STRUCTURAL CORRELATES OF CROSS-REACTIVE AND INDIVIDUAL IDIOTYPIC DETERMINANTS ON MURINE ANTIBODIES TO $\alpha-(1 \rightarrow 3)$ DEXTRAN

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Since their first description over 20 years ago, idiotypes have been used extensively to study various aspects of the immune system (1, 2). Idiotypes, antigenic determinants associated with immunoglobulin-variable regions, have been found on $\text{V}_\text{H}$ and $\text{V}_\text{L}$ regions, or both (3-11), and have been localized to both antigen-binding and framework regions (12-17). Those idiotypes shared by immunoglobulins known to be structurally different are designated IdX, cross-reactive or public idiotypes, whereas those apparently restricted to one or a few closely related types of molecules are designated IdI, individual or private idiotypes.

In spite of these general descriptive associations, the molecular bases for idiotypes have not been precisely defined. This lack of molecular correlation stands in striking contrast to the precise structural correlations for the constant region allotypes of rabbit and human immunoglobulins where allotype-positive and -negative proteins differ by only one or a few amino acid residues. Closely related sets of structurally defined idiotype-positive and -negative proteins have not previously been described.

As part of an examination of the murine repertoire of anti-$\alpha-(1 \rightarrow 3)$ dextran antibodies, a number of dextran-binding hybridoma proteins have been produced. A comparison of amino acid sequences (18) and idiotype expression of these proteins has allowed us to determine the molecular basis of anti-dextran idiotypes as a result of two features. First, the light chains from two dextran-binding myeloma proteins, MOPC104E and J558, are identical by amino acid sequence analysis. Furthermore, the light chains of these proteins and the 10 hybridoma proteins are identical by isoelectric focusing. If these 12 light chains are identical, idiotypic variability is encoded only in heavy chains. Second, some idiotype-positive and idiotype-negative heavy chains are different by only one or a few adjacent amino acids. Accordingly, precise correlations between IdX and IdI have been made with amino acid sequence variations in certain portions of the $\text{V}_\text{H}$ regions.

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1 Abbreviations and nomenclature used in this paper: CFA, complete Freund's adjuvant; HGPRT, hypoxanthine guanine phosphoribosyl transferase; $I_{50}$, 50% inhibition; IdI, individual or private idiotype(s); IdX, cross-reactive or public idiotype(s); IEF, isoelectric focusing.

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Materials and Methods

Mice. Male and female retired breeders of the strains BALB/cJ and BALB/c Cum were obtained from The Jackson Laboratory, Bar Harbor, Maine, and The Cumberland View Farms, Clinton, Tenn., respectively. (BAB-14 × BRVR)F1 mice were bred in our animal facility (Washington University, St. Louis, Mo.). The BAB-14 females were gifts of Dr. L. A. Herzenberg (Stanford University, Palo Alto, Calif.). BRVR male mice were purchased from the Laboratory Animal Facility, State University of New York at Buffalo, Buffalo, N. Y.

Antigens and Immunizations. Primary anti-α-(1 → 3) dextran responses were generated by intraperitoneal injection of 100 μg B1355 dextran (Northern Regional Research Laboratory, Peoria, Ill.) in complete Freund’s adjuvant (CFA). Hyperimmune responses were elicited by two monthly injections of 100 μg of B1355 in incomplete Freund’s adjuvant followed 1 mo later by three intraperitoneal injections of 2 × 10^9 *Escherichia coli* B (Calbiochem.-Behring Corp., American Hoechst Corp., San Diego, Calif.) that had been heat killed at 90°C for 30 min before use (19).

Somatic Cell Hybridization. The procedure used was essentially that of Galfre et al. (20). Briefly, ~10^6 spleen cells from dextran-immunized mice were fused by polyethylene glycol (Carbowax 1,500 [1,500 mol wt, Fisher Scientific Co., Pittsburgh, Pa.) to at least 10^6 hypoxanthine guanine phosphoribosyl transferase (HGPRT)-deficient MPC-11 (γ-2b k) plasmacytoma cells, line 45.6T61.7 (21) or a nonsecreting variety of MOPC-21 (γ-I,k), line NS1/1- Ag4-1 (22). These cells were generously supplied by Dr. M. Scharff (Albert Einstein College of Medicine, New York) and by Dr. C. Milstein (Medical Research Council, Cambridge, Mass.). Cells were cultured in 24-well culture dishes in HAT selection medium (23). Dextran-binding proteins were detected in culture supernates either by isoelectric focusing or radioimmune assays. Hybrids were cloned in culture supernates either by isoelectric focusing or radioimmune assays. Hybrids were cloned in soft agar over 3T3 feeder layers (24) and grown in BALB/c mice as ascites tumors.

Idiotype Assays. The assays for three separate idiotype determinants, IdX, IdI(M104), and IdI(J558), have been described previously (25). Analyses were inhibition-type, solid-phase radioimmunoassays performed in microtiter plates (Cooke Laboratory Products, Div. Dynatech Laboratories, Inc., Dynatech Corp., Alexandria, Va.).

IdX. Goat anti-M104 was absorbed with T183 (μ-K), HOPC-1 (γ-μλ), and normal serum by solid-phase and soluble absorption. The resulting antiserum, called anti-IdX, was used at a 1:20,000 dilution to bind 125I-J558, a related dextran-binding protein with identical λ-chains.

IdI(M104). Rabbit anti-M104 was absorbed with T183, HOPC-1, normal serum, and J558. The resulting antiserum, called anti-IdI(M104), was used at a 1:100 dilution to bind 125I-M104. It was necessary to include 10 μg of T183 and J558 in the assay to achieve the desired specificity.

IdI(J558). Rabbit anti-J558 was absorbed with M315(μλ), HOPC-1, and M104 to generate an antiserum called anti-IdI(J558). This reagent was used at a 1:2,000 dilution to bind 125I-J558.

The relative concentration of these determinants in dextran-binding proteins was measured by calculating the amount of test protein needed for 50% inhibition (I_50) of the radiolabeled proband molecules from binding to the plastic-adsorbed anti-idiotype. This value, when divided into the I_50 value for unlabeled proband, gives a measure of idiotype expression. A value of 1.0 denotes idiotype identity between test protein and the proband; <1.0 indicates nonidentity.

Results

Anti-α-(1 → 3) Dextran Antibodies. Table 1 summarizes the origins and characteristics of the anti-α-(1 → 3) dextran antibodies used in this study. The 10 hybrid-derived antibodies are all μλ-immunoglobulins and result from fusions of spleens from 9 individual animals. By isoelectric focusing, the λ-light chains of the hybrid proteins are indistinguishable from those of M104 and J558 (data not shown). The hybrid proteins that result from fusions with MPC-11 in particular have variable amounts of plasmacytoma-derived K chains in the IgM molecules, but the μ-K pairs do not bind dextran (26) and probably have little effect on idiotype determinations (see below).
**TABLE I**

**Anti-α-(1 → 3) Dextran Antibodies**

| Protein | Origin | Hybrid partner* | Immunization‡ | Class |
|---------|--------|-----------------|---------------|-------|
| M104    | BALB/c | —               | —             | IgM   |
| J558    | BALB/c | —               | —             | IgA   |
| Hdex 1  | (BAB-14 × BRVR)F₁ | MPC-11 | dextran/E. coli | IgM   |
| Hdex 2  | BALB/c | M21             | dextran/E. coli | IgM   |
| Hdex 3  | BALB/c | MPC-11         | dextran/E. coli | IgM   |
| Hdex 4  | BALB/c | MPC-11         | dextran/E. coli | IgM   |
| Hdex 5  | (BAB-14 × BRVR)F₁ | MPC-11 | dextran/E. coli | IgM   |
| Hdex 6  | BALB/c | M21             | dextran/E. coli | IgM   |
| Hdex 7  | BALB/c | M21             | dextran 1°, | IgM   |
| Hdex 8  | BALB/c | M21             | dextran 1°, | IgM   |
| Hdex 9  | BALB/c | dextran 1°,     | IgM   |
| Hdex 10 | BALB/c | MPC-11         | dextran/E. coli | IgM   |

* HGPRT-deficient MPC-11 (γ-2bK) or nonsecreting variant of MOPC-21 (γ-1K) that synthesizes but does not secrete K chains.

‡ Hybrids Hdex 1 and 5 were derived from a single (BAB-14 × BRVR)F₁ mouse 7 d after hyperimmunization with two monthly injections of 100 μg of dextran B1355 in CFA followed 1 mo later by three intravenous injections of 2 × 10⁵ E. coli at 2 d intervals. The other hybrids were derived from individual BALB/c mice: Hdex 2, 3, 4, 6, and 10 were obtained 1-3 d after the dextran/E. coli protocol, and Hdex 7, 8, and 9 were obtained 5-6 d after a single injection of 100 μg of dextran in CFA.

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**Fig. 1.** Diversity patterns of V₉ regions of α-(1 → 3) dextran-binding myeloma and hybridoma proteins. The complete V₉ amino acid sequences of these 12 proteins are presented elsewhere (18, 31). Protein M104 is used for comparison; solid lines for the V segments (1-99) and J segments (102-117) denote identity with M104. Differences are indicated by the one-letter code of Dayhoff (30). The D-segment sequences (100-101) are given for each protein.

**Amino Acid Sequence Patterns of Dextran-binding Proteins.** Details of the total amino acid sequences of the V₉ regions of M104, J558, and the 10 dextran-binding hybridoma proteins are presented elsewhere (18). Fig. 1 summarizes the major features of these sequences and shows that the diversity patterns divide the V₉ regions into
three segments: the V segment (residues 1–99), the D segment (100 and 101), and the J segment (102–117). There are four different V segments that differ from one another at two to seven positions, and three J segments that differ from each other at from two to five positions. There are nine different pairs of amino acids in the D segment with three duplicated sequences. The V and J segments are encoded by separate gene segments analogous to the V- and J-gene segments coding for mouse light-chain-variable regions (27–29). 

The genetic basis for the D segment is unknown; however, it must arise from DNA not directly contiguous with either the V- or J-gene segments. The fact that the D segment occurs at the junction of the V- and J-gene segments suggests that the D segment represents yet another gene segment coding for the classical variable region. Diversity in D-gene segments may arise by some process of somatic variation or it may be encoded by germ-line D-gene segments.

**Idiotypic Characteristics of Dextran-binding Proteins.** The dextran-binding proteins were analyzed for their expression of three idiotypes previously described by Hansburg et al. (25), and the results along with a summary of the sequence data are shown in Fig. 2 and Table II. The IdX, expressed equally by M104 and J558, is found on 11 of the 12 hybridoma proteins. One protein, Hdex 8, shows partial expression of IdX, whereas Hdex 10 is IdX negative. In addition to the IdX, other determinants have been described previously that were found on M104, but not J558 [IdI(M104)] and on J558, but not M104 [IdI(J558)]. These IdI determinants are also found among some of the hybridoma proteins. Two hybridoma proteins, Hdex 8 and Hdex 7, are equivalent to M104 in expressions of IdI(M104). One hybridoma protein, Hdex 9, expresses IdI(J558) fully, whereas two others, Hdex 1 and Hdex 2, show partial expression of this idiotype. This pattern of expression of IdX and IdI determinants among the monoclonal antibodies is consistent with that seen in serum anti-α-(1 → 3) dextran antibodies. The bulk of both 7S and 19S serum antibodies react with anti-IdX, whereas only a minority express either IdI(M104) or IdI(J558) (25).

It is unlikely that the existence of plasmacytoma-derived K chains in some of the hybridoma proteins seriously alters our estimates of idiotypic relatedness. Even if it should be found that μ-K pairs do not express a determinant normally expressed by μ-λ-pairs, the effect is minimized here by normalizing idiotype expression to λ-concentration.

**Localization of the Amino Acids Important for IdX Expression.** A search was made to correlate amino acid sequence with the IdX determinant. Table II shows that it is unlikely that the D segment is involved directly with IdX expression; the IdX-positive proteins have nine different D segments that contain residues with side chains that differ considerably in size, charge, and bonding abilities. Likewise, the J segments do not contain residues that determine IdX expression because all three J segment prototypes are associated with IdX-positive proteins and the IdX-negative protein contains J1 as do several IdX-positive proteins. Thus, it is differences in the V segment that most likely determine IdX expression. The four prototype V segments and their IdX expression are summarized in Fig. 3. Comparison of the V4 (Hdex 10) IdX-negative sequence with the IdX-positive segments, V1, V2, and V3 shows that the residues at positions 54 and 55 correlate with IdX expression. All 10 IdX-positive

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2 Early, P., H. Huang, M. Davis, K. Calame, and L. Hood. An immunoglobulin heavy chain is generated from three segments of DNA: VH, D, and JH. *Cell.* In press.
proteins have asparagine at residues 54 and 55 with a carbohydrate side chain at position 55, whereas the IdX-negative V4 sequence of Hdex 10 lacks the carbohydrate and has lysine residues at positions 54 and 55. Note that differences at positions other than 54 and 55 do not modify expression of IdX (e.g., V3 of Hdex 9). Interestingly, a serine for asparagine interchange at position 54 results in partial expression of IdX (e.g., V2 of Hdex 8). This suggests that both positions 54 and 55 are involved in the IdX determinant. The influence of the carbohydrate side chains on IdX expression is as yet unknown.

Localization of the Amino Acids Important for IdI Expression. Table II clearly shows that the IdI(M104) and IdI(J558) determinants are dependent upon D-segment residues. A comparison of the M104 and J558 proteins alone demonstrates this point. These two proteins, whose light chains have been shown to be identical by amino acid sequence analysis (32), differ only in their D segments. Thus, anti-IdI(J558), which discriminates between J558 and M104 by a factor of 1,000, must recognize the influence of arginine and tyrosine at residues 100 and 101 in J558. This is confirmed by the Hdex 9 protein, which fully expresses the IdI(J558) determinant and also has arginine and tyrosine at residues 100 and 101, despite a different V segment (V3). Interestingly, change of arginine 100 to asparagine (Hdex 1 and 2) results in partial reactivity in the anti-IdI(J558) assays. Thus, the dominant contributor to the idiotype is tyrosine 101, with significant involvement of arginine 100. Similarly, the IdI(M104) determinant involves the D segment, tyrosine-aspartic acid. Both M104 and Hdex 8 express the IdI(M104) determinant equally and exhibit identical D segments. In addition, Hdex 7 with alanine instead of tyrosine at position 100 also shows full expression of IdI(M104). This again suggests that position 101 is the predominant contributor to the determinant. However, two IdI(M104)-negative proteins, Hdex 3 and 4, also have aspartic acid at position 101 but differ from IdI(M104)-positive proteins at position 100. This suggests that some amino acids at position 100 (tyrosine and alanine) allow the expression of IdI(M104), whereas others such as arginine and lysine prohibit it. It may be significant that both arginine and lysine are positively charged amino acids.

Discussion

Antibodies to α-(1 → 3) dextran have been thought to be relatively restricted in heterogeneity because of simple and widely shared isoelectric focusing (IEF) patterns (25, 33) and of the existence of an idiotype shared by the majority of anti-dextran antibodies (25, 34). However, by making a correlative study of IEF patterns with a set of four idiotypes developed with the existing dextran-binding myeloma proteins, M104, J558, and UPC102, it became apparent that more heterogeneity existed than was anticipated (33). The anti-idiotype reagents included goat anti-M104, absorbed to remove only isotypic activity, that detected determinants expressed by all three myelomas and thus was an IdX. Three additional reagents prepared by removing anti-IdX activity recognized only the individual myelomas, and thus were labeled anti-IdI(M104), -IdI(J558), or -IdI(UPC102) (25). It was evident that anti-dextran myeloma proteins contained both shared and unique variable-region determinants.

All four of these antisera detected proteins present in the sera of conventionally immunized responder mice. The IdX was found to be associated with 60–80% of 7S and 19S anti-dextran antibodies. This idiotype reagent is probably similar to the anti-
### Table II

Comparison of Variable-Region Structure with Idiotype Expression

| Variable region* | Idiotype expression‡ |  |
|------------------|----------------------|--|
|                  | IdX (SDF§)           | IdI(M104) (SDF) | IdI(J558) (SDF) |
| V                | D                    | J               |               |
| M104             | YD 1                 | 1.0 (1.1)       | 1.0 (1.2)     | <0.001 NC    |
| Hdex 8           | YD 1                 | 0.2 (1.3)       | 0.9 (1.2)     | <0.001 NC    |
| Hdex 7           | AD 1                 | 0.8 (1.3)       | 1.1 (1.5)     | <0.001 NC    |
| J558             | RY 1                 | 1.2 (1.2)       | <0.002 NC     | 1.0 (1.1)    |
| Hdex 9           | RY 1                 | 1.4 (1.1)       | 0.002 (1.5)   | 1.5 (1.1)    |
| Hdex 1           | NY 3                 | 1.0 (1.1)       | 0.005 (2.1)   | 0.10 (1.2)   |
| Hdex 2           | NY 1                 | 2.1 (1.2)       | 0.002 (1.5)   | 0.11 (1.1)   |
| Hdex 3           | RD 1                 | 1.6 (1.2)       | 0.01 (1.2)    | <0.001 NC    |
| Hdex 4           | KD 2                 | 1.6 (1.1)       | 0.008 (1.4)   | <0.001 NC    |
| Hdex 5           | SN 2                 | 1.5 (1.0)       | 0.002 (1.2)   | 0.002 (2.2)  |
| Hdex 6           | SH 1                 | 1.4 (1.3)       | 0.002 (1.3)   | 0.009 (1.5)  |
| Hdex 10          | VN 1                 | <0.001 NC       | <0.001 NC     | 0.002 (1.2)  |

* See Fig. 1 for explanation of variable-region segments. The one-letter amino acid code of Dayhoff (30) is used to define the D segments.
‡ See Materials and Methods.
§ Standard deviation factor. Log SDF = SD of log (idiotype expression).
∥ Not calculable.

J558 idiotype described by Blomberg et al. (34) that inhibited 80% of anti-dextran plaque-forming cells. Also, amino acid sequences of pooled IdX-negative and IdX-positive dextran-binding serum antibodies were identical for at least the first 30–50 residues (35). This demonstrated the limited heterogeneity of $V_N$ segments in anti-dextran antibodies and that the IdX reagent recognized differences in the C-terminal portion of the $V_N$ region of these antibodies. The IdI were expressed to a much smaller degree than the IdX and appeared to be associated with antibodies that also expressed the IdX activity. The frequency of expression for individual idiotypes followed the order $M104 > J558 > UPC102$, but the sum of the antibodies bearing these IdI determinants accounted for only a minority (~10%) of the anti-dextran antibodies (25). This left the majority of antibodies defined either by an IdX alone or by the absence of any detectable idiotype.

The ability to produce α-(1 → 3) dextran-binding hybridoma proteins has not only enhanced our study of the anti-dextran repertoire, but also has allowed us to study the molecular bases of idiotypes. This is possible primarily for two reasons. First, the two myeloma proteins, M104 and J558, are known to share the identical λ-light chain (32) which by isoelectric focusing appears common to all the hybridoma proteins. Therefore, the light chain should be a constant factor in idiotype expression, thus

Fig. 2. Relative inhibitory patterns of dextran binding proteins in radioimmune assays for (A) IdX, (B) IdI(M104), and (C) IdI(J558). Inhibitory patterns for 12 dextran binding proteins in idiotype assays as described in Materials and Methods. Distinctive patterns are emphasized by solid lines. NSIg represents the inhibitory capacity of an immunoglobulin fraction of normal mouse serum.
simplifying characterization of sequence correlates for the idiotypes. It should be emphasized that this supposition requires experimental confirmation by direct sequence analysis of the λ-chains. Secondly, the heavy chains share a large degree of sequence identity with only one or two amino acid differences between some idiotypic-positive and -negative VH regions.

Three separate idiotype determinants on dextran-binding immunoglobulins correlate well with hypervariable-region structure. The IdX correlates with two amino acids and/or associated carbohydrate in the second hypervariable region, and the two IdI determinants are dependent upon D-segment structure in the third hypervariable region.

The finding that the dextran idiotypes are dependent upon one or two amino acids is consistent with the probable molecular bases of other serological markers of immunoglobulins, notably, Gm markers of γ-chains, the Inv markers on K chains, and the Oz and Kern markers of λ-chains (36–38). Likewise, the rabbit γ-chain markers a 11–12 and a 14–15 reflect single amino acid substituents (39, 40). Studies by Vrana et al. (11) on the light-chain idiotypes of inulin-binding myeloma proteins limit the location of an Idl and IdX determinant to a few amino acids each.

Other families of immunoglobulins have been characterized idiotypically. In a study of human IgM cold agglutinins, Williams et al. (41) were the first to show that proteins with similar binding specificities possess both shared and unique antigenic determinants. Furthermore, myeloma and hybridoma proteins that bind inulin, levan, galactan, and poly-(L-glutamic acid, L-alanine, L-tyrosine) (10, 42–44) have been studied extensively and demonstrate both IdX and IdI specificities. Like dextran antibodies, conventionally raised antibodies to these antigens exhibit IdX determinants; however, IdI determinants are rarely detected. This has led to the concepts that IdX determinants reflect germ-line genes, and that IdI determinants reflect random somatic mutation. Our data show how IdI determinants can occur in natural antibody; however, the mechanism by which the D-segment diversity arises is not yet understood.2 Whether the IdI determinants in other idiotype systems will localize to the D segment awaits further study.

There are several important points to be made from these data concerning the use of idiotypes as structural markers for genetic studies. (a) The anti-IdX reagent is
specific for only two adjacent amino acids and/or carbohydrate in the V segment although the antiserum was absorbed only for isotype activity and with normal serum. This suggests that either the two amino acids and/or carbohydrate constitute the major antigenic determinant of the V segment or that normal serum immunoglobulins contain structures similar to the remaining determinants of M104. (b) Three of the four dextran V segments express the IdX determinant. Thus, this highly specific idioptic determinant is shared by more than one germ-line gene if, indeed, the distinct V-segment sequences are encoded by different germ-line V-gene segments. (c) Idiotypic determinants have been shown to depend on V and D segments. It is conceivable that some idiotypic determinants may involve J segments. Whereas V- and possibly D-segment idiotypes may indeed be specific for dextran-binding proteins, potential J-segment idiotypes may be shared by proteins binding to a number of antigens. For example, the J1 sequence has been shown to be present in myeloma proteins binding phosphocholine and galactan (18). Accordingly, it is possible that J segments are shared widely among immunoglobulin groups and that data that demonstrate an unusually high degree of idiotype sharing may be a result of anti-J-segment activity. (d) The individual idiotypes in the dextran system are dependent upon the D segment, which may or may not involve a germ-line DNA sequence: one possibility is that the diversity in this segment arises from a somatic variation mechanism. If so, it may be difficult to correlate the IdI determinants with distinct germ-line DNA sequences. Hence, the IdI markers may have uncertain significance in terms of gene mapping, linkage, and expression.

The localization of idiotypic determinants to V, D, and potentially even J segments has implications for studies that have used idiotypes as probes to analyze V-region composition and inheritance (17, 45, 46), to map V-region gene order through the analysis of recombinant animals (14, 45, 47), and to compare the nature of T cell receptors (48, 49) or effector factors (50, 51) to immunoglobulins, to name a few. Clearly, correct interpretations of results that involve idiotypes are dependent upon precise definition of the structure of each determinant and knowledge of the genetic bases for their inheritance.

Summary

For the first time V-region amino acid sequence differences have been correlated with the expression of cross-reactive and individual idiotypes through an analysis of 12 dextran-binding proteins. This correlation has been possible because of the apparent sequence identity of the corresponding lambda chains. Expression of a cross-reactive idiotype was localized to two residues and/or a carbohydrate in the second hypervariable region of the heavy chain. Two individual idiotypes correlate with the two amino acids within the third hypervariable region that comprises the D segment of the dextran-binding proteins. These results demonstrate that idiotype reagents can recognize two amino acid differences within V and D segments of classical variable regions. In anti-dextran antibodies, cross-reactive idiotypes involve V-region determinants, whereas individual idiotype determinants correlate with D-segment variation.

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