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Idiotypic selection of an antibody mutant with changed hapten binding specificity, resulting from a point mutation in position 50 of the heavy chain

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Somatic mutation occurs at a low rate in the rearranged antibody V region genes of the hybridoma line B1-8.δ1 which expresses an antibody with specificity for the hapten 4-hydroxy-3-nitro-5-iodo-phenylacetyl (NIP). A mutant was selected which had lost a binding site-related idiotope but retained most of its other idiotypic determinants. The mutant had concomitantly lost NIP binding and acquired specificity for dinitrophenylated bovine serum albumin. It carried a single point mutation in position 50 of the heavy chain, resulting in the replacement of an arginine by a glycine.

Key words: structural basis of antibody specificity/somatic mutation/idiotypic selection/internal image

Introduction

The antibody repertoire expressed in B lymphocytes newly arising in the bone marrow is further diversified by somatic point mutations in the antibody variable (V) region genes (Bernard *et al.*, 1978; Gearhart and Bogenhagen, 1983), occurring stepwise in the course of the expansion of those cells (McKean *et al.*, 1984; Rudikoff *et al.*, 1984; Sablitzky *et al.*, 1985). The somatic mutants are selected by antigens and perhaps also in the idiotypic network (Jerne, 1974) and, in addition to selection for high binding affinity, negative selection could occur through suppression of the wild-type. It is thus of importance to understand how somatic point mutations affect antigen binding and idiotypic properties of antibodies. The present work represents a continuation of our own efforts in this direction. We have previously shown that a single point mutation in the D region of a hapten-binding antibody can profoundly change the specificity of that antibody in the idiotypic network, leaving its hapten-binding specificity intact (Radbruch *et al.*, 1985). We now demonstrate that a single amino acid substitution in the second hypervariable region (CDR2) of the heavy chain, of the same antibody, results in a profound change of its hapten binding specificity, while most of the idiotypic determinants of the antibody are left intact. The mutant has also lost, however, certain binding site-related idiotypic determinants and, since it was selected for the latter property, its isolation also represents a direct demonstration that a change of hapten binding specificity based on a single somatic point mutation can be selected not only by the hapten itself but also by idiotypic interaction.

Results

The experimental system and isolation of idiotope mutants

The hybridoma line B1-8.δ1 expresses an IgD antibody (antibody B1-8.δ1) which is of C57BL/6 origin, carries λ1 light (L) chains and binds the hapten 4-hydroxy-3-nitro-5-iodophenylacetyl (NIP) (Neuberger and Rajewsky, 1981). Antibody B1-8.δ1 is a class switch variant of the IgM antibody B1-8 (Reth *et al.*, 1978) which carries an identical V region. The B1-8 V region is encoded by germ line sequences except for an aspartic acid residue at the V-D border (Bothwell *et al.*, 1981, 1982). A series of monoclonal anti-B1-8 antibodies are available each of which defines an idiotypic determinant or idiotope on the B1-8 V region (Reth *et al.*, 1979; Rajewsky *et al.*, 1981). Some of these idiotopes are related to the hapten-binding site in that binding of the corresponding anti-idiotypic antibody to B1-8 can be inhibited by the hapten (group 1 idiotopes). An example of such an idiotope is idiotope Ac146, recognized by the anti-idiotope antibody Ac146. Other idiotopes (group 2) appear to be further apart from the hapten-binding site. The binding of the corresponding anti-idiotope antibodies to B1-8 is inhibitable by hapten-carrier complexes but not by the hapten alone. An example of such an idiotope is idiotope Ac38, recognized by the anti-idiotope antibody Ac38.

Antibodies Ac38 and Ac146 as well as most of the other group 1 and 2 anti-idiotopes inhibit each other in their binding to the

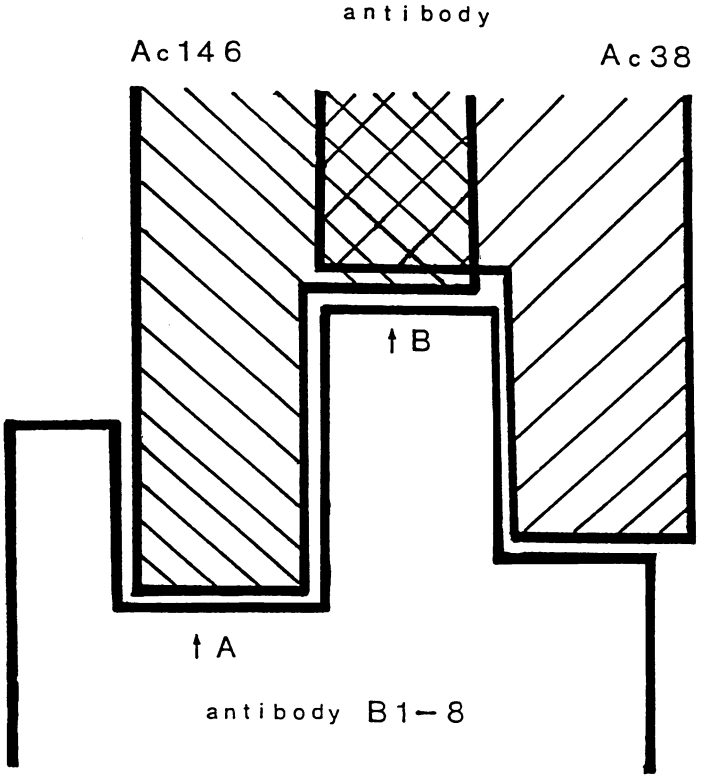

Fig. 1. Schematic and hypothetical interaction of the V region of antibody B1-8.δ1 with the binding site-related anti-idiotope antibody Ac146 and the anti-idiotope antibody Ac38. **(A)** Hapten binding cleft. **(B)** V region surface for which antibodies Ac146 and Ac38 compete. This is thought to include part of the B1-8.δ1 D region. For further explanation see text.

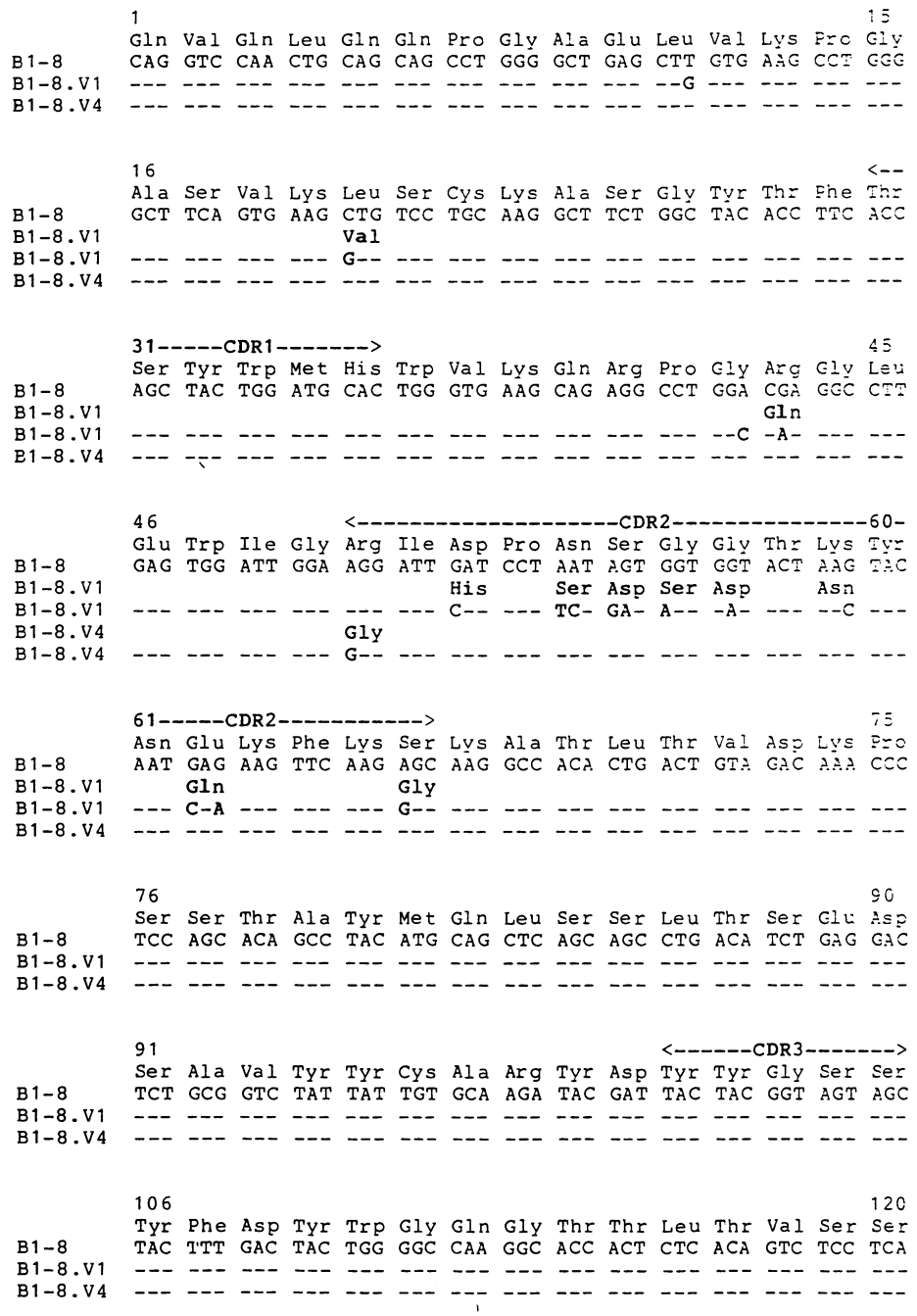

Fig. 2. Nucleotide sequence of the VDJ segments of B1-8, B1-8.V1 and B1-8.V4. The corresponding amino acid sequences are also shown.

B1-8 V region. Therefore, it would appear that they bind to partially overlapping idiotypic determinants which either include (Ac146; group 1 idiotopes) or exclude (Ac38; group 2 idiotopes) the hapten-binding site (Figure 1).

On the basis of the scheme in Figure 1 we can undertake a structure–function analysis of the B1-8 V region in terms of hapten binding and idiotypic specificity by isolating and characterizing B1-8 mutants which have either lost idiotope Ac38 and retained idiotope Ac146 or vice versa. This can be done by a variation of the method developed by Holtkamp *et al.* (1981), allowing mutant isolation by fluorescence activated cell sorting (FACS). The binding of a given fluorescent anti-idiotope antibody to the B1-8.δ1 target cell is inhibited by an excess of an unlabelled second anti-idiotope antibody. Mutants which have selectively lost the target idiotope of the second antibody are stained under these conditions by the first antibody and can be sorted out by FACS (Brüggemann *et al.*, 1982).

An $Ac38^-$,$Ac146^+$ mutant isolated in this way (B1-8.V3) was consistent with the model in Figure 1 in that it had lost most B1-8 idiotopes and retained hapten binding. It carried a single point mutation at position 103 of the heavy chain, i.e. in the D region, leading to a glycine to aspartic acid exchange (Radbruch *et al.*, 1985). An $Ac146^-$,$Ac38^+$ mutant (B1-8.V1) had also been isolated previously. As predicted from the model in Figure 1, it had lost hapten binding and retained most of its group 2 idiotopes (Brüggemann *et al.*, 1982). However, in structural terms the situation was complex since the mutant was the product of a recombinational event involving another V_H gene so that it carried 10 amino acid exchanges in the heavy chain, most of them localized in CDR2 (Dildrop *et al.*, 1982; Krawinkel *et al.*, 1983; see also

Table I. Analysis of transfected J558L cells for cytoplasmic Ig expression by immunofluorescence

	Plasmid	Transfection efficiency	$IgM^+/Ac38^{+a}$	$IgM^+/Ac146^{+a}$	$Ac38^+/Ac146^-$
Transient:	pSV-Vμ1	2–5%	78/78	98/98	n.d.
	pSV-V4	1–2%	75/75	0/135	69/69[b]
Stable:	pSV-Vμ1	10^{-5}	$10^2/10^2$	n.d.	0/10[4c]
	pSV-V4	10^{-6}	$10^3/10^3$	n.d.	$10^4/10^{4b}$

For transient expression the cells were tested 40–48 h after transfection.
[a]Cells were double stained by a sandwich test using anti-idiotypic antibodies Ac38 (IgG1) and Ac146 (IgG1) as first and FITC-labelled anti-IgG1 and TRITC-labelled anti-IgM as second antibodies. The numbers represent transfected cells, identified in the fluorescence microscope as IgM positive and subsequently analysed for idiotope expression.
[b]Cells were double stained using anti-idiotypic antibodies Ac38 (IgG1) and Ac146.2b (IgG2b) as first and FITC-labelled anti-Ig1 and TRITC-labelled anti-IgG.2b as second antibodies.
[c]Determined by a fluorescence inhibition assay. Cells were first incubated with 10 mg/ml unlabelled antibody Ac146 and then stained with TRITC-labelled antibody Ac38 (IgG1). No fluorescent cell was detected under these conditions among 10^4 IgM-positive cells.

Figure 2). Because of this complication we have isolated a second $Ac146^-$,$Ac38^+$ mutant of B1-8.δ1, by six rounds of FACS, starting from a population of 9×10^7 B1-8.δ1 cells. The cloned mutant cells, designated B1-8.V4, stained brightly with fluorescent antibody Ac38 but were negative for the Ac146 determinant. They secreted an antibody (antibody B1-8.V4) whose functional and structural properties are described below and compared with those of the mutant B1-8.V1.

Structure of the mutant B1-8.V4

The mutant cell line B1-8.V4 secretes a typical dimeric IgD antibody which is biochemically very similar to the wild-type molecule B1-8.δ1 (Neuberger and Rajewsky, 1981). On SDS–polyacrylamide gels both mutant and wild-type antibodies appear as molecules of 160 kd. Upon reduction, an H chain of 61 kd and an L chain of 22.5 kd can be seen on the gels. In isoelectric focusing, using a pH gradient from 3.5 to 10, the L chains of mutant and wild-type were indistinguishable, whereas a tryptic fragment of the mutant H chain containing its entire VDJ region (Brüggemann *et al.*, 1982) was slightly more acidic than the corresponding wild-type fragment (data not shown). These results suggested that B1-8.V4, similar to B1-8.V1, carried a mutation in the V region of the heavy chain.

In order to identify the mutation, the active, rearranged VDJ gene of B1-8.V4, located as in the wild-type (Sablitzky *et al.*, 1982) on a 4.3-kb *Eco*RI restriction fragment, was isolated from a genomic library, subcloned as a 2.3-kb *Eco*RI/*Bam*HI fragment into phage M13 and sequenced. The sequence appears in Figure 2 where it is compared with the sequences of the wild-type and of the mutant B1-8.V1. The mutant carries a single point mutation in codon 50, changing an A to a G, and at the amino acid level, an arginine to a glycine residue.

Does this single point mutation confer the mutant phenotype to the B1-8.V4 antibody? To verify this point, we modified an expression vector containing the VDJ gene of B1-8 in front of the Cμ gene (pSV-Vμ1; Neuberger, 1983) by replacing this VDJ gene with that of the mutant B1-8.V4. The new plasmid, designated pSV-V4, was then transfected into J558L cells, a myeloma expressing λ1 chain identical to those expressed by the B1-8 wild-type (Bothwell *et al.*, 1982), in the absence of an H chain. As shown by fluorescence analysis (Table I), the transfected gene was transiently expressed in ~2% of the cells. These cells produced an IgM antibody positive for idiotope Ac38 and negative for idiotope Ac146. The latter was also true for stable transfectants. In cells transfected with the (wild-type) pSV-Vμ1 plasmid, IgM antibodies positive for both idiotopes were

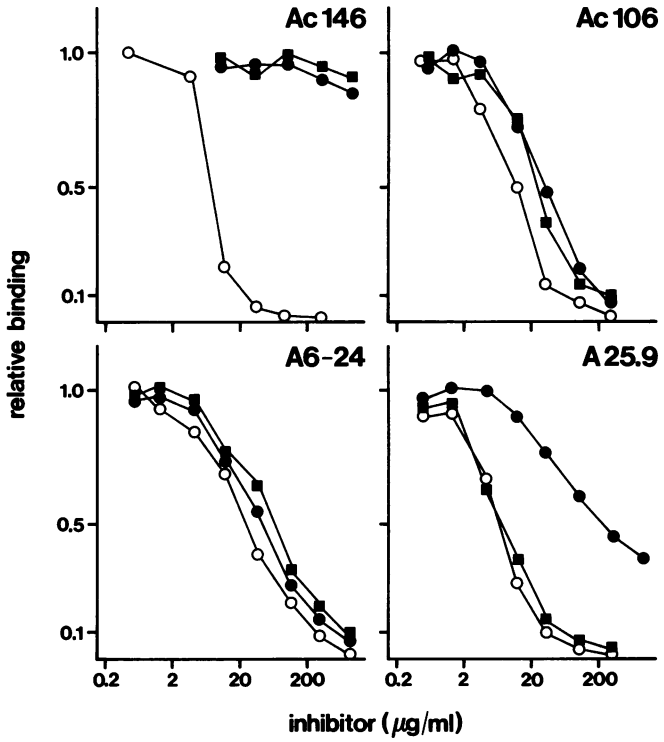

Fig. 3. Idiotypic analysis of B1-8.δ1 and the variants B1-8.V1 and B1-8.V4. Plates were coated with the various anti-idiotope antibodies (the group 1 anti-idiotope Ac146 and the group 2 anti-idiotopes A6-24, Ac106 and A25.9) and the binding of radiolabelled B1-8.δ1 antibody (ordinate) was inhibited by various concentrations (abscissa) of antibodies B1-8.δ1 (○), B1-8.V1 (●) and B1-8.V4 (■).

expressed. These data demonstrate that the B1-8.V4 VDJ region, and therefore the point mutation in position 50 of the H chain, is responsible for the mutant phenotype of antibody B1-8.V4.

Idiotypic and hapten binding specificity of antibody B1-8.V4

The idiotypic specificity of the B1-8.V4 mutant antibody was assayed in binding inhibition experiments in which the binding of radioactively labelled B1-8 wild-type antibody to various plastic plate-bound anti-idiotope antibodies was carried out in the presence of either unlabelled wild-type or unlabelled mutant antibody. The results of this analysis for some of the anti-idiotopes are shown in Figure 3 which also depicts results obtained with the B1-8.V1 mutant for comparison. The two mutants behave very similarly in the analysis in that they have lost the binding site-

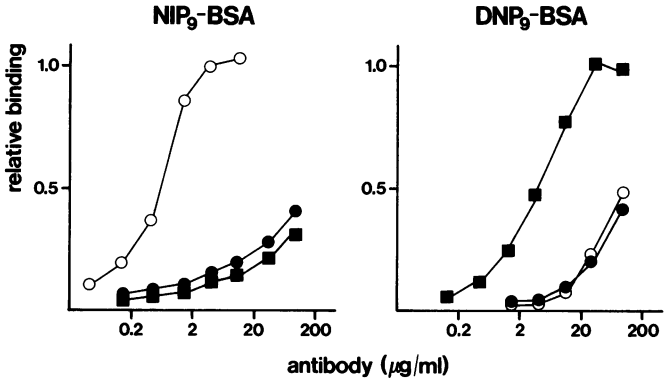

Fig. 4. Binding specificity of antibody B1-8.δ1 and the variants B1-8.V1 and B1-8.V4. Plates were coated with NIP_9–BSA (left) and DNP_9–BSA (right) and the binding of B1-8.δ1 (○), B1-8.V1 (●) and B1-8.V4 (■) at various concentrations (abscissa) was measured by radiolabelled anti-λ1 antibody Ls136 (ordinate).

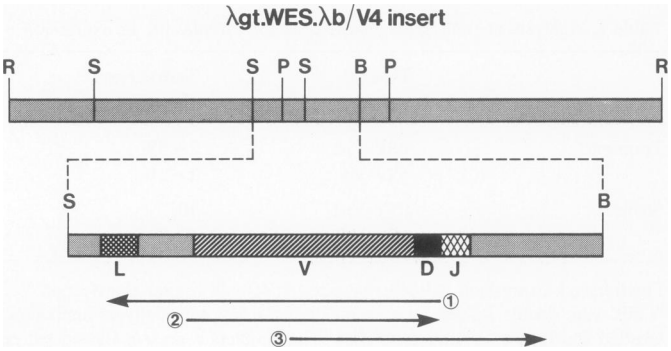

Fig. 5. Strategy for sequence analysis of the VDJ region of B1-8.V4. Partial restriction analysis of the 4.3-kb *Eco*RI fragment isolated from the genomic library of B1-8.V4. A 2.3-kb *Eco*RI/*Bam*HI fragment was subcloned into M13. A *Sau*3A/*Bam*HI fragment containing the VDJ gene of B1-8.V4 is enlarged to show the sequence strategy.

related (group 1) idiotope (Ac146) and largely preserved three idiotopes of group 2 (Ac106, A6-24 and A25.9). The mutants have also lost a second binding site-related idiotope (As79) but preserved two others (Ac22 and A39-40), as well as the group 2 idiotopes Ac38 (for which they were selected) and A6-24 (data not shown).

The binding of wild-type and mutant antibodies to the hapten NIP [coupled to the plastic plate surface through the carrier molecule bovine serum albumin (BSA)] is depicted in Figure 4. The mutants have both essentially lost their NIP binding capacity. Binding assays in liquid phase have shown that the affinity for NIP is 1×10^{-7} for B1-8.δ1 wild-type (Neuberger and Rajewsky, 1981), $\sim 2 \times 10^{-5}$ M for B1-8.V1 (Brüggemann *et al.*, 1982) and below 1×10^{-5} for B1-8.V4 (Brüggemann, 1984). The mutants also showed very low binding to the related haptens 4-hydroxy-3-nitro-phenylacetyl (NP) and 4-hydroxy-3,5-dinitro-phenylacetyl (NNP) to which the wild-type antibody binds well (data not shown). Somewhat to our surprise, the mutant B1-8.V4 exhibited distinct binding to plates coated with 2,4-dinitrophenyl (DNP)–BSA conjugates, which was not observed for B1-8.δ1 wild-type or B1-8.V1 (Figure 4). The fine specificity of this binding reaction is unknown at present, since we could not detect binding of the B1-8.V4 antibody to DNP–lysine in liquid phase assays so that the affinity of the antibody for this compound must be $> 10^{-5}$ M (data not shown). However, the result demonstrates that the B1-8.V4 mutant has acquired binding specificity for a ligand to which the wild-type antibody does not detectably bind.

Discussion

Point mutation and recombination of expressed immunoglobulin genes are rare events in transformed plasma cells

Three spontaneous antibody mutants of the hybridoma cell lines B1-8.δ1 have been isolated so far. Two of them, B1-8.V3 (Radbruch *et al.*, 1985) and B1-8.V4 (this paper), carry single point mutations. The third one, B1-8.V1 (Brüggemann *et al.*, 1982), selected for the same phenotype as B1-8.V4, was generated by a recombinational event involving the expressed and a neighbouring non-expressed V_H gene, presumably gene conversion (Dildrop *et al.*, 1982; Krawinkel *et al.*, 1983). In all cases, selection started from a population of $\sim 10^8$ hybridoma cells in which the mutants were present at a very low frequency, presumably in the range of 10^{-7} to 10^{-8} per cell. Furthermore, analysis by fluorescence microscopy has shown that, in populations of B1-8.V3 and B1-8.V4 cells, revertants to the wild-type phenotype occur at a frequency below 1×10^{-6} (A.Radbruch and M. Brüggemann, unpublished data). This low frequency of spontaneous mutants resulting from both point mutation and recombinational events contrasts sharply with the high rate and the selectivity by which point mutations are introduced into antibody V region genes in activated B cells (McKean *et al.*, 1984; Sablitzky *et al.*, 1985) and, perhaps, pre-B cells (Wabl *et al.*, 1985). We conclude that the mechanism of somatic hypermutation is turned off in the B1-8.δ1 hybridoma line. This is presumably also true for other transformed plasma cell lines such as the hybridoma line SP6 in which V region mutations appear to be rare (Köhler and Shulman, 1980). The high rate of phenotypic reversion observed in the myeloma line S107 (Teillaud *et al.*, 1983) may reflect the interplay of duplicated V_H genes (M. Scharff, personal communication). We speculate that in the B cell lineage somatic hypermutation occurs only at certain stages of differentiation and is turned off at the plasma cell stage.

A single amino acid exchange in CDR2 of the H chain results in loss of hapten binding and generates a new binding specificity

On the basis of sequence and binding site comparisons, Reth *et al.* (1981) predicted that the amino acid residues in positions 31, 35, 50, 52 and 99 of the B1-8 H chain play a key role in hapten binding. It is striking to see that in the B1-8.V4 mutant, which has lost NIP binding specificity, one of these five positions (position 50) is indeed affected. One may note in this context that a second of the five positions, position 52, is mutated in the mutant B1-8.V1. These results support the concept of Ohno *et al.* (1985) that only a few positions in the V region determine the antibody binding specificity of antibodies. The arginine to glycine exchange in B1-8.V4 represents a major structural alteration affecting not only charge but perhaps also the shape of the binding site. The latter could explain why the V region of the mutant accommodates, in contrast to the wild-type, a DNP–carrier complex. Be this as it may, it is clear from the data that a single point mutation in CDR2 of the H chain can profoundly change the specificity of an antibody, similar to point mutations in CDR1 of the H chain (Diamond and Scharff, 1984) and CDR3 of both H (Cook *et al.*, 1982; Radbruch *et al.*, 1985) and L chains (Azuma *et al.*, 1984).

Structure of a binding site-related idiotope and the nature and potential function of the internal image of a hapten

Both the B1-8.V1 and the B1-8.V4 mutants were selected not

for the loss of hapten binding but for the loss of a binding site-related idiotope, namely idiotope Ac146. The fact that hapten binding was lost together with the idiotope in both cases suggests that the latter (and also idiotope As79 which was concomitantly lost) must be closely associated with the hapten-binding site. However, there is clear evidence that the hapten-binding site alone does not define the full extent of the Ac146 idiotope (Rajewsky and Takemori, 1983; Radbruch *et al.*, 1985). Particularly striking in this respect is the finding that an antibody lacking the aspartic acid in position 100 of B1-8 also lacks the Ac146 idiotope although it binds the NIP hapten with equal affinity to the B1-8 antibody (Cumano and Rajewsky, 1985). Our interpretation of these superficially conflicting results is given in Figure 1. The interaction of anti-idiotope antibodies with the idiotypic target involves extended overlapping surface areas, in keeping with a large body of experimental evidence (reviewed by Rajewsky and Takemori, 1983; see also Sablitzky and Rajewsky, 1984). by selecting for a mutant which has lost reactivity with one anti-idiotope but retained that with another, we select for mutations in the contact area specific for the former antibody. In the combination of the anti-idiotope antibodies Ac146 and Ac38 the contact area on B1-8 selectively seen by antibody Ac146 is the hapten-binding site. That this is a major point of contact of this antibody is underlined by the ability of antibody Ac146 to selectively induce NIP binding λ1 chain-bearing antibodies when used as an immunogen (Takemori *et al.*, 1982; Tesch *et al.*, 1983). The structure on antibody Ac146 interacting with the B1-8 hapten binding site could be called the internal image of the hapten in the sense of the network hypothesis (Jerne, 1974). It is an imperfect image, since most anti-NP antibodies do not react with the Ac146 anti-idiotope, and it represents only one of the determinants of the latter involved in its idiotypic interaction with antibody B1-8.δ1. Nevertheless, as the present data demonstrate, it could play a role in the selection of somatic antibody mutants with changed hapten binding specificity.

Materials and methods

Cell lines and cell culture

The myeloma line J558L (Oi *et al.*, 1983) secretes λ1 light chains in the absence of a H chain and was kindly given to us by Dr V.Oi. This line as well as the cell line B1-8.δ1 (Neuberger and Rajewsky, 1981) and variants derived from it were either cultured in RPMI 1640 medium (Seromed, Munich) supplemented with 10% newborn calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) or grown intraperitoneally in pristane-primed (C57BL/6×BALB/c)F1 mice. Cells were cloned by limiting dilution (0.3 cells/well) without feeder cells.

Monoclonal antibodies and serology

Antibodies B1-8.δ1 and B1-8.V1 (both IgD, λ1) were purified from ascites fluid by adsorption to (NIP−cap)−Sepharose and subsequent elution with 10^{-3} M NIP−cap in phosphate-buffered saline (PBS) as previously described (Brüggemann *et al.*, 1982; Radbruch *et al.*, 1985). B1-8.V4 antibodies were purified by adsorption to (DNP−cap)−Sepharose (a gift of Dr M.Cramer) and elution with 10^{-3} M DNP−cap in PBS. Hapten conjugates (NIP_9−BSA and NP_8−BSA as well as NNP_{11}−BSA, TNP_{10}−BGG and DNP_9−BSA which were gifts from Dr M.Cramer) were prepared as described previously (Brüggemann *et al.*, 1982). Anti-idiotope antibodies A6-24 (IgG2a, κ), A25.9.7 (IgG1, κ) (Rajewsky *et al.*, 1981), Ac146.γ2a (IgG2a, κ) (Müller and Rajewsky, 1983) as well as Ac146, Ac38, Ac106 and anti-λ1 (Ls136) antibodies (all IgG1, κ) (Reth *et al.*, 1979) were purified from ascites fluid by $(NH_4)_2SO_4$ precipitation and ion exchange chromatography on DEAE−cellulose (Reth *et al.*, 1979). Wild-type and variant proteins were compared serologically by radioimmunoassay (Reth *et al.*, 1979). Microtiter plates (Cooke, Alexandria, USA) were coated with either anti-idiotope antibodies (10 μg/ml) or hapten−protein conjugate (100 μg/ml) and either the binding of B1-8.V1 and B1-8.V4 to the coats was detected using radiolabelled Ls136 (direct binding assay) or various concentrations of antibodies B1-8.V1 and B1-8.V4 were used to compete the binding of radiolabelled B1-8.δ1 to the coats (binding inhibition assay).

Immunofluorescence and cell sorting

The preparation of fluorochrome-conjugated antibodies has been described earlier (Kearney and Lawton, 1975). For isolation of variants in a fluorescence activated cell sorter (FACS I, Becton Dickinson, Mountain View, CA), cells were stained with 0.02 mg fluorescein-conjugated Ac38/ml in the presence of 5 mg Ac146/ml (Brüggemann *et al.*, 1982). For the initial enrichment of the B1-8.V4 variant 1×10^8 wild-type cells were sorted. Variants were isolated after six successive rounds of sorting in which the brightest cells (0.5−3%) of the population were selected.

Gel electrophoresis of secreted Ig

Cells were labelled biosynthetically with [^{14}C]amino acid lysate (Brüggemann, 1984). Secreted Ig was purified by adsorption to Ac38−Sepharose and subjected to electrophoresis on a 10% SDS−polyacrylamide gel under reducing conditions. After autoradiography, gel pieces containing light chains were cut out and subjected to isoelectric focusing over a pH range from 4 to 8 (Brüggemann *et al.*, 1982; Brüggemann, 1984).

DNAs

The J_H probe, a *Bam*HI/*Eco*RI restriction fragment containing the murine IgH exons J_{H3} and J_{H4}, was a gift of M.Boersch-Supan (Boersch-Supan *et al.*, 1985). It detects a 4.3-kb and a 6.6-kb restriction fragment in the genome of B1-8.V4 (U.Krawinkel, data not shown). The rearranged VDJ gene of B1-8.V4 is located on the 4.3-kb fragment whereas the 6.6-kb fragment originates from the genome of the X63.Ag8 fusion partner (Sablitzky *et al.*, 1982). Sequencing primers were kindly provided by K.Otto and B.Müller-Hill. Plasmid pSV-Vμ1 (Neuberger, 1983) encodes the H chain of an NP-specific antibody expressing two idiotopes Ac38 and Ac146. Plasmid pSV-V4 is a derivative of pSV-Vμ1 where the original VDJ gene has been replaced by a 4.3-kb *Eco*RI fragment carrying the VDJ gene of B1-8.V4 5′ to Cμ.

DNA techniques

High mol. wt genomic DNA was extracted from B1-8.V4 cells (Blin and Stafford, 1977) and digested to completion with *Eco*RI. Fragments of ~4.3 kb size were enriched by agarose (0.8%) gel electrophoresis and ligated to the arms of λ phage λgt.WES.λb (Leder *et al.*, 1977). Following *in vitro* packaging (Hohn and Murray, 1977) the resulting library of $\sim 6 \times 10^5$ recombinant phages was screened by *in situ* hybridization (Benton and Davis, 1977) to the J_H probe. Hybridizing plaques were picked and re-screened. Four out of eight hybridizing λ clones were analysed by restriction mapping and shown to contain the rearranged VDJ gene of B1-8.V4 (data not shown) which was then subcloned as a 2.3-kb *Eco*RI/*Bam*HI fragment into M13. DNA sequence analysis was performed by the dideoxy chain termination method (Sanger *et al.*, 1977) using specific primers as indicated in Figure 5.

Transfection

For transient expression 1 μg of plasmid DNA was used to transfect 10^7 J558L cells using the DEAE dextran method (Banerji *et al.*, 1983). Stable transfectants were obtained using the electroporation method described by Potter *et al.* (1984).

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