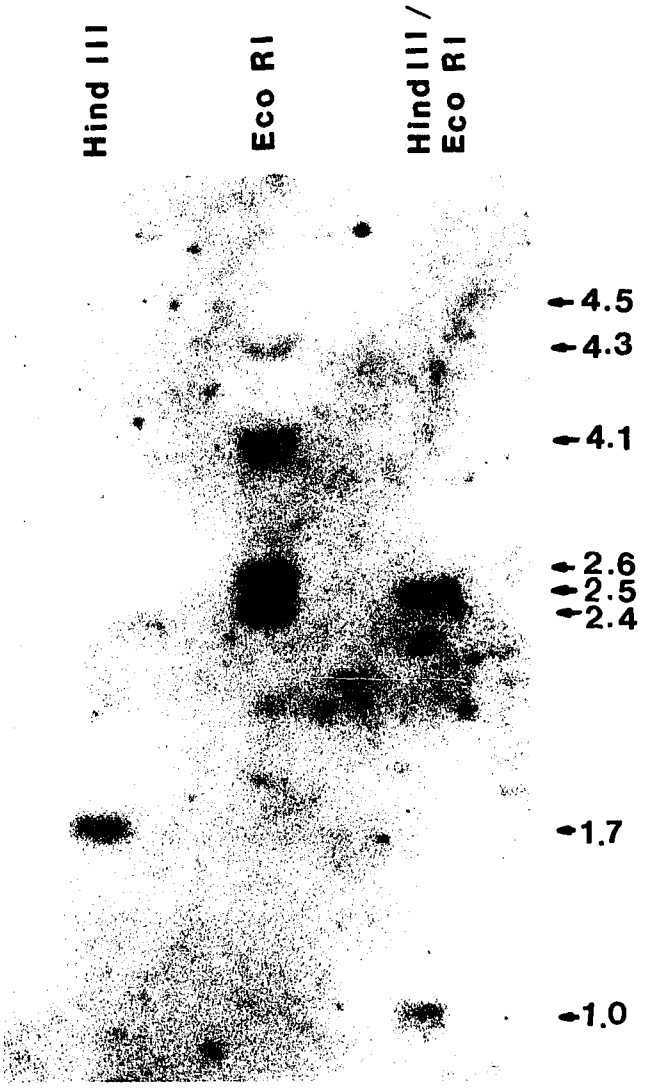


Figure 4: Southern blot analysis of normal BALB/c mice liver DNA.
Three samples of 10 μg BALB/c mice liver DNA were digested with EcoRI, HindIII and a double digest with EcoRI/HindIII, and electrophoresed through a 0.8% agarose gel, southern blotted, and hybridized to the ^{32}P nick-translated V_K10 light chain probe.

digested with HindIII only shows two bands hybridizing to the probe at 4.5 kb and 1.7 kb. Liver BALB/c DNA digested with EcoRI only shows four bands in positions 4.3, 4.1, 2.6 and 2.4 kb. The double digest EcoRI/HindIII of the BALB/c liver DNA reveals two bands at 2.5 kb and 1.0 kb. The fact that the number of bands in the double digest is smaller than in the single digests, suggests that each or some of the bands in the double digest present more than one type of fragment of V_K DNA. Hybridization of the probe to the digested eukaryotic liver DNA demonstrates a simple pattern of hybridization suggesting that this probe is probably not hybridizing to genes other than those belonging to the V_K10 light chain gene family.

3. Northern blot analysis of the hybridomas

The usage of V_K10 germline gene has been studied in the hybridomas by Northern blot analysis of cytoplasmic poly(A)$^+$ RNAs. Intact cytoplasmic RNA has been extracted from series 1, 2, 3 of the hybridomas and from the A48 myeloma. 200 ng of mRNA of each sample were electrophoresed through a 0.8% formaldehyde containing agarose gel, Northern blotted, and hybridized under highly stringent conditions with ^{32}P nick-translated V_K10 probe.

Figure 5 presents the hybridization results. A48 is a prototype myeloma expressing the A48 Id using the V_H441-4 heavy chain gene, two markers by which the monoclonal antibodies for this work were selected. From published protein sequence of the A48 myeloma it is known to use the V_K10 light chain gene. A48 mRNA has been used as a positive control. The 3-23-3 and MOPC-460 mRNAs use the V_K4 and V_K1 light chain gene respectively, both are being used as negative controls. With the exception of

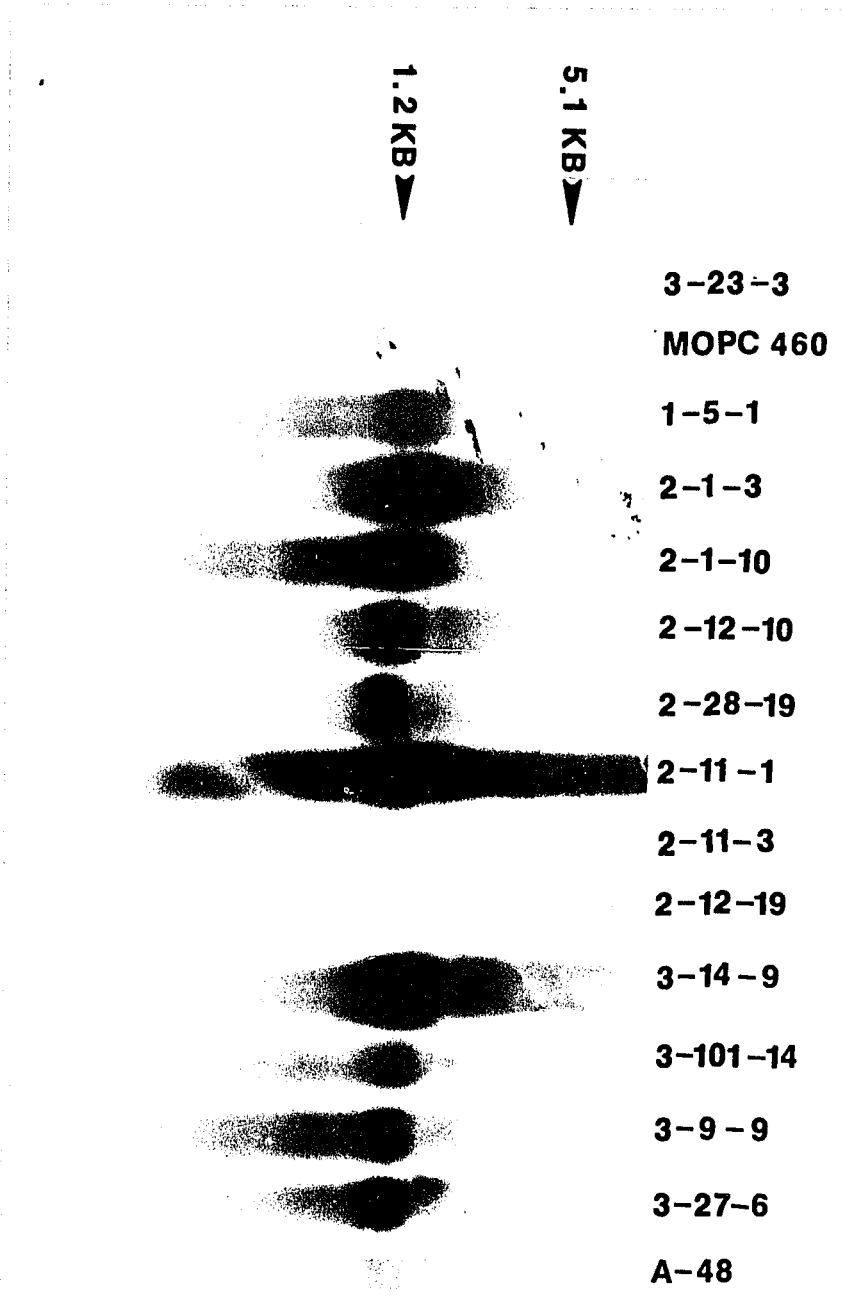


Figure 5: Northern blotting analysis of series 1 to 3 hybridomas.
200 ng of oligo(dT)-selected RNA was electrophoresed through a 0.8% formaldehyde containing agarose gel, Northern blotted, and hybridized to the ^{32}P-nick translated V$_K$10 probe.

two monoclonal antibody RNAs, the 2-11-3 and the 2-12-19, the V_K10 probe hybridized to mRNA species found in all the monoclonal antibodies, the size of which is consistent with that of mature immunoglobulin light chain mRNA, 1.2 kb. The differences in the intensity of the band are related to the relative level of immunoglobulin mRNA production, and sequence homology to the V_K10 probe. The prototype A48 is probably a low producer of V_K10 mRNA whereas the 2-11-1 and the 3-14-9 hybridomas appear to be the highest producers of V_K10 mRNA hybridizing to the probe.

Thus, there appears to be a preferential utilization of κ light chains related to the V_K10 family of the fructosan binding hybridomas expressing the A48-RI as presented in this work.

4. V_H sequence analysis of monoclonal antibodies bearing the A48 regulatory idiotype

To adequately address the issue of somatic mutation in the V_H441-4 heavy chain gene we established in our laboratory a technique of cloning ds-cDNA molecules corresponding to the full length expressed rearranged V_H gene.

After cloning the ds-cDNA into the pUC-9 plasmid, both DNA strands were sequenced by the Maxam and Gilbert method (Figure 3). To preferentially label the 5' end of each strand, cleavage with the appropriate restriction enzyme was carried out. Alkaline phosphatase treatment removed the 5' end phosphates. Those free ends were then kinased by kinase and $\gamma^{32}P$ ATP. A second restriction enzyme digest released the 5' end labeled immunoglobulin DNA from the plasmid. Digested DNA was

run on a gel. The immunoglobulin band was electroeluted and used for the sequencing reaction.

The clean labeled DNA fragment was chemically modified. The base specific reactions for sequencing the 5' end labeled DNAs were G, G + A, C + T, C, A > C. To read the actual sequence, reacted DNAs were run on a denaturing polyacrylamide gel. This method yields data with no ambiguities. In addition, results were confirmed by sequencing the two complementary strands.

The sequences of five heavy-chains of the monoclonal antibodies are presented in Figure 6. V_H441-4 and V_HX24 are germline genes, their sequences were reported by Hartman et al. (1984). V_HX24 family is the smallest murine V_H family identified, located at the 5' terminus of the murine V_H locus, and consists of the two members - the V_H441-4 and V_HX24 which are highly homologous V_H genes, those two germline genes contain more than 93% nucleotide sequence homology.The V_H441-4 germline gene is utilized by two myelomas - A48 and UPC10, expressing the A48 regulatory idiotype, and bind polyfructosan (Auffray, C. et al. 1981). The V_HX24 is the germline gene used by the galactan binding myeloma XRPC24 (Galactan is a $\beta_{1\rightarrow6}$ linked galactose molecule).

The series one hybridoma 1-5-1 is generated from BALB/c mice injected at birth with 10 μg A48 and challenged one month later with bacterial levan. 2-1-3, 2-1-10, and 2-28-9 belong to series two hybridomas generated from BALB/c mice injected at birth with anti-A48 Id antibodies and challenged one month later with bacterial levan. 3-14-9 a series three hybridoma, was obtained from an adult BALB/c mouse which was hyperimmunized with a syngeneic polyclonal anti-A48 Id-KLH conjugate

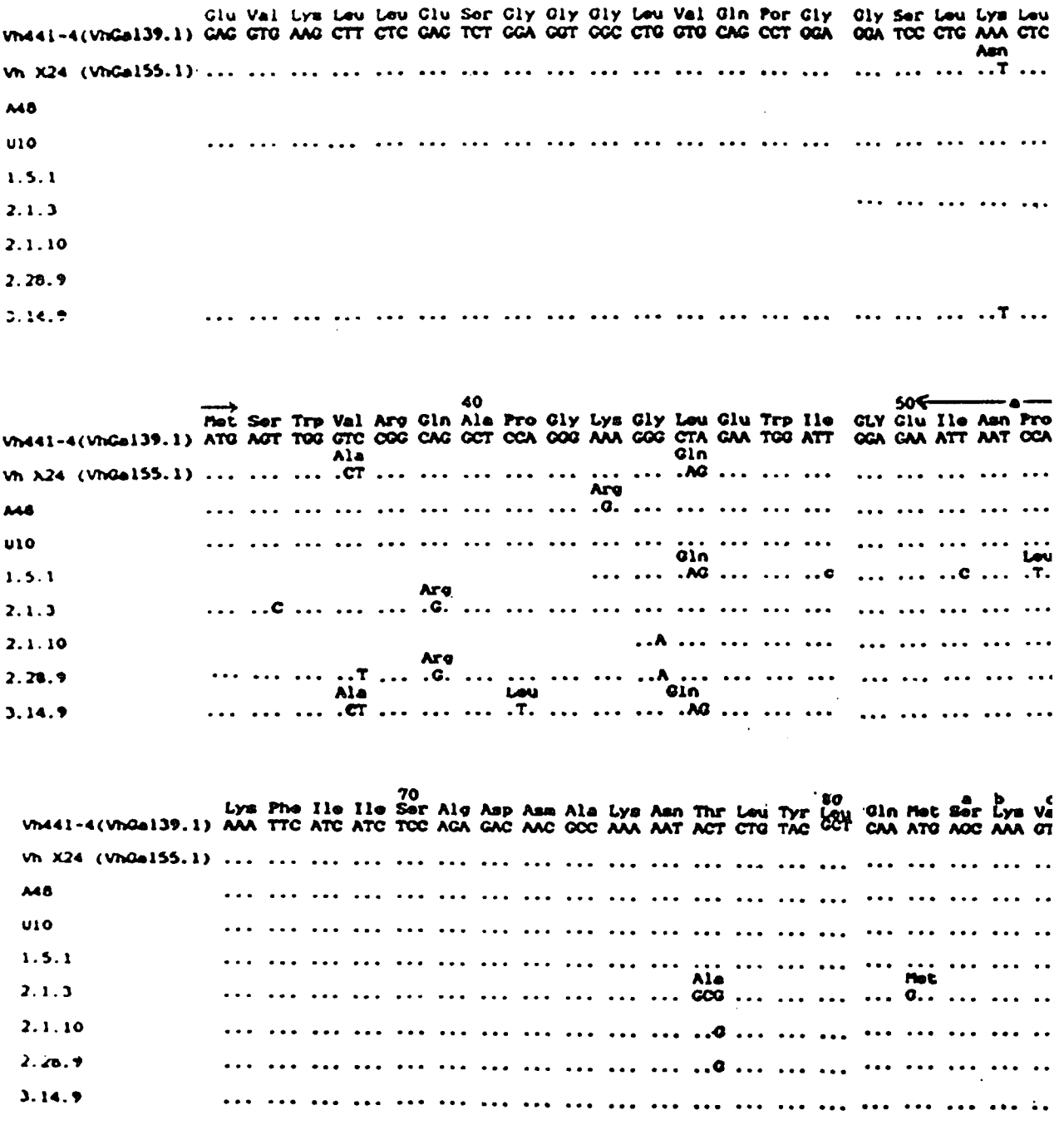
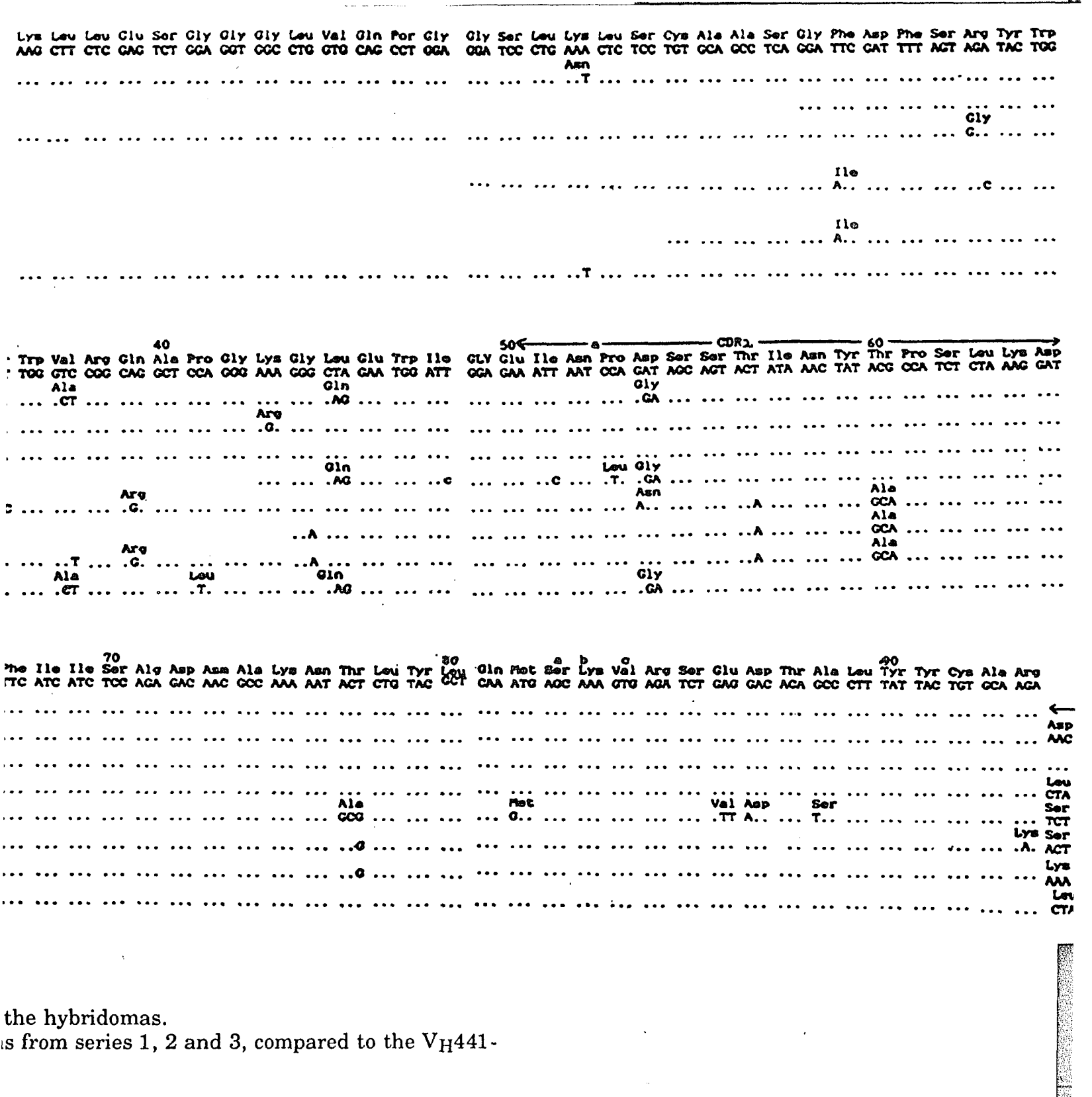


Figure 6: V_H sequences of the hybridomas.
V_H sequences of hybridomas from series 1, 2 and 3, compared to the V_H441-4 and X24 germline genes.



the hybridomas.
is from series 1, 2 and 3, compared to the V_H441-

and challenged after completion of the immunization schedule with bacterial levan.

A48 uses the J_{H3} sequence while UPC10 uses the J_{H2} genetic configuration, 1-5-1 and 3-14-9 use the J_{H4} rearrangement, and the 2-1-10 present the J_{H1} sequence suggesting a random usage of J_H segment, and thus no possible contribution of J_H gene to regulatory idiotype expression or antigen binding specificity.

1-5-1 and 3-14-9 hybridomas do express the same J_{H4} segment as well as identical 12 nucleotide-long D segments. This D segment is completely different from that used by A48 and UPC10, or any of the other hybridomas sequences, or any of those utilized by Legrain's six A48 Id^+ hybridomas (Legrain P. et al. 1985b). The 2-1-10 hybridoma's six nucleotide sequence D region which is ACT GGG has a shift in the reading frame and is compared to the Q-52 D sequence used by the A48 and UPC10 prototype myelomas. The Q-52D sequence is AAC TGG GAC GTG GCG, suggesting a common origin for the 2-1-10 D segment and the A48 D segment.

1-5-1 and 3-14-9 hybridomas share great homology also in the V regions, only several nucleotide differences are noted between their expressed V-gene and the consensus V_H441-4 germline gene utilized by A48 and UPC10. The most striking changes are in positions 37, 45, and 54, the respective changes are from Val, Leu, and Asp in the V_H441-4 gene to Ala (result only for the 3-14-9 V_H gene), Gln and Gly in the sequences of 1-5-1 and 3-14-9 hybridomas. All these nucleotide differences delineate the V_H441-4 gene from its closely related family member, the V_HX24 gene. Hence, these differences are in fact indicative of the two V_H genes as being derived from the closely related V_HX24 germline gene, and not the V_H441-4 gene. Overall, the 1-5-1 and 3-14-9 V_H sequences show few

nucleotide changes from the V_HX24 germline gene sequence which is consistent with their being IgM antibodies which generally have little somatic mutation.

More changes are identified in the VH region. Three hybridomas, the 2-1-3, 2-1-10, and 2-28-9, share common changes in three positions: a silent mutation in position 57, a change from T to A. An amino acid change in position 60 from Thr (ACG) to Ala (GCA) and a change in position 77 from T to G. As for the binding specificities the 2-1-3 binds rye levan, bacterial levan and Inulin. Whereas the 2-1-10 and 2-28-9 hybridomas bind rye levan and bacterial levan only, a fact which indicates that those changes are not the only factors affecting the binding specificity and the contribution of the V_L to the binding specificity is of great importance.

5. Analysis of V_H genes according to their binding ability to IDA-10

Analysis of the available sequences according to the hybridomas' ability to bind IDA-10 a monoclonal antiidiotope which binds to A48, is presented in Figure 7. The monoclonal antibodies in Figure 7 are presented in decreasing order of their ability to bind IDA-10. The 2-1-10 hybridoma represents about 10% of IDA-10 binding compared to UPC10 myeloma and 3-14-9 hybridoma (data not presented).

Amino acid sequences from positions 55 to 62 might play an important role in the IDA-10 binding. The strong binders: UPC10, 3-14-9, 1-5-1 and 2-28-9 monoclonal antibodies, share the amino acid sequence SSTINYTP in position, 55-65 amino acid, where amino acid T is in position 61. While 2-1-3, 2-1-10 hybridomas, the weak binders share the sequence SSTINYAP, where amino acid A is in position 61. The XRPC24 myeloma

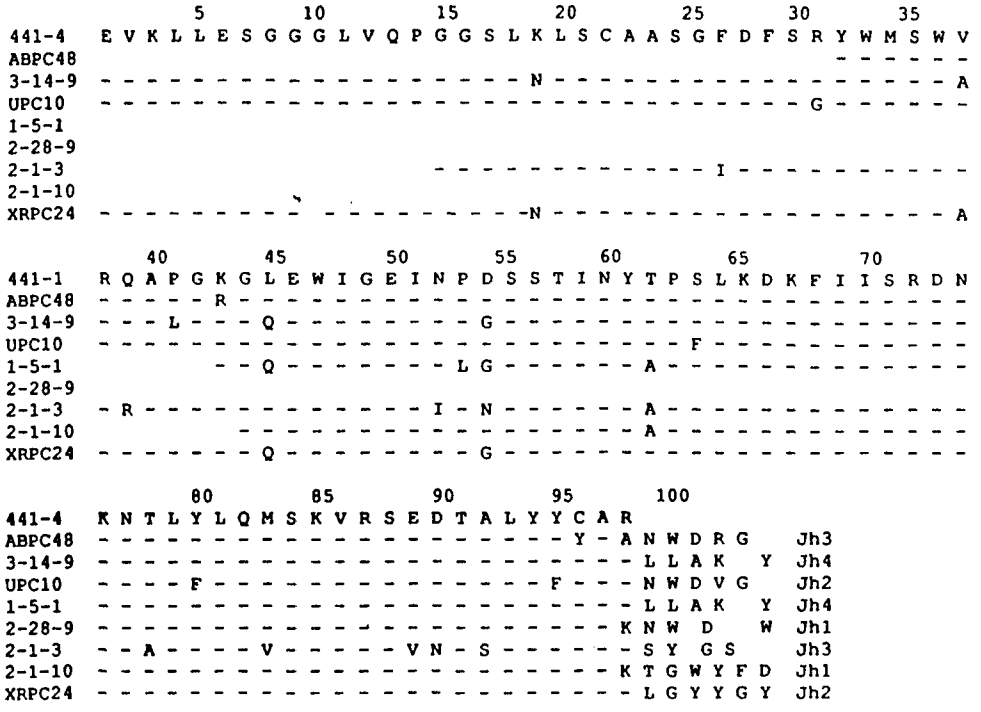


Figure 7: V_H genes according to their binding ability to IDA-10
Sequences of monoclonal antibodies presented in decreasing order of their ability to bind IDA-10. The 2-1-10 hybridoma at the bottom represents about 10% of IDA-binding compared to UPC10 myeloma

which is a nonbinder, whilst sharing the sequence of the first group, namely the strong binder, does not utilize the light V_K10 but uses the V_K4 light chain.

6. V_K sequence analysis of monoclonal antibodies bearing the A48 RI

The sequencing strategy for the V_K light chain gene is analogous to that used for the V_H sequencing, except for the fifteenmer oligonucleotide 5' -GAT ACA GTT GGT GCA A - 3' which is complementary to the 5'-end of the mRNA of the κ light chain constant region position 116-111. The rearranged V_K10 gene derived from the 2-28-9 hybridoma has been sequenced. The 2-28-9 hybridoma has been generated by injected of 10 μg A48 at birth to BALB/c mice and challenged one month later with 20 μg levan. The sequence obtained is compared in Figure 8 to the partial V_K sequences published by Lagrain, P. et al. (1985a).

The consensus sequence is the derived consensus sequence for V_K10 gene. ABPC48 is the A48 myeloma bearing the A48 Id, and strongly binds to bacterial levan and from previous protein sequences known to use the V_K10 light chain gene. 3-10-14, 3-10-16 and 3-23-3 are the three hybridomas generated in a similar manner as the hybridomas presented in my work, 3-10-4 has been generated from BALB/c mice hyperimmunized with polyclonal anti-A48 Id/KLH and challenged one month later with 20 μg levan and binds Inulin. 3-10-16 was generated from BALB/c mice immunized with IDA10-KLH conjugate and binds bacterial levan, 3-23-3 was generated from BALB/c mice immunized with IDA23-KLH conjugate and binds Inulin and very weakly bacterial levan.

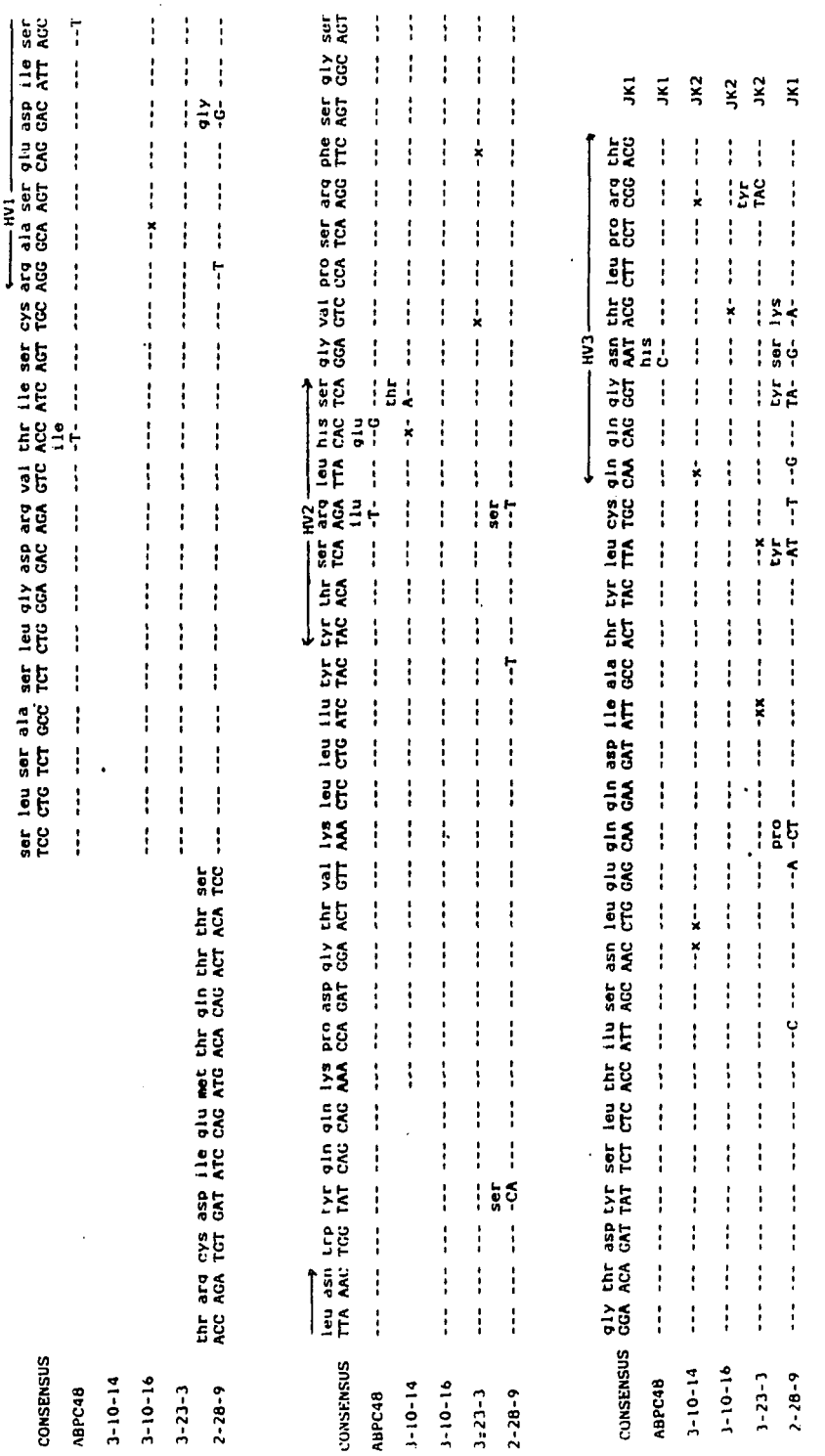


Figure 8: V_L sequences of hybridomas expressing the A48-RI. V_L sequences of antibodies expressing the A48 RI compared to the consensus sequence.

The comparison of the V_K10 sequence obtained from the 2-28-9 hybridoma, to the published sequences reveals great homology suggesting that these chains are the products of genes derived from a unique V_K germline gene which is used by hybridomas utilizing the V_H441-4 heavy chain gene bearing the A48, and bind levan.

An interesting observation is that although the 5' end of the J_{K1} germline gene is tryptophane (TGG), an arginine (CGG) is found at position 96 in all the monoclonal antibodies utilizing the J_{K1} gene i.e., the consensus sequence, ABPC48, and 2-28-9. The nucleotide sequence at position 96 corresponds to the V_K - J_{K1} junction. Arginine at that location instead of tryptophane is consistent with the interpretation that the first base arginine residue originated from the V_K gene.

Discussion

The observations presented in this communication on monoclonal antibodies bearing the A48 regulatory idiotype, indicate that the majority of the monoclonal antibodies are encoded by V_H genes derived from the X24 germline gene family and V_L genes derived from the V_K10 family. Idiotypes represent phenotypic markers of the variable region genes, which encode the antigen specific receptors of B and T cells. Idiotypic links reliably interconnect the B and T cell clones comprising the immune system in spite of their tremendous diversity which manifests itself in their recognition of foreign as well as self antigens (Hood, L. et al. 1985).

The network theory views the immune system as a web of variable domains, whose major function is recognition of self-idiotypes which should culminate in the maintenance of cellular and molecular homeostasis through idiotype-antiidiotype interactions (Jerne, N.K. 1974; Burnet, F.M. 1959). Accordingly the major pattern of this constantly occurring idiotype-antiidiotype clonal interaction is suppression, which alone is able to maintain the clones in a steady state of dynamic equilibrium. Antigen, idiotype, or antiidiotype can upset this equilibrium leading to the proliferation of clones specific for antigens (Ab_1), which in turn, will stimulate anti-Id clones (Ab_2). This scenario represents a new stable steady state configuration. Thus, the network theory envisions the immune system existing in a succession of stable states which are repeatedly attained after its resident clones undergo transitional states of disequilibrium caused by antigen stimulation.

The initial interaction in the cascade between idiotype and antiidiotype is viewed as a crucial one since it is at this point that the clones bearing RI are regulated and can achieve dominance.

To study immunochemical and molecular properties of antibodies bearing A48 regulatory idiotopes, hybridomas were prepared from mice immunized with A48 protein or anti-A48-Id antibodies and then challenged with bacterial levan. Low doses of antiidiotypic antibodies expanded B cell precursors bearing the corresponding Id. Anti-Id antibodies administered to neonatal or adult mice preferentially expanded Ab_1 or Ab_1-like clones. The syngeneic origin of the antiidiotypic antibodies makes it highly likely that those clones were activated through regulatory idiotopes. Since only regulatory idiotopes play a role in clonal expansion while the conventional idiotopes become mere by-products of the system because of their non-inheritable nature. Therefore the normally silent A48-Id was expressed by a large proportion of anti-BL antibodies in BALB/c mice immunized at birth with either the A48 monoclonal protein or anti-A48-Id antibodies, and challenged one month later with levan.

The existence of regulatory idiotypes implies that self-recognition plays an important role in clonal interactions and that cross-reactive regulation can lead to the dominance of some clones and suppression of others, depending on concentration, affinity, and pattern of cross-reactivity of RI recognized by anti-idiotypic antibodies or idiotype specific regulatory T cells. The existence of regulatory idiotopes on antibodies produced by cells expanded by anti-Id suggested that such idiotopes were V_H and V_L germline gene markers.

Since one of the criteria to select the hybridomas for this work was their usage of V_H441-4 heavy chain gene, it was of great significance to

determine the type of light chain those monoclonal antibodies utilize. From protein sequence data two myeloma proteins which are known to bind polyfructosan and express the A48 regulatory idiotype, the UPC10 and MOPC-173 use the V_K10 light chain gene (Kabat, E.A. et al. 1983). Therefore it appeared important to determine whether or not the idiotope recognition of anti-Id antibodies requires the association of the V_H441-4 with V_K10 light chain gene. Identification of structural correlates of idiotopes was the major issue addressed in my work. Furthermore, from previous reports it appears that in heavy and light chains, combinatorial association plays an important role in the expression of idiotypic specificities.

The significance of pairing V_H and V_L genes as shown in the A48RI system, in which the monoclonal antibodies use the V_H441-4 gene in association with the V_K10 gene is reported in another idiotypic system by Paul, F.R. et al. (1986) in which they show that only a certain pairing of light and heavy chains will generate an antibody expressing an idiotype. In that system an interstrain cross-reactive idiotype (CRI), CRI_D, associated with anti-P-azobenzenearsonate antibodies of the A/J strain of mouse and distinguishable, by some but not all of its idiotopes, from the major anti-P-azobenzenearsonate idiotype (CRI_A). Molecules carrying the CRI_D idiotype have heavy chain variable segment sequences that are identical or nearly identical to that of the germline encoded heavy chain variable sequence of CRI_A. Their light chain variable sequences are very similar to those present in a third idiotypic family, CRI_C, that is a minor CRI in the A/J strain but a major CRI in BALB/c. This appears to represent a form of combinatorial diversity, in which the heavy and light chain variable genes of two unrelated idiotypic families interact to form a third family, all

involving antibodies of the same antigen-binding (anti-P-azobenzenearsonate) specificity (Robbins, P.F. et al. 1986).

Our data strongly support this idea (Victor-Kobrin, C. et al. 1987). Poly(A)$^+$ enriched heavy-chain mRNA from the hybridomas derived from clones stimulated with anti-A48-Id antibodies, hybridized to the V_H441-4 probe under stringent conditions (Victor-Kobrin, C. et al. 1985a). In a similar way light chain mRNA analysis by Northern blot revealed preferential usage of the V_K10 gene. In Fig. 5 Northern blot analysis of series 1 to 3 hybridomas showed that with the exception of mRNA extracted from the 2-11-3 and the 2-12-19 hybridomas, the V_K10 gene hybridized to all the monoclonal antibodies. All those hybridomas presented here as V_K10 positive were previously (Victor-Kobrin, C. et al. 1985a), found to be V_H441-4 positive, and the two V_K10 negative hybridomas were found to be V_H441-4 negative as well.

These data support the notion that V_H genes derived from the V_H441-4 germline gene paired with V_L genes derived from the V_K10 family yield Id-positive antibodies. These results fit another piece into the large puzzle of the generation of antibody diversity in general, and specifically in the V_K10 family.

Recombination experiments, and expression of recombinants of the V_H441-4 rearranged gene and the V_K10 gene might give an exclusive answer to the question of idiotype expression, and the importance of pairing the V_H441-4 gene and V_K10 gene in expressing the A48 regulatory idiotype (Tonegawa, S. et al. 1977).

A key prediction of the regulatory idiotype concept is that clones exhibiting different antigenic specificities can be coordinately activated by virtue of shared regulatory idiotopes (Paul, W.E. et al. 1982). The antigen

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We define "cross-regulation" as a humoral and cellular mechanism controlling discrete sets of clones via recognition of self-regulatory idiotopes. This concept emphasizes that there is a major difference between the expression of clones expanded via recognition of foreign antigen and the clonal proliferation that occurs after recognition of self idiotope.

Antigenic stimulation should invoke a low degree of cross-regulation since the activated clones have a very restricted antigenic specificity and can express a multitude of idiotopes depending on the number of distinct epitopes. The activation of clones induced by anti-Id would be Id restricted but would exhibit a broader spectrum of specificities. This spectrum is probably limited to a group of antigens reacting with antibodies encoded by the active V genes of a single V_H gene family. Cross-regulation also implies a competition between clones, leading to the dominance of those that express regulatory iditopes and the suppression of other clones. Our data strongly support this idea since we observed that A48-Id and UPC10-Id, minor idiotopes of a conventional anti-levan response, became dominant idiotypes in animals receiving minute amounts of Id-bearing or anti-Id antibodies at birth. The ability to expand clones with various antigen specificities by anti-Id antibodies, particularly in ontogeny, demonstrates that cross-regulation mediated by the recognition of self-regulatory idiotypes can shape the immune repertoire. This also shows that the equilibrium established between clones in the immune system, which is based on idiotypic links, is weak, since minute amounts of Id or anti-Id could upset it and lead to the clonal dominance of a minor Id.

The emergence and evolution of the immune and idiotypic repertoires of the immune system are closely intertwined, given that idiotypes are after all the antigenic markers of the antibody molecule's

variable region. Those factors which can affect the expression of these two repertoires will differ according to the maturity of the immune system. At this point I would like to distinguish between those forces affecting the primary or early repertoire versus those influencing the expression of the later secondary repertoire.

The primary immune and idiotypic repertoires are present at birth and are established during the early stages of B cell differentiation (i.e., the pre-B cell stage) when these cells lack any membrane-associated idiotype bearing immunoglobulin receptors which can interact with any external antigens or anti-idiotypic molecules. Therefore, at this early stage, the repertoire is determined solely by the genetic programming which is executed to generate the functional immunoglobulin variable regions. Those molecular mechanisms which can influence this programming process will exert the largest influence on the development of the primary immune and idiotypic repertoire.

An examination of the molecular processes involved in the generation of functional variable regions for immunoglobulin heavy and light chains, reveals several potential points where changes can be introduced into the germline encoded repertoire. The variable regions of the immunoglobulin heavy and light chains are encoded by three discrete genomic segments which through a series of recombinatorial processes are juxtaposed to create the intact variable region. The V_H, D and J_H segments, and the V_L and J_L segments encode the variable regions for the heavy and light chains, respectively. Each of these five types of segments occur in multiple copies in the genome, with each copy encoding a different amino acid structure. Therefore, even though restrictions might exist in the pairing of different heavy and light chain variable regions, a

tremendous source of variable region diversity can result from the random combinatorial association of the different genomic segments which encode the respective heavy and light chain variable regions. Furthermore, additional somatic diversity can be introduced as a result of the recombination events which juxtapose the various variable region genomic segments. The recombination junctions between these various genomic segments exhibit great nucleotide diversity as a result of both the flexibility allowed in this system as to the choice of the exact point at which the recombination will occur in order to maintain the correct reading frame as well as the "de novo" addition of nucleotides which has been frequently observed in this region. Both of these factors which contribute to the "junctional diversity" seen in this system, play an important role in determining the coding region of the third hypervariable regions (CDRIII) of the heavy and light chains. Because it is in CDRIII where these recombinatorial junctions occur in the antibody molecule, this region not only plays a pivotal role in forming the antibody combining site but also has been shown to be important in the expression of idiotypes in several systems.

Legrain, P. et al. (1985a,b) indicate the importance of the third hypervariable region of V_H in correlation with the A48-Id and the antigen binding specificity. Two series of monoclonal antibodies have been obtained from BALB/c mice immunized against two anti-ABCP48 antiidiotypic antibodies. They were divided into two serologically different classes. Class I antibodies bind only the immunizing antibody class II antibodies display a broad binding capacity to various antiidiotypic antibodies, and some bind levan, as does ABPC48. Northern blot analyses and partial mRNA sequencing show that all class II antibodies express the V_H gene coding for

ABPC48 and UPC10 anti levan antibodies associated with a variety of D and J segments, similar to those shown in my experiments. The third hypervariable region of the sequenced antibody with anti levan activity is structurally related to that of ABPC48 and UPC10 antibodies but has a different genetic origin. This study indicates that the identification of idiotype-related antibodies arising from antiidiotypic immunization may be misleading, if based on their antigen-binding properties; and it stresses the importance of structural approaches for the analysis of regulatory mechanisms ruling immune responses.

Another potential source of diversity for the primary immune and idiotypic repertoires might be the occurrence of somatic mutations in those genomic sequences encoding the hypervariable regions of the immunoglobulin heavy and light chains (Selsing, E. and Storb, V. 1981). Although the exact extent to which this mechanism does play a role at this stage of B cell maturity is quite unclear because few mutations are generally observed in the variable regions of IgM antibodies when their sequences were compared to the germline gene sequences from which they were derived.

Finally, it should be mentioned that because the antigen binding sites and the expression of idiotypic determinants are both generated from the interactions of the variable regions of the heavy and light chains, whatever limited combinatorial association of heavy and light chain variable regions is permitted to occur, will still increase the diversity of the primary immune and idiotypic repertoire.

Like A48-RI expression it was demonstrated by molecular characterization of antibodies bearing the Id-460 (Elaine, A.D. et al. 1986) that immunoglobulin idiotype determinant expression can be the result of

the expression of nonidentical but highly homologous genes. The heavy chain immunoglobulin genes encoding a variety of antibodies specific for DNP (2,4-dinitrophenyl) or Pasteurella pneunotropic and bearing the dominant idiotype of MOPC-460, ID-460, were cloned and sequenced. The V_H genes encoding the M460 and D35 (DNP binding) antibodies were found to be homologous but not identical to the V_H gene encoding the LB8 (P. pneumotropica binding) monoclonal antibody. Two of at least eight genes in the V_H460 cross-hybridizing gene family can encode the Id-460 positive antibodies. The V_H460 gene family overlaps with the gene family described by V_H36-60 and more completely describes this germline V_H gene family. It has also been demonstrated that in those antibodies their is a genetic requirement for V_{K1} expression in order to observe the expression of Id-460 in anti-DNP antibody responses. In general in that work Elaine, A.D. et al. (1986) demonstrated that immunoglobulin idiotype determinant expression can be the result of the expression of nonidentical but highly homologous genes in the V_H460 cross-hybridizing V_H gene family and also in the V_K1 cross-hybridizing V_L family.

If the A48 regulatory idiotype is a marker of the V_H441-4 gene, and may be of the V_K10 gene, one may ask how can we explain the diverse array of antigen specificities among the A48 regulatory idiotype bearing hybridomas. The answer for that question probably lays in the precise sequencing of the V_H and V_L genes utilized by those hybridomas. Different binding specificities might be related to the expression of the same V_H gene in the context of different D (diversity) or J_H segments or of somatic mutations present in the V_H region as well as slightly different light chains which might subtly alter the combining site such that, although the

antibodies still recognize the fructosan determinant, the precise specificity differs from that of A48 and UPC10.

Nucleotide sequencing analysis of the V_H441-4 heavy chain genes and V_K10 light chain genes of the A48-RI system, clearly reveals changes in the rearranged gene structures when compared to the germline gene (Fig. 6). Changes might be a result of somatic mutation, or the result of junctional diversity, which originates during B cell development and maturation. Some of the changes result in silent mutation, whereas most of the changes are replacement mutations. Amino acids Leu. and Asp. in positions 45 and 54 respectively, are characteristic to the V_H441-4 agene, while Gln. and Gly., in the same respective positions, are characteristic of the V_HX24. From the sequencing data it seems that the A48 and UPC10 myelomas, the 2-1-3 and 2-1-10 hybridomas originated from the V_H441-4 gene whereas the 1-5-1 and 3-14-9 hybridomas originated from the V_HX24, a germline gene which is characteristic of the galactan binding antibodies. The usage of those two different heavy chain germline genes is not surprising since these two germline genes share very high homology > 93%. Three hybridomas 2-1-3, 2-1-10, and 2-28-9, which use the V_H441-4 gene, share common changes in three positions, a silent mutation in position 57, a change from T to A, an amino acid change in position 60 from Thr. (ACG) to Ala. (GCA), and a change in position 77 from T to G, suggest a common ancestor for these three rearranged genes.

The D regions are very different, 2-1-10 hybridoma represent a shift in the reading frame of D compared to the Q-52 gene used by A48 and UPC10. 1-5-1 and 3-14-9 both utilize the same D gene and both use the J_H heavy chain gene. In addition, the variable region genes of the 1-5-1 and the 3-14-9 share great homology. In spite of the great homology those two

hybridomas present different binding specificities. 1-5-1 binds bacterial levan, whereas 3-14-9 binds bacterial levan and Inulin, suggesting contribution of the V_K10 light chain to the different binding specificity.

The usage of J segment is random and in general is homologous to other Js, suggesting no contribution of J or D (as previously discussed) to the expression of RI or binding specificity. Nucleotide sequence changes affect the antigen binding specificities, in particular if the changes are introduced into the hypervariable regions.

A possible important sequence which might determine the expression of the A48-RI is in positions 55 to 62 of the heavy chain gene. In Fig. 7 sequences of the hybridomas are presented according to their ability to bind IDA-10 an anti-A48 monoclonal antibody. The comparison of the hybridomas indicates that there is perfect homology between the strong binder in position 55 to 62, whereas the weak binders have a different sequence in which Ala. in position 60 substitutes the Thr., which is characteristic of the strong binders. Position 55-62 correlates to an area in the CDR-2 of the gene, and fits the fact that the binding of IDA-10 to A48 is inhibited by levan, whose binding is correlated with the hypervariable region. The XRPC24, which does not bind IDA-10, but still has sequence homologies to that of the strong binders, uses another V_K gene - the V_K1 gene, compared to the V_K10 used by the strong binders.

The results of partial light chain sequencing of A48 and of antibodies induced by antiidiotypic immunization, reveals nearly perfect homology, the great homology of the 2-28-9 light chain from series 2 antibodies (Fig. 8), suggests that these chains are products of genes derived from a unique V_K germline gene. Like in the usage of the V_H gene also in the usage of the V_L gene, there is no preferential usage of J_K in the different monoclonal

antibodies, the usage of J_K is random. Most of the changes compared to the consensus sequence are introduced in the hypervariable regions 2 and 3. These observations, in the heavy and light chain sequences, indicate that the heavy and light hypervariable regions might contribute to define the structure of A48 idiotopes.

Remarkably, the V_K sequences identified in the A48 Id^+ monoclonal antibodies are products of genes derived from the same germline gene as the V_K genes used by the anti-arsonate antibodies (Siegelman, M. et al. 1981), and the V_K genes utilized by anti oxazolone antibodies (Kaartinen, M. et al. 1983). Anti-arsonate antibodies anti-oxazolone antibodies, and A48 cross-reactive idiotypes express different idiotypes whose structural bases are determined mainly by one or a limited number of H-L pairing. The fact that those three families of antibodies with different idiotypic determinants and different antigenic specificities use identical or highly homologous V_K genes demonstrates strongly the economy of the genetic system that is able to construct, from common elements, molecules with completely different functions.

My study was aimed both at adding to, and consolidating the current understanding of idiotype-derived events. I accomplished this by examining, at the molecular level, the light and heavy chain immunoglobulin regulating idiotypic expression in the A48 RI system, within the context of its antigen-binding specificity to various polyfructosan determinants.

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