# MOLECULAR CHARACTERIZATION OF ANTIBODIES BEARING Id-460

## II. Molecular Basis for Id-460 Expression

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We have previously described an experimental system for studying the cellular and molecular basis of idiotype expression (1–5). BALB/c mice produce anti-dinitrophenyl (DNP)[1] antibody expressing a recurrent idiotypic determinant, Id-460. Two genes controlling the expression of this determinant map to the heavy and kappa chain variable regions ($V_H$ and $V_{\kappa 1}$). Thus, the determinant is likely to be a combinatorial determinant involving both $V_H$ and $V_{\kappa 1}$ domains. Id-460 is also found in normal serum, where much of it binds *Pasteurella pneumotropica* (*P. pneumotropica*), and none to DNP. Furthermore, *P. pneumotropica* immunization induces production of non–DNP-binding, Id-460$^+$ antibodies. To understand the structural basis for this shared idiotypy, we have undertaken a molecular analysis of monoclonal Id-460$^+$ antibodies. We have previously described the development of molecular probes for and the structure of the genes encoding the MOPC-460 (M460) protein, the prototypic DNP-binding antibody in the Id-460 system. We have also reported the structure of two Id-460$^+$ hybridomas, LB8, which binds *P. pneumotropica*, and D35, which is DNP specific. These three proteins are encoded by closely related $V_H$ and $V_\kappa$ gene segments, but differ in other gene segments (5). In the present experiments we use these probes to establish the molecular and genetic relatedness of other Id-460$^+$ monoclonal antibodies in an attempt to determine the molecular basis of shared idiotypy by antibodies of differing antigen binding specificity.

We have produced a panel of monoclonal antibodies selected for Id-460 expression. We obtained these Id-460$^+$ monoclonal antibodies in two ways: by anti-Id-460 immunization or by DNP immunization. In this report, we demonstrate that all of the Id-460$^+$ hybridomas tested share immunoglobulin (Ig) gene rearrangements and DNA sequence homologies for both $V_H$ and $V_L$ with M460.

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[1] *Abbreviations used in this paper:* Ars-CRI, crossreactive idiotype in arsonate-immunized A/J mice; DNP, dinitrophenyl; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; Id-460, idiotype associated with the MOPC-460 protein; KLH, keyhole limpet hemocyanin; M460, MOPC-460; M467, MOPC-467; M315, MOPC-315; OVA, ovalbumin; PC, phosphorylcholine; T15, idiotype associated with the TEPC-15 myeloma.

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Most notably, all hybridomas recovered from the anti-Id-460 immunization possessed the rearranged M460 $V_H$ gene and a $V_{\kappa 1A}$ or $V_{\kappa 1C}$ gene. The Id-460 determinant in these monoclonal antibodies is therefore clearly a $V_H$-$V_L$ conformational or combinatorial determinant. Furthermore, we demonstrate that antigen binding specificity in Id-460$^+$ antibodies cannot be predicted by the rearrangement of any one particular gene segment, but may be reflected in $D_H$ junctional sequences or somatic mutational changes in $V_H$ or $V_\kappa$.

## Materials and Methods

*Mice, Immunizations, and Hybridomas.* Mice were obtained from various sources and maintained as previously described (1). Three BALB/c mice were immunized with rabbit anti-Id-460 antibody (1) for production of Id-460$^+$ monoclonal antibody of various antigen binding specificities. Each mouse received 10 μg of rabbit anti-Id-460 emulsified in an equal volume of complete Freund's adjuvant and injected intraperitoneally in each of four footpads. The boosting immunization was given 3 mo later with 5 μg of rabbit anti-Id-460 injected intravenously. Fusion of spleen cells to SP2/0 was performed 7 d after the boosting immunization, as previously described (3). Hybridoma supernatants were screened for Id-460$^+$ antibody in a competitive inhibition radioimmunoassay (1). Each spleen averaged ~10 Id-460$^+$ hybridomas. Six were chosen for study: 2AB5 from the first spleen, Id16 from the second, and Id24, Id25, Id29, and Id30 from the third. Specificity for DNP or *P. pneumotropica* was tested as previously described (3, 4).

DNP-binding hybridomas were produced from two BALB/c mice immunized as previously described (5). The Id-460$^+$ hybridoma D35, and the Id-460$^-$ hybridomas D10, D20, and D24, were chosen for study. The three Id-460$^-$ hybridomas were produced from a spleen fused 4 d after the boosting immunization while D35 was from a spleen fused 7 d after the boosting immunization.

The A/J 36–60 and BALB/c 1210.7 hybridomas were kindly provided by R. Near (Massachusetts Institute of Technology, Cambridge, MA). MOPC-467 (M467), M460, MOPC-315 (M315), and hybridoma G-1 were as previously described (1).

*Immunoassays.* An enzyme-linked immunoadsorbent assay (ELISA) for Id-460 as previously described (4) was used to determine the degree of idiotypic similarity. To ensure that our comparisons of idiotypic similarities in hybridoma supernatants were due to qualitative differences and not quantitative differences, we determined the concentration of antibody in tissue culture supernatants by kappa light chain quantitation. This was determined in an ELISA consisting of anti-kappa antibody–coated microtiter plates and alkaline phosphatase–conjugated anti–mouse Ig.

*Molecular Techniques.* All DNA preparation and RNA preparation techniques, Southern blots, probes, and DNA sequencing are as previously described (5). The synthesis of cDNA for primer extension sequencing of heavy and light chains was as follows. 150 μg of total mRNA from hybridomas or myelomas was ethanol precipitated and resuspended in 45 μl of 0.5 M Tris-HCl, pH 8.3, 0.1 M $MgCl_2$, 0.1 M KCl, 0.2 mM dNTP, 0.4 mM dithiothreitol (DTT), 4 mM sodium pyrophosphate, and 50 ng [$^{32}$P]ATP kinase-treated primer. Primers were kinased for 1 h at 37°C with 4 U of polynucleotide kinase (Biolabs) in 5 mM Tris-HCl, pH 9, 1 mM $MgCl_2$, and 5 mM DTT, and γ [$^{32}$P]ATP. The RNA-primer mixture was heated at 65°C for 10 min. 5 μl of AMV reverse transcriptase was added and the extension reaction was allowed to proceed for 1 h at 42°C. Extended DNA were denatured for 5 min at 90°C before size fractionation on 8% acrylamide gels. Gels were autoradiographed for 1 h on Kodak XAR film and distinctly migrating bands were cut from the gel, soak eluted twice overnight in 0.3 M NaCl, 0.01 M Tris, pH 7.8, and 0.001 M EDTA, and ethanol precipitated. Primer-extended DNA fragments were then sequenced by the Maxam and Gilbert method.

Probes for primer extension sequencing specific for the gamma, mu, and kappa constant regions and the heavy chain joining ($J_H$) region were obtained from the DNA synthesis laboratory of the Howard Hughes Medical Institute at Massachusetts General Hospital.

The primer specific for the 5′ end of $J_{H2}$, $J_{H3}$, and $J_{H4}$ was the 14-mer, d(5′CCTTGGCCCCAGTA). The gamma constant region primer was the 15-mer, d(5′GGCCAGTGGATAGAC); the mu constant region primer was the 18-mer, d(5′GCTCTCGCAGGAGACGAG), and the kappa constant region primer was the 27-mer, d(5′CTGCTCACTGGATGGTGGGAAGATGGA). An alpha constant region primer was kindly provided by Dr. M. Weigert (Fox Chase Cancer Institute, Philadelphia, PA) and was an 18-mer having the sequence d(5′GGGAGTGTCAGTGGTAG).

## Results

*Analysis of Id-460 Expression by Monoclonal Antibodies of Various Antigen Binding Specificities.* The in vivo increase in expression of Id-460-bearing antibodies in induced responses to the immunogens DNP-ovalbumin (OVA) and *P. pneumotropica* has been demonstrated serologically. Antibodies bearing Id-460 have also been found in the pre–laboratory immunization (normal) serum of these mice, of which an average of 50% are *P. pneumotropica* binding. We prepared hybridomas secreting Id-460$^+$ antibody in response to antigen DNP or to anti-Id-460. We assume that the anti-Id-460–induced hybridomas are representative of Id-460$^+$ B cells present in the animals before our experimental immunization, some of which should be specific for *P. pneumotropica*. In all previous studies of Id-460$^+$ Ig there has been no overlap between Id-460$^+$ antibodies specific for *P. pneumotropica* and those specific for DNP.

Id-460$^+$ antibodies of particular interest are resulting from anti-Id-460 immunizations: 2AB5, a *P. pneumotropica*–binding IgM; Id29 and Id16, which may have some specificity for DNP; and Id24, Id25, and Id30, which are of unknown specificity. After DNP immunization, hybridomas were selected for DNP binding and subsequently for Id-460 expression. Four were chosen for study: D35, which we have described previously (5), and D10, D20, and D24, which are DNP binding, Id-460$^-$. All four are IgG. LB8, as previously reported (3), was produced from 2-d in vitro LPS-stimulated spleen cells. It is an IgM, Id-460$^+$ and *P. pneumotropica*–binding antibody. Hybridomas produced by these methods and their characteristics are listed in Table I along with relevant myelomas and hybridomas from other sources.

To determine the degree of similarity in overall determinant expression of these Id-460$^+$ antibodies, we performed a titration of hybridoma supernatants in a competitive inhibition ELISA assay. As shown in Fig. 1, close to 100% inhibition was observed with three of these four antibodies. In most assays, LB8 inhibits to >75% (3), but in this assay it inhibited only ~50% of the binding of anti-Id-460 to M460. This suggests that the major determinant(s) detected originally by the heterologous anti-Id-460 is common to all of the antibodies tested here, although LB8 may lack one such epitope. However, the shapes of the inhibition curves vary and appear to represent two groups: 2AB5, which inhibits similarly to M460, and the less well-inhibiting group of D35 and LB8 antibodies. Although the heterologous anti-Id-460 is directed against the DNP-binding portion of M460 protein, the inhibition curves do not correlate with antigen binding specificity. The inhibition assay results suggest that (*a*) there are minor qualitative differences in the expression of the Id-460 determinant, and (*b*) expression of idiotype is not directly related to specificity for antigen (DNP or *P. pneumotropica*).

TABLE I
*Hybridomas and Myelomas*

| Cell | Immunization | Fusion | Isotype | Id-460 | Specificity | | |
|---|---|---|---|---|---|---|---|
| | | | | | DNP | *P. pneumo.* | Other |
| MOPC-460* | Myeloma | — | IgA | + | + | − | − |
| LB8‡ | LPS | 2 d postculture | IgM | + | − | + | − |
| D35 | DNP-KLH | 7 d postboost | IgG1 | + | + | − | − |
| 2AB5 | Anti-Id-460 | 7 d postboost | IgM | + | − | + | − |
| Id29 | Anti-Id-460 | 7 d postboost | IgG1 | + | +/− | − | − |
| Id16 | Anti-Id-460 | 7 d postboost | IgG1 | + | +/− | − | − |
| Id25 | Anti-Id-460 | 7 d postboost | IgM | + | − | − | − |
| Id30 | Anti-Id-460 | 7 d postboost | IgM | + | − | − | − |
| Id24 | Anti-Id-460 | 7 d postboost | — | + | − | − | − |
| D10 | DNP-KLH | 4 d postboost | IgG | − | + | − | − |
| D20 | DNP-KLH | 4 d postboost | IgG1 | − | + | − | +§ |
| D24 | DNP-KLH | 4 d postboost | IgG1 | − | + | − | +§ |
| 1210.7 | Ars-KLH | Hyperimmunized | IgG1 | − | NT‖ | NT | Ars |
| G-1 | PC-KLH | Hyperimmunized | IgG1 | − | + | NT | PC |
| MOPC-315 | Myeloma | — | IgA | − | + | − | TNP |
| MOPC-467 | Myeloma | — | IgA | − | − | +¶ | NT |

* All hybridomas and myelomas are derived from BALB/c mice.
‡ The hybridomas were generated as described in Materials and Methods.
§ These monoclonal antibodies may have weak binding affinity for other haptens such as TNP, NP, NIP, and oxazolone.
‖ Not tested.
¶ MOPC-467 recognizes a different determinant on *P. pneumotropica* than LB8 or 2AB5 antibodies (4).

*Id-460$^+$ Antibodies Require the Rearrangement and Expression of a $V_{H460}$ Crosshybridizing Gene.* At least three of the $V_H$ genes within the $V_{H460}$ family contain an Eco RI site at codon 83 within the $V_H$ region (5). An Eco RI site has not been detected at this position in the $V_H$ genes for any other families thus far described (6). This characteristic site thus provides a convenient and diagnostic fragment for detection of rearranged Eco RI–containing $V_H$ genes to $J_H$ genes in Southern blots of genomic hybridoma DNA. Since the Eco RI site 3′ to the $J_H$ region is a known distance from $J_{H4}$ (7), fragment sizes of $V_H$ gene rearrangements within the $V_{460}$ family to $J_{H1}$, $J_{H2}$, $J_{H3}$, and $J_{H4}$ can be predicted for $J_H$-hybridizing Eco RI fragments in Southern blots, i.e., 2.4, 2.2, 1.8, and 1.4 kb, respectively (Fig. 2*a*). As shown in Fig. 2*b*, many hybridomas produce $J_H$-hybridizing Eco RI fragments of the predicted small size for rearrangement of a $V_H$ gene containing an internal Eco RI site to $J_{H2}$, $J_{H3}$, or $J_{H4}$. The rearranged heavy chain genes from M460 and D35 were cloned and shown to rearrange $V_H$ genes to $J_{H4}$, and a 1.4 kb $J_H$-hybridizing fragment was detected. Likewise, LB8 was demonstrated to rearrange a $V_H$ gene to $J_{H2}$, and a 2.1 kb fragment was detected. These Eco RI $J_H$-hybridizing fragments serve as convenient markers in genomic Southern blots. The 1.4 kb fragment was observed in many DNA, suggesting similar $V_H$ gene rearrangements in hybridomas 2AB5, Id29, Id16, Id25, Id30, and D20 to $J_{H4}$.

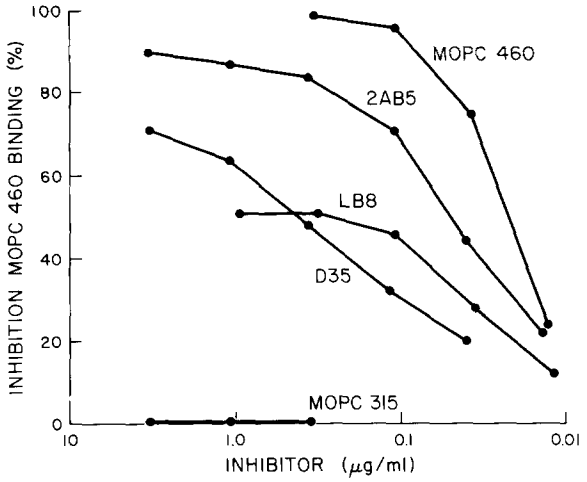



FIGURE 1. Qualitative differences in Id-460 expression by monoclonal antibodies. Equivalent quantities of hybridoma antibodies, 2AB5 and LB8 (*P. pneumotropica* specific) and D35 (DNP specific) were titrated against purified M460 and M315 proteins (DNP specific) in a competitive inhibition ELISA assay to detect expression of Id-460. Qualitative differences in Id-460 expression are apparent by the slopes and positions of individual titration curves.

The Id24 DNA possessed a 2.1 kb fragment, suggesting a $J_{H2}$ rearrangement. Hybridoma 1210.7 is known to have a $J_{H3}$ rearrangement (8); D24 possesses a similarly sized fragment. Hybridoma G-1 also appears to rearrange $J_{H3}$, confirming the known protein sequence (P. Gearhart, unpublished results).

However, the correlation in these genomic Southern blots between small $J_H$ Eco RI fragments and $J_H$ rearrangement could be misleading since aberrantly rearranged and $DJ_H$ rearrangements also occur within these antibody-producing cells (9). Thus, firm conclusions concerning the rearrangement of an Eco RI-containing $V_H$ gene to any $J_H$ require Southern blot data demonstrating hybridization of a probe specific for the $V_{H460}$ gene family to the same restriction fragment as a $J_H$-specific probe. This would be accomplished by using restriction enzymes that do not cut specific sequences within the $V_H$ gene.

Two different restriction enzymes, Xba I and Hind III, were chosen to digest these genomic hybridoma DNA because these restriction sites appear at different distances 5′ to the internal Eco RI site in the two cloned $V_H$ genes ($V_{H460}$ and $V_{HLB8}$) and because of convenient and clearly mapped sites 3′ and within the $J_H$ region. In the M460 cloned gene, the Xba I site is 3.4 kb 5′ to the internal Eco RI site, with the Hind III site 0.65 kb 5′ to Eco RI. In the LB8 cloned gene the Hind III site is 5.0 kb 5′ to the internal Eco RI site and Xba I is 0.63 kb 5′ to it. Fragment size predictions can be made for rearrangement of either the $V_{H460}$ or $V_{HLB8}$ gene to any of the four $J_H$ (Table II).

Fig. 3, *a* and *b* shows Southern blot analysis of Xba I- or Hind III–digested DNA from hybridomas and myelomas. The same filters used for hybridization with a $J_H$-specific probe were washed and reused for hybridization with the probe specific for the crosshybridizing $V_{H460}$ gene family to verify the uniquely rearranged $J_H$-hybridizing fragments as those containing a $V_H$ gene from this family (starred fragments). Table III lists the size rearrangements of $J_H$-hybridizing

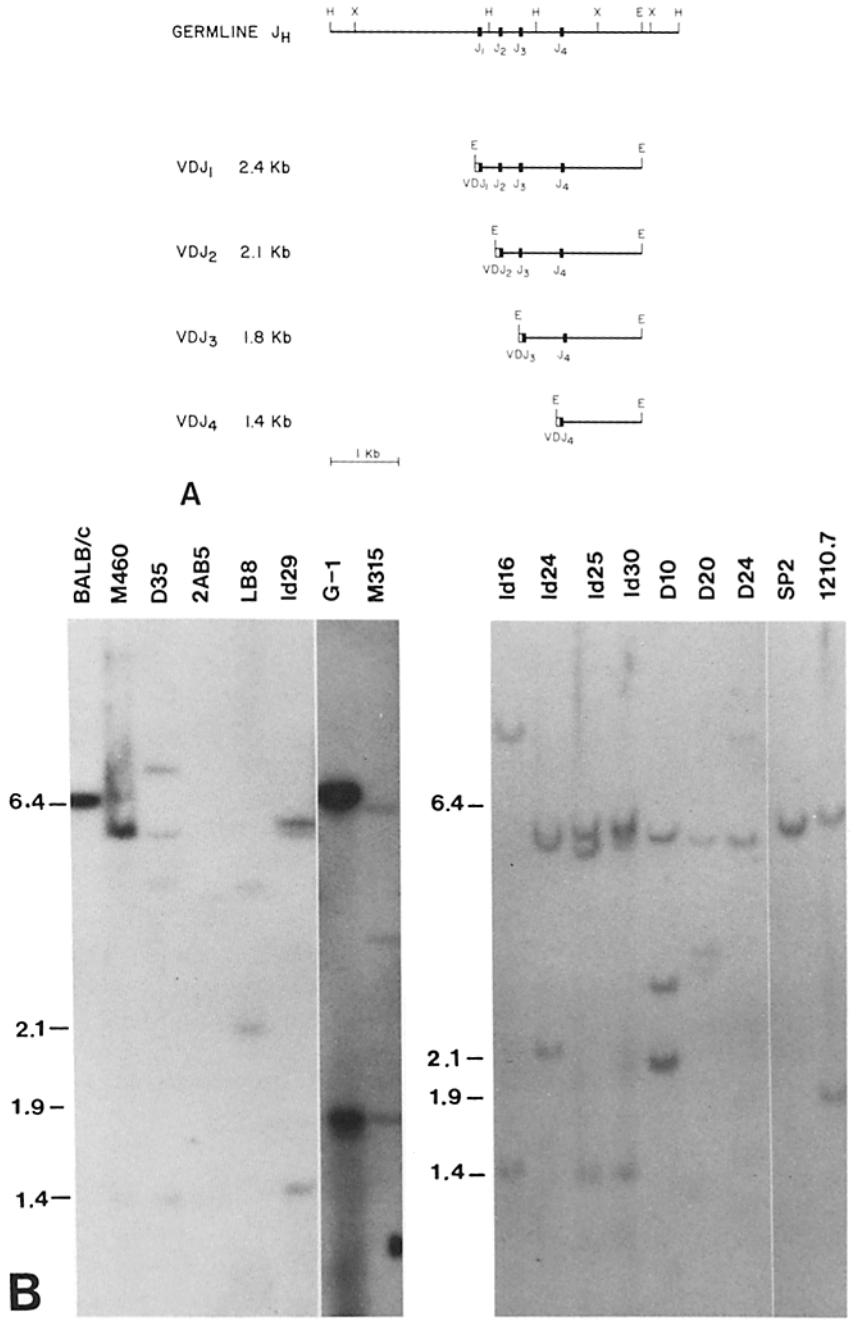



FIGURE 2. (*A*) Germline map of $J_H$ region (7) is presented for comparison with Eco RI fragments produced when a $V_H$ region containing an internal Eco RI site at codon 83 is rearranged to any of four J germline genes. Predicted sizes of Eco RI $J_H$–containing fragments are listed. Enzyme code: H, Hind III; X, Xba I, and E, Eco RI. (*B*) Southern blot analysis of Eco RI $J_H$–hybridizing fragments in the genomic DNAs of indicated monoclonal antibody–producing cell lines. BALB/c liver and SP2 DNA serve as controls for the germline fragment and nonproductively rearranged fragments from the hybridoma fusion partner. $J_H$ hybridizing bands at 2.4, 2.1, 1.8, or 1.4 kb suggest rearrangement to $J_{H1}$, $J_{H2}$, $J_{H3}$, or $J_{H4}$, respectively.

TABLE II

*Predicted Size Rearrangements for $V_{H460}$ and $V_{HLB8}$ to any $J_H$*

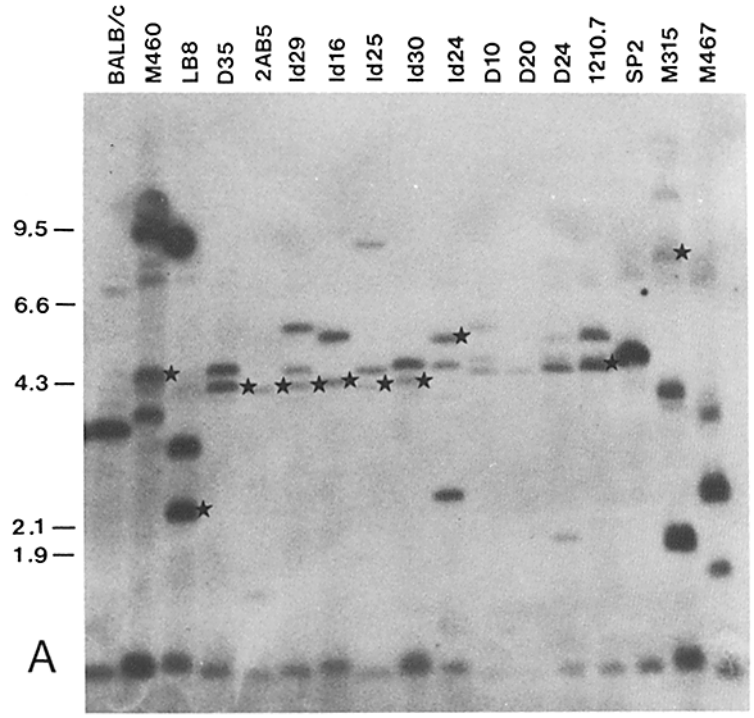
| | | Xba I fragments | Hind III fragments |
|---|---|---|---|
| | | *kb* | *kb* |
| $V_{H460}$ | $J_{H1}$ | 5.5 | 0.8 |
| | $J_{H2}$ | 5.2 | 1.3 |
| | $J_{H3}$ | 4.8 | 0.9 |
| | $J_{H4}$ | 4.3 | 2.5 |
| $V_{HLB8}$ | $J_{H1}$ | 2.5 | 5.2 |
| | $J_{H2}$ | 2.2 | 5.6 |
| | $J_{H3}$ | 1.8 | 5.2 |
| | $J_{H4}$ | 1.2 | 6.8 |

fragments in these hybridomas (fragments hybridizing with both probes are underlined). From this analysis many of the hybridomas, 2AB5, Id29, Id16, Id25, and Id30, appear to have similar Xba I (4.4 kb) and Hind III (2.6 kb) fragments and to correspond precisely to the predicted size of the $V_{H460}$ gene rearranged to $J_{H4}$. Therefore, it is reasonable to suggest that hybridomas 2AB5, Id29, Id16, Id25, and Id30 express this rearranged gene. The fragments observed for Id24 are consistent with the rearrangement of the $V_{H460}$ gene to $J_{H2}$. Rearrangements observed for the other hybridomas (Id-460$^-$) will be discussed later.

The uniqueness of the rearranged fragments observed in Southern blots and the similarity in size between these Id-460$^+$ antibody-producing cells strongly suggested that the products of these rearrangements were the expressed Ig heavy chain protein. However, formal confirmation of the expression of these genes was made through Northern blot analysis for most of the hybridomas (not shown). As expected, M315, LB8, Id16, Id29, and D35 mRNA hybridized to $V_{H460}$. Hybridomas Id30, Id24, Id25, and 2AB5 were not tested. D20 (Id-460$^-$ control) did not hybridize to the $V_H$-specific probe but was shown to be expressing Ig of the gamma isotype by its hybridization with a gamma-specific probe. This probe also confirmed that the expressed antibody in Id16, Id29, and D35 are of the IgG isotype. Isotype determinations done previously by Ouchterlony immunodiffusion with the secreted Ig were in complete agreement.

Hence, for hybridomas that were selected by the heterologous anti-Id-460 reagent, it is clear from the observed gene rearrangements and expression data that: (*a*) there is a crosshybridizing $V_{H460}$ gene rearrangement in the genomes of

FIGURE 3. (*A*) Southern blot analysis of immunoglobulin heavy chain (*A*) Xba I or (*B*) Hind III $J_H$-hybridizing fragments in the genomic DNA of indicated monoclonal antibody–producing cell lines. BALB/c liver DNA serves as control for germline fragments and SP2 DNA for the nonproductively rearranged fragments from the hybridoma fusion partner. Starred fragments indicate hybridization with both $J_H$- and $V_{H460}$-specific probes. (*B*) Southern blot analysis of immunoglobulin heavy chain (*A*) Xba I or (*B*) Hind III $J_H$-hybridizing fragments in the genomic DNA of indicated monoclonal antibody–producing cell lines. BALB/c liver DNA serves as control for germline fragments and SP2 DNA for the nonproductively rearranged fragments from the hybridoma fusion partner. Starred fragments indicate hybridization with both $J_H$- and $V_{H460}$-specific probes.


BALB/c
M460
LB8
D35
2AB5
Id29
Id16
Id25
Id30
Id24
D10
D20
D24
1210.7
SP2
M315
M467
9.5
6.6
4.3
2.1
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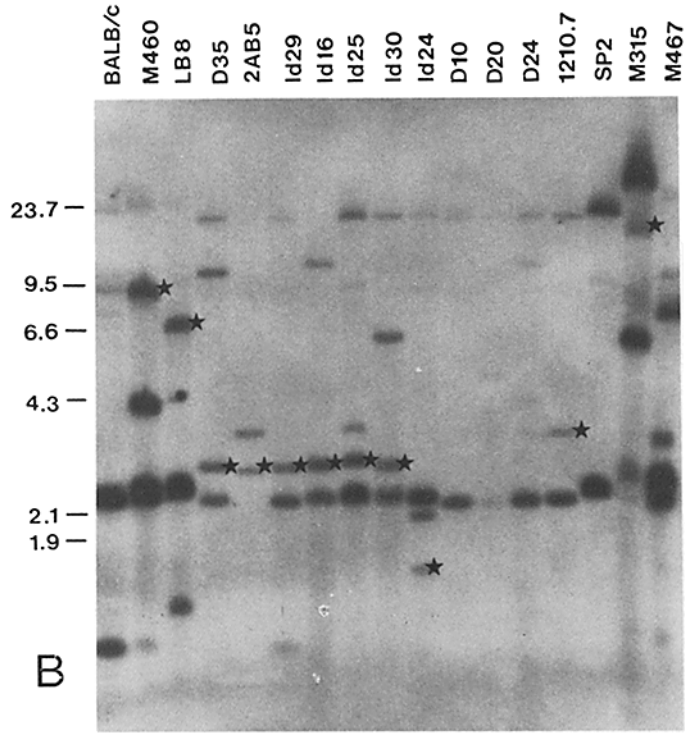
BALB/c
M460
LB8
D35
2AB5
Id29
Id16
Id25
Id30
Id24
D10
D20
D24
1210.7
SP2
M315
M467
23.7
9.5
6.6
4.3
2.1
1.9
B

TABLE III

*Heavy Chain Gene Rearranged Fragments in Monoclonal Antibody–producing Cells*

| Cell line | Xba I fragments | | | | Hind III fragments | | | | $V_H$ | $J_H$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | Germline | SP2 | Unique | | Germline | SP2 | Unique | | | |
| M460 | 0.7 | | 9.5 | 4.4 | 2.4 | | 7.2 | 3.8 | $V_{H460}$ | $J_{H4}$ |
| LB8 | 0.7 | | 3.1 | 2.2 | 2.4 | | 5.6 | 1.3 | $V_{HLB8}$ | $J_{H2}$ |
| D35 | 0.7 | 4.9 | | 4.4 | 2.4 | 16.5 | 8.6 | 2.6 | $V_{H460}$ | $J_{H4}$ |
| 2AB5 | 0.7 | | 4.4 | 1.4 | | | 3.3 | 2.6 | $V_{H460}$ | $J_{H4}$ |
| Id29 | 0.7 | 4.9 | 6.2 | 4.4 | 2.4 | | | 2.6 | $V_{H460}$ | $J_{H4}$ |
| Id16 | 0.7 | 4.9 | 5.9 | 4.4 | 2.4 | | 10.5 | 2.6 | $V_{H460}$ | $J_{H4}$ |
| Id25 | 0.7 | 4.9 | | 4.4 | 2.4 | 16.5 | 3.3 | 2.6 | $V_{H460}$ | $J_{H4}$ |
| Id30 | 0.7 | 4.9 | | 4.4 | 2.4 | 16.5 | 5.2 | 2.6 | $V_{H460}$ | $J_{H4}$ |
| Id24 | 0.7 | 4.9 | 5.7 | 2.4 | 2.4 | 16.5 | 2.1 | 1.5 | $V_{H460}$ | $J_{H2}$ |
| D10 | 0.7 | 4.9 | 5.9 | 5.1 | 2.4 | 16.5 | | 1.9 | — | — |
| D20 | 0.7 | | 2.6 | 2.0 | 2.4 | | 4.0 | 4.7 | — | — |
| D24 | 0.7 | 4.9 | 5.7 | 2.1 | 2.4 | 16.5 | 11.0 | 4.1 | — | — |

Underlined fragments are those which hybridize with both the $V_{H460}$ and $J_H$ probes.

all Id-460$^+$ hybridomas; (*b*) the size of all $VDJ_H$ rearrangements, with the exception of LB8, correspond to the predicted size rearrangement of $V_{H460}$; (*c*) $J_{H2}$ and $J_{H4}$ segments are rearranged in Id-460$^+$ hybridomas; and (*d*) these segments are the expressed heavy chain genes in all the Id-460$^+$ hybridomas tested.

These results support the previous genetic analysis of Id-460 expression (1) and show that expression of Id-460 requires a rearrangement of a $V_{H460}$ cross-hybridizing $V_H$ gene; however, not all antibodies having $V_H$ regions encoded by a member of the $V_{H460}$ family are capable of producing Id-460$^+$ antibodies, e.g., the lambda light chain–bearing M315 myeloma (Fig. 3, *a* and *b*) and the G-1 hybridoma (not shown). Furthermore, the arsonate-specific $V_{\kappa 2}$-bearing hybridoma, 1210.7, rearranges the $V_{H460}$ gene to $J_{H3}$ but only marginally expresses Id-460 (T. Marion, unpublished results). $J_H$ segment usage does not appear to affect expression of Id-460 since both $J_{H2}$ and $J_{H4}$ were found to be rearranged and expressed (Fig. 4) in Id-460$^+$ monoclonal antibodies. However, the formal possibility exists that $J_{H1}$ or $J_{H3}$ affect Id-460 expression. Finally, Id-460 expression could be influenced by junctional regions surrounding $D_H$.

To further characterize these heavy chains in terms of their $D_H$ or surrounding junctional regions, we performed cDNA sequence analysis of the junctional regions of eight Id-460$^+$ monoclonal antibodies. If $D_H$ were important in the formation of the Id-460 determinant, similarities in $D_H$ size or sequence might be expected in the panel of hybridomas. As shown in Fig. 4, both $D_H$ sequence and size varied extensively between these antibodies. Thus, there is no evidence at present to suggest the involvement of $D_H$ junctional sequences in the expression of Id-460.

*Expression of the Id-460 Determinant Is Dependent upon Rearrangement and Expression of $V_{\kappa 1A}$ or $V_{\kappa 1C}$.* The expression of Id-460 in the response to DNP is dependent on the ability to produce $V_{\kappa 1}$ light chains. Therefore, we next determined the rearrangement and expression of a $V_{\kappa 1}$ light chain gene in the

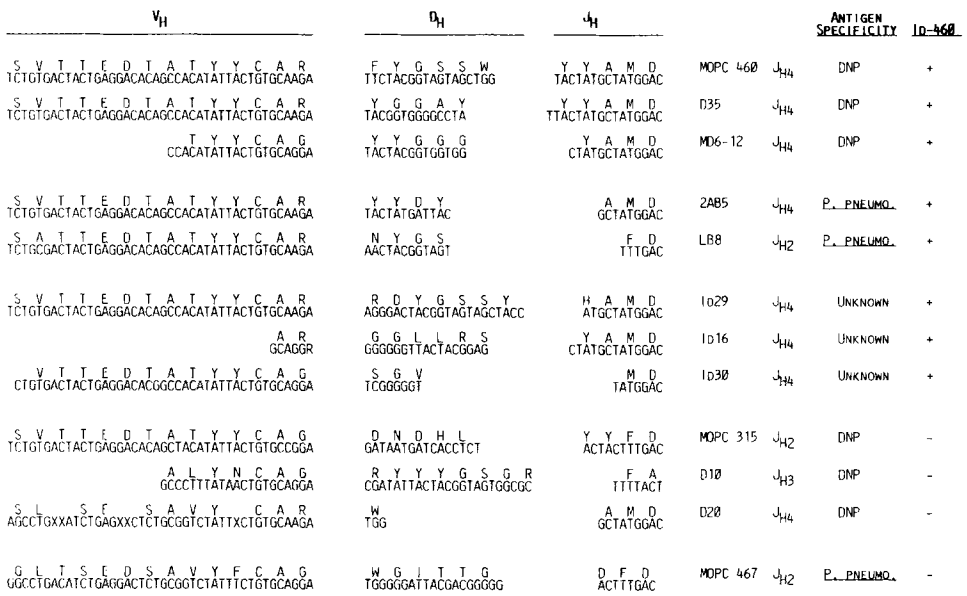


FIGURE 4. Immunoglobulin heavy chain cDNA sequences from monoclonal antibody-producing cells MD6–12, Id29, Id16, Id30, D10, D20, and M467. A $J_{H2-4}$-specific primer, along with gamma 1, mu, and alpha constant region–specific primers (as described in Materials and Methods) were used to promote cDNA synthesis on mRNA from Id-460$^+$ and Id-460$^-$ cells as indicated. Antigen specificities of the proteins are also indicated. Extended cDNAs were sequenced by Maxam and Gilbert method and are aligned so as to illustrate junctional sequence and size differences between rearranged heavy chain genes. DNA sequences from M460, LB8, and D35 were obtained from genomic clones as described previously (5) and were confirmed by limited cDNA sequences only in $VDJ_H$ junctional regions. The DNA sequence of 2AB5 was obtained from a genomic Eco RI clone derived by P. Brodeur (unpublished results) and sequenced from the Eco RI site at codon 83 and the Nae I site 3′ to $J_{H4}$ on both strands.

Id-460$^+$ and Id-460$^-$ hybridomas. Using a 5′ flanking region probe to a $V_{\kappa 1}$ gene we were able to detect only two $V_{\kappa 1}$ crosshybridizing Bam HI fragments in germline DNA: $V_{\kappa 1A}$ and $V_{\kappa 1C}$. By Southern blot analysis of Bam HI–digested hybridoma DNA using $JC_\kappa$- and $V_{\kappa 1}$-specific probes, rearrangements of $V_{\kappa 1A}$ genes or $V_{\kappa 1C}$ genes were demonstrated in all of the Id-460$^+$ hybridomas in comparison with blots of M460 ($V_{\kappa 1A}$) and M467 ($V_{\kappa 1C}$) DNA. Fig. 5 shows the Bam HI-digested hybridoma DNA fragments detected by a $JC_\kappa$ probe. M460 expressed a $V_{\kappa 1A}$ gene, based on protein sequence data, and exhibited a 10.5 kb $V_{\kappa 1}$- and $JC_\kappa$-hybridizing rearranged DNA fragment. Hybridomas 2AB5, Id16, and Id25 produced $V_{\kappa 1}$- and $JC_\kappa$-hybridizing bands of that size. M467, by protein sequence, expressed a $V_{\kappa 1C}$ gene and, by Southern blot, yielded a rearranged hybridizing fragment of 12 kb. Five hybridomas, D35, LB8, Id29, Id24, and Id30, rearranged a $V_{\kappa 1C}$, as shown by $V_{\kappa 1}$- and $JC_\kappa$-hybridizing fragments at 12 kb. All Id-460$^-$ hybridomas showed no rearranged $JC_\kappa$-hybridizing fragments that also hybridized with the $V_{\kappa 1}$ probe. The negative control was the G-1 hybridoma, which expresses a lambda light chain. Confirmation of the expression of $V_{\kappa 1}$ by some of the hybridomas was determined by Northern blot analysis (not shown).

Since $J_\kappa$ may play a role in the expression of Id-460, we used the technique of

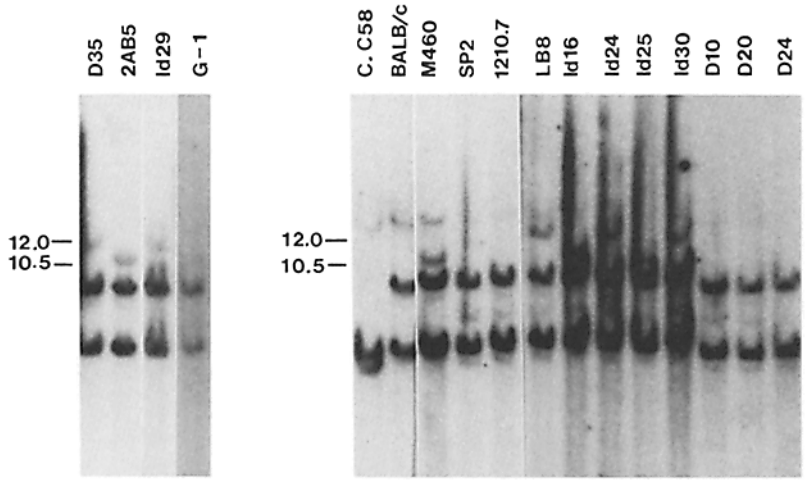


FIGURE 5. Immunoglobulin light chain gene analysis in monoclonal antibody-producing cells. Genomic DNA from the indicated cell lines was analyzed by Southern blot for $V_{\kappa 1}$-hybridizing Bam HI restriction fragments. BALB/c liver DNA and SP2 served as controls for the germline configuration of Bam HI restriction fragments and the nonproductively rearranged fragments of the hybridoma fusion partner. Hybridization of the $V_{\kappa 1}$ probe with a fragment of 12 kb indicates the rearrangement of a $V_{\kappa 1C}$ gene whereas hybridization of the probe to produce a 10.5 kb band demonstrates the rearrangement of a $V_{\kappa 1A}$ gene. D10, D20, and D24 are reproducibly negative for $V_{\kappa 1}$ hybridization to unique $JC_{\kappa}$-hybridizing fragments. G-1 is known to express a lambda light chain (P. Gearhart, unpublished), and 1210.7 expressed a $V_{\kappa 2}$ gene.

| $V_K$ | $J_K$ | | | Antigen specificity | Id-460 |
|---|---|---|---|---|---|
| L G V F F C S Q S T H V P<br>ICTGGGAGTTTTTTTCTGCTCTCAAAGTACACATGTTCC | L T F G A G T K L E L K R<br>GCTCACGTTCGGTGCTGGGACCAAGCTGGAGCTGAAACGG | MOPC 460 | $V_{K1A}$, $J_{K5}$ | DNP | + |
| V P<br>GTTCCT | W T F G G G T K L E I K R<br>TGGACGTTCGGTGGAGGCACTAAGCTGGAAATCAAACGG | D35 | $V_{K1C}$, $J_{K1}$ | DNP | + |
| L G V Y Y C F Q G S H V P<br>TCTGGGAGTTTATTACTGCTTTCAAGGTTCACATGTTCCTC | R T F G G G T K L E I K R<br>GGACGTTCGGTGGAGGCACCAAGCTGGAAATCAAACGG | LB8 | $V_{K1C}$, $J_{K1}$ | P. PNEUMO. | + |
| F C S Q G T H V P I<br>TTTCTGCTCTCAAGGTACACATGTTCCTAT | F T F G S G T K L E I K R<br>ATTCACGTTCGGCTCGGGGACAAAGTTGGAAATAAAACGG | 2AB5 | $V_{K1A}$, $J_{K4}$ | P. PNEUMO. | + |
| L G V Y F C S Q S T H V P<br>TCTGGGAGTTTATTTCTGCTCTCAAAGTACACATGTTCCT | W T F G G G T K L E I K R<br>TGGACGTTCGGTGGAGGCACCAAGCTGGAAATCAAACGG | Id29 | $V_{K1C}$, $J_{K1}$ | UNKNOWN | + |
| P<br>CCG | L A F G A G T K L E L K R<br>CTCGCGTTCGGTGCTGGGACCAAGCTGGAGCTGAAACGG | Id16 | $V_{K1A}$, $J_{K5}$ | UNKNOWN | + |
| V Y Y C F Q G S H V P<br>GTTTATTACTGCTTTCAAGGTTCACATGTTCC | W T F G G G T K L E I K R<br>GTGGACGTTCGGTGGAGGCACCAAGCTGGAAATCAAACGG | MOPC 467 | $V_{K1C}$, $J_{K1}$ | P. PNEUMO. | - |

FIGURE 6. Immunoglobulin light chain cDNA sequences from monoclonal antibody-producing cells. A $C_{\kappa}$-specific primer was used to promote cDNA synthesis on mRNA from Id-460$^+$ and Id-460$^-$ cells as indicated. Extended cDNA were sequenced by Maxam and Gilbert method. $V_{\kappa 1}$ assignments were made on the basis of Southern blot analysis. Antigen specificities of these proteins are also listed.

cDNA sequencing of $C_{\kappa}$-primed total mRNA to determine which $J_{\kappa}$ was rearranged in the hybridomas and to confirm the expression of $V_{\kappa 1}$. The sequences generated by this technique (Fig. 6) demonstrate the use of $J_{\kappa 1}$, $J_{\kappa 4}$, and $J_{\kappa 5}$, and thus rule out the possibility of a particular $J_{\kappa}$ as an important element in the expression of Id-460.

Of 16 myelomas and hybridomas in Table IV, all 9 Id-460$^+$ monoclonal antibodies were demonstrated to use a $V_{H460}$-crosshybridizing gene and $V_{\kappa 1}$-crosshybridizing gene. Neither $J_H$ or $J_{\kappa}$ gene segment usage appears to play a

TABLE IV
*Expressed Genes in Monoclonal Antibody–producing Cells*

| Cell | $V_H$ | $J_H$ | $V_L$ | $J_\kappa$ | Id-460 | Antigen specificity |
|---|---|---|---|---|---|---|
| MOPC-460 | $V_{H460}$* | 4 | $V_{\kappa 1A}$ | 5 | + | DNP |
| D35 | $V_{H460}$ | 4 | $V_{\kappa 1C}$ | 1 | + | DNP |
| LB8 | $V_{HLB8}$ | 2 | $V_{\kappa 1C}$ | 1 | + | *P. pneumotropica* |
| 2AB5 | $V_{H460}$ | 4 | $V_{\kappa 1A}$ | 4 | + | *P. pneumotropica* |
| Id29 | $V_{H460}$ | 4 | $V_{\kappa 1C}$ | 1 | + | ‡ |
| Id16 | $V_{H460}$ | 4 | $V_{\kappa 1A}$ | 5 | + | ‡ |
| Id25§ | $V_{H460}$ | 4 | $V_{\kappa 1C}$ | NT‖ | + | Unknown |
| Id30 | $V_{H460}$ | 4 | $V_{\kappa 1C}$ | NT | + | Unknown |
| Id24§ | $V_{H460}$ | 2 | $V_{\kappa 1C}$ | NT | + | Unknown |
| D10 | — | 3 | — | NT | − | DNP |
| D20 | — | 4 | — | NT | − | DNP |
| 1210.7 | $V_{H460}$ | 3 | $V_{\kappa 2}$ | NT | − | Ars |
| G-1 | — | 3 | $V_\lambda$ | — | − | PC + DNP |
| MOPC 315 | $V_{H315}$ | 2 | $V_\lambda$ | — | − | DNP |
| MOPC 467 | — | 2 | $V_{\kappa 1C}$ | 1 | − | *P. pneumotropica*¶ |

* $V_{H460}$ corresponds to the rearrangement of the 1210 germline $V_H$ gene. $V_{HLB8}$ corresponds to the rearrangement of the SB32 germline $V_H$ gene.
‡ These hybridomas may have low binding affinity for DNP.
§ These Id-460$^+$ hybridomas, although not formally tested for transcripts of $V_{H460}$ and $V_{\kappa 1}$ crosshybridizing genes, are predicted by Southern blot analyses and serologic results to functionally express these genes.
‖ Not tested.
¶ The antigenic determinant on *P. pneumotropica* that MOPC-467 recognizes is different than the determinant recognized by LB8 or 2AB5.

major role in the expression of Id-460. Furthermore, the rearrangement and expression of either a $V_{H460}$-crosshybridizing gene or a $V_{\kappa 1}$-crosshybridizing gene did not result in the expression of Id-460. Therefore, there is a strict requirement for rearrangement and expression of both a $V_{H460}$- and $V_{\kappa 1}$-crosshybridizing gene for the expression of Id-460 in our panel of proteins.

*Specificity for Antigen Cannot Be Predicted from the Use of Particular Gene Segments or Junctional Sequences in Id-460$^+$ Hybridomas.* Within the panel of Id-460$^+$ monoclonal antibodies specific for DNP or *P. pneumotropica* or of unknown antigen specificity, gene segment rearrangements and sequences were compared to identify regions that may be responsible for antigen binding.

First we studied $V_H$ gene usage between the hybridomas of differing antigen binding characteristics. Monoclonal Id-460$^+$ antibodies of all three binding categories (e.g., M460, 2AB5, and Id30) were demonstrated to rearrange $V_{H460}$ by Southern blot analysis (Figs. 2 *b* and 3, *a* and *b*). Therefore, antigen specificity does not localize to $V_H$ alone. Somatic mutation may play a role in determining the antigen specificity of Ig encoded by this $V_H$ gene but this cannot be ascertained by these studies.

*P. pneumotropica*–binding antibodies LB8 and 2AB5 use $V_{HLB8}$ and $V_{H460}$, respectively. By Southern blot analysis and limited DNA sequence information they should be ~90% homologous. Therefore, specificity for this antigen does not correlate with a single $V_H$ gene rearrangement. Homologies between discon-

tinuous germline sequences within $V_H$ or somatic mutations may be responsible for the *P. pneumotropica* binding characteristic.

Also, antigen specificity is not predicted by $J_H$ rearrangement (Table III). All Id-460$^+$, DNP-binding monoclonal antibodies rearrange $J_{H4}$ to $V_{H460}$ (D35, Id29, Id16 and M460). An antibody with specificity for *P. pneumotropica* can also use $J_{H4}$ when rearranged to $V_{H460}$ (2AB5). Furthermore, antibodies specific for neither antigen, Id25 and Id30, rearrange the $V_{H460}$ gene to $J_{H4}$. Hence, the rearrangement of $J_{H4}$ does not predict specificity.

Rather, the possibility exists that the $D_H$ segment or junctional sequences between $V_H$, $D_H$, and $J_H$ can predict antigen specificity. The invariant use of a TCX codon at position 95 in the $VD_H$ junction has been found in hyperimmune, arsonate-binding, crossreactive idiotype (CRI) positive antibodies (10). We explored the interesting notion of localization of antigen binding characteristics to the $D_H$ segment by primer extension sequencing of hybridoma RNA with either C region or $J_H$ region primers. The $D_H$ junctional sequences shown in Fig. 4 were compared between the monoclonal antibodies within the antigen-specific groups.

$D_H$ sequence comparisons then were made between those Id-460$^+$ antibodies binding DNP: M460, D35, and MD6–12 (a fetal liver hybridoma from J. Kearney, unpublished results), and low affinity DNP-binding proteins Id16 and Id29. No obvious sequence similarities were observed, but the size of the junctional region (between invariant codons for alanine in $V_H$ and aspartic acid in $J_H$) is somewhat conserved at 9–11 codons. *P. pneumotropica*–binding antibodies 2AB5 and LB8 also show no similarities in $D_H$ sequence. Interestingly, the size of the junctional sequence in these antibodies is 6–7 codons and is smaller than those in the DNP-binding antibodies. $D_H$ junctional region size may thus play a role in determining antigen binding specificity of these antibodies.

Finally, it was important to determine whether antigen binding specificity was in any way related to $V_{\kappa 1}$ or $J_\kappa$ usage. Since M460, which binds DNP, rearranges a $V_{\kappa 1A}$ and $J_{\kappa 5}$, and M467, which binds *P. pneumotropica*, rearranges a $V_{\kappa 1C}$ and $J_{\kappa 1}$, it was possible that antigen specificity is related to light chain rearrangement and can be localized to either $V_{\kappa 1}$ type or $J_\kappa$ usage. However, within the group of Id-460$^+$ antibodies, $V_{\kappa 1A}$ can be used by DNP-binding antibodies M460 and Id16, and can also be used by the *P. pneumotropica*-binding antibody, 2AB5. Also, $V_{\kappa 1C}$ can be used by the DNP-binding antibody D35 and by the *P. pneumotropica*–binding antibody LB8. Therefore, antigen specificity cannot be localized to either of the $V_{\kappa 1}$ genes (Fig. 5). Similarly, $J_\kappa$ sequences appear to have no correlation with antigen binding specificity in these monoclonal antibodies (Fig. 6). Thus, at present, no conclusive relationship between light chain gene usage and antigen binding specificity can be drawn from these data. Specificity for small haptens has been considered to be a function of a small number of contact residues throughout the variable region of Ig (reviewed by Potter [11]) and is probably not a function of continuous sequence. Since the $V_\kappa$ sequence information in these studies is limited, the role of somatic mutation cannot be ascertained for changes in residues that may be important in antigen contact.

*Some DNP-binding, Id-460$^-$ Monoclonal Antibodies Use Nonhomologous $V_H$ and $V_\kappa$ Genes.* The antibody response to DNP is known to be heterogeneous. Specificity

for DNP does not require expression of a member of the $V_{H460}$ family. Of the three Id-460$^-$, anti-DNP antibodies generated from a single DNP-boosted mouse, each appeared by Southern blot to rearrange a different $V_H$ gene (Figs. 2 and 3). Hybridoma D20 contains a rearranged gene that hybridizes to the 5′ flanking sequence of $V_{HLB8}$ (not shown), although this is a nonfunctional rearrangement. D24 and D10 use completely different $V_H$ genes that do not crosshybridize with $V_{H460}$.

Sequence information in the $V_H$ region (Fig. 4) gives more insight into these antibodies. The limited cDNA sequences of D10 and D20 show little homology to the 3′ end of $V_{HLB8}$ or $V_{H460}$, and thus express a $V_H$ gene completely unrelated to this family. Therefore, none of the three DNP-binding, Id-460$^-$ antibodies use $V_{H460}$-crosshybridizing genes. Also, they express at least two different $J_H$: D10 uses $J_{H3}$ while D20 uses $J_{H4}$. D10 and D20 use $D_H$ segments that have no sequence or size similarities. Finally, Southern blot analysis of the light chain gene rearrangements shows neither common rearrangements nor rearrangement of $V_{\kappa 1}$ light chain genes (Fig. 5). These limited molecular data confirm the heterogeneous nature of antibodies found in the late, hyperimmune response to DNP. Therefore, we can conclude that Id-460$^-$ antibodies appearing late in the response to DNP do not arise from the B cells that gave rise to the Id-460$^+$ antibodies.

## Discussion

Id-460$^+$ antibodies identified by serological tests were studied for the structural basis of idiotypy and antigen binding specificity and were found to be closely related on the genetic level. Monoclonal Id-460$^+$ antibodies are encoded by (*a*) $V_{H460}$ or $V_{HLB8}$ and (*b*) $V_{\kappa 1A}$ or $V_{\kappa 1C}$ (5). Analysis of heavy and light chain genes from a panel of hybridomas revealed $V_{H460}$-crosshybridizing and $V_{\kappa 1}$-crosshybridizing genes rearranged and expressed in all Id-460$^+$ cells independent of antigen binding specificity. Most of the hybridomas rearranged the $V_{H460}$ gene to $J_{H4}$, although rearrangements to $J_{H2}$ were also observed. $D_H$ sequence and length varied in all the hybridomas. Examination of light chain genes demonstrated rearrangement and expression of $V_{\kappa 1}$ with $J_{\kappa 1}$, $J_{\kappa 4}$, or $J_{\kappa 5}$. Thus, the common gene segments used by all the Id-460$^+$ hybridomas were $V_{H460}$- and $V_{\kappa 1}$-related genes (Table IV), confirming serologic data that demonstrated Id-460 as a $V_H$-$V_L$-associated determinant. The anti-Id-460 reagent recognized antibodies expressing closely related $V_H$ and $V_L$ segments regardless of the $D_H$, $J_H$, and $J_\kappa$ gene segments used in this panel of hybridomas. Recently, both the T15 idiotype (12) and Ars-CRI (10) have been reported to be affected in their expression by changes in the $D_H$ segments encoding antibodies that are otherwise genetically identical. Although $D_H$ gene sequences and sizes varied in our panel of monoclonal Id-460$^+$ antibodies, certain sequences in this segment may affect the expression of Id-460; since Id-460$^-$ antibodies were not specifically analyzed, this question cannot be resolved.

Finally, one can correlate the use of $V_{\kappa 1}$ genes with the behavior of the proteins in the immunoassay for Id-460. M460 and 2AB5, which inhibit similarly, both use $V_{\kappa 1A}$, while LB8 and D35, which use $V_{\kappa 1C}$, inhibit similarly to one another

but differently from 2AB5 and M460. LB8, the only protein to use the $V_{HLB8}$ gene segment, is deficient in one (or more) epitope.

The relationship of genetic structure to antigen binding specificity was not so easily defined in these studies. In arsonate-binding, $CRI^+$ and related hybridomas, an invariant TCX codon at position 95 could be partially responsible for arsonate-binding (10) in hyperimmune antibodies. Because of conserved use of $V_{H460}$ and $V_{\kappa 1}$ and the varied use of $J_H$ and $J_\kappa$ in the panel of Id-$460^+$ hybridomas, the role of the $D_H$ segment in antigen binding specificity was examined. When the $D_H$ regions and junctions with $V_H$ and $J_H$ were compared among DNP-specific and *P. pneumotropica*–specific hybridomas, no consensus sequences were evident. However, the $D_HJ_H$ junction region size appeared to loosely predict antigen specificity in the ID-$460^+$ antibodies studied. $D_H$ segment junctional size may be important in antigen binding specificity but does not appear to affect expression of Id-460.

These studies have not addressed the role of somatic mutation in generating different antigen binding specificities in Id-$460^+$ antibodies. Just as one somatic mutation in the heavy chain variable region of the S107 myeloma can change the specificity of this T15 idiotype–positive antibody from phosphorylcholine to double-stranded DNA (13), somatic mutation(s) may be responsible for the shift in Id-$460^+$ antibodies from *P. pneumotropica* to DNP binding specificity. Since no single monoclonal antibody has been isolated having a high degree of specificity for both antigens, it could be that acquisition of specificity for DNP requires the loss of specificity for *P. pneumotropica*. We have evidence in at least three monoclonal Id-460 antibodies, one of which agglutinates *P. pneumotropica*, for some low degree of binding to DNP. Furthermore, it is interesting that LB8, which is an IgM antibody specific for *P. pneumotropica*, has no somatic mutations from its germline $V_H$, while D35 (IgG) and M460 (IgA) antibodies are both somatic mutants of their germline $V_H$ gene. Extensive sequencing studies are necessary to analyze somatic mutations further. McKean et al. (14) have demonstrated common amino acid changes in the light chains of antibodies specific for influenza hemagglutinin and suggest on the basis of Southern blot analysis that these hybridomas are related and have acquired sequential somatic mutations. Antigen clearly plays an important selective role in the generation of these antibodies. Future studies in our system will examine the lineage relationships between cells producing Id-$460^+$ antibodies of the DNP binding specificity and those of the *P. pneumotropica* binding specificity, within individual animals, and the role of somatic mutation in generating the disparate specificities.

Our heterologous anti-Id-460 reagent appears to accurately detect murine antibodies encoded by the $V_H$ and $V_\kappa$ genes described. Although the antiidiotype reagent was produced by rabbit immunization with M460 and was subsequently affinity purified on M460 for DNP-binding site–directed anti-Id-460, it detected more hybridomas (from anti-Id-460–hyperimmunized mouse spleens) that have either unknown antigen specificity or specificity for *P. pneumotropica* than DNP-binding hybridomas. Most of the Id-$460^+$ hybridomas from this fusion had little or no specificity for DNP (only 2 of 20 demonstrated specificity for DNP and this binding was very weak). The nature of this finding, however, is not so unexpected when one considers the in vivo serum repertoire of antibodies in the

mouse before laboratory immunization. Id-460$^+$ antibodies in normal serum appear not to have specificity for DNP although the absorption assay used to examine this point could only have detected such antibodies if they comprised >10% of the Id-460$^+$ Ig. ~50% of the Id-460$^+$ serum antibody could be absorbed out on *P. pneumotropica*, with the remaining 50% of unknown antigen binding specificity. It is interesting to note that a fetal liver hybridoma, MD6–12, isolated by J. Kearney (unpublished results) is Id-460$^+$ and binds DNP, and that a neonatal DNP-specific hybridoma, TF2–36, isolated by S. Riley (15), uses $V_H$ and $V_\kappa$ genes with close sequence homology to those encoding M460. Although we cannot detect such antibodies in normal mouse serum, it is clear that the representation of DNP-specific antibodies using this particular $V_H$ and $V_\kappa$ gene combination is high, at least in very young mice. Determination of the precursor frequencies of Id-460$^+$, antibody-producing cells of the DNP-specific and the *P. pneumotropica*–specific types awaits investigation with our anti-Id-460 reagent.

Examination of qualitative differences between Id-460$^+$ antibodies (Fig. 1) and observed gene rearrangements (Table IV) suggests that the anti-Id-460 reagent can discriminate between Id-460$^+$ molecules with $V_{\kappa 1A}$ and those with $V_{\kappa 1C}$ light chains. In fact, the titration curve of DNP hyperimmune monoclonal antibody D35 matches that of DNP hyperimmune serum, and suggests the use of $V_{\kappa 1C}$ in most antibodies produced in a secondary response to DNP. This, in addition to the finding of restriction site polymorphisms flanking the $V_{\kappa 1C}$ gene in C58 mice (16) that do not make Id-460$^+$ anti-DNP antibody responses, suggests that $V_{\kappa 1C}$ may be the most important $V_\kappa$ gene in this response.

Finally, one of the most interesting points to emerge from this analysis is that the use of $V_{H460}$:$V_{\kappa 1A\,or\,C}$ appears to be much higher than would be predicted on a purely stochastic basis. Given that there are at least 100 $V_H$ genes and 300 $V_\kappa$ genes, one would estimate that this combination would appear about once per $10^4$–$10^5$ B cells. Instead, we find that such antibodies comprise ~0.1–1% of serum Ig, and ~1% of LPS-activated B cell hybridomas (3). Furthermore, ~50% of the normal serum Id-460, and ~50% of Id-460$^+$ hybridomas from LPS-stimulated spleen cells are specific for *P. pneumotropica*. These data are consistent with strong positive selection for the expression of this particular pair of genes in the total B cell repertoire, perhaps by environmental antigen, since germ-free mice express lower levels of Id-460 (3). Thus, it seems unlikely that the results of Manser et al. (17), suggesting random utilization of $V_H$ gene segments in the arsonate system, will be generalizable to all idiotypic systems. Furthermore, we see evidence for selection in the expression of Id-460 in anti-DNP antibody responses (1, 2). Given that there are multiple combinations of V genes that can give rise to anti-DNP antibodies, it is remarkable that, at the single B cell level, one gets transient and essentially complete dominance of the anti-DNP antibody response by Id-460$^+$ anti-DNP antibody-forming cells. These results thus provide strong albeit indirect evidence that both antigen- and idiotype-specific regulatory cells are operating to influence V gene expression in the BALB/c mouse.

## Summary

Id-460$^+$ immunoglobulins can be induced in vivo by immunization with dinitrophenyl (DNP) or *P. pneumotropica* and form two nonoverlapping groups of

antibodies with respect to antigen binding specificity. In this study, using Id-460$^+$ antibodies of differing antigen binding specificities, we compared on the molecular genetic level the five gene segment combinations ($V_H$, $D_H$, $J_H$, $V_L$, and $J_L$) that encode the variable regions of these idiotype-positive immunoglobulins. The Id-460 determinant appears to be a conformational or combinatorial determinant encoded by $V_{H460}$ and $V_{\kappa 1}$ crosshybridizing genes. $D_H$, $J_H$, and $J_\kappa$ gene segments appear to have no measurable effect upon expression of Id-460. Finally, antigen binding specificity does not appear to simply localize to any particular gene segment but may in part be the result of somatic mutation and/or $VDJ_H$ junctional sequences, whose length correlates roughly with antigen binding specificity.

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