Analysis of Idiotypic and Anti-Idiotypic Antibodies as Models of Receptor and Ligand

D. Scott Linthicum,1 Michael B. Bolger,2 Paul H. Kussie,1 George M. Albright,1 T. Andrew Linton,1 Sarah Combs,1 and Danilo Marchetti1

Antibodies to small bioactive ligands and peptides may mimic the binding characteristics of the natural receptor; in turn, the anti-idiotypic antibodies generated against the binding sites of such anti-ligand antibodies may mimic some aspects of small bioactive ligands and peptides. Among the several levels of investigation of such antibody–receptor networks are (a) the quantitative structure–activity relationships of ligand binding to antibody as compared with natural receptor; (b) the molecular modeling of antibody–receptor binding sites and the genomic basis for such structures; and (c) the characteristics of the molecular mimicry exhibited by "mimic-topes" on anti-idiotypic antibodies. To illustrate the analysis encountered at each of these levels, we discuss here antibody and anti-idiotype systems that are directed to small neuroactive ligands and their receptors.

Additional Keyphrases: receptor binding · peptides · idiotypes · ligands · mimicry

Numerous investigators have recently begun to utilize the vast molecular repertoire of antibody molecules as "artificial" receptors and "mimics" of ligand for a wide variety of bioactive compounds and receptors. Because antibodies can be produced against small ligands with exquisite structural specificity, they can often demonstrate binding properties similar to those of the natural receptor. In addition, antibodies directed against ligands can also serve as antigens themselves, giving rise to anti-idiotypic antibodies, some of which, in turn, can mimic the binding characteristics of the original ligand. Before we explore some of the findings of such studies let us set the stage for the terminology and concepts in this fast-paced and sometimes confusing field.

The basic structure of the immunoglobulin molecule is a symmetrical glycoprotein comprising four polypeptide chains (two heavy chains and two light chains) formed into globular regions, or "domains." The amino terminal portion (F(ab) fragment) of the molecule forms the antigen-binding site. Analysis of the amino acid sequence of the light and heavy chains in this region reveals that one-half of the light and one-half of the heavy chain is relatively constant and the rest is variable (Fv). There are three noncontiguous regions of hypervariability within each variable region; Kabat and Wu (1) accurately predicted that the antigen-binding site was formed in three dimensions by the positioning of these six hypervariable regions consisting of three from the light chain and three from the heavy chain. The remainder of the molecule forms the "framework" region. The arrangement of these six hypervariable regions is termed the "complementarity determining region" (CDR) because they form a molecular "complement" of the antigen, as was confirmed by subsequent x-ray diffraction studies of Fab and light-chain dimer crystals (2). More recent analyses of the variable region (V) genes involved in immunoglobulin synthesis elucidated the genetically coded and noncoded basis for the formation of the CDRs (3). Recent molecular modeling studies examined the role of CDRs in antigen binding in detail (4) and are discussed below.

Because the hypervariable regions or CDRs of an antibody molecule are unique amino acid sequences encoded by specific V-region genes and used only by a specific set of B lymphocyte clones, they also represent the appearance of a unique and therefore new antigenic set of determinants not previously present in the organism. Thus these "new" antigenic determinants are "idiosyncratic" to a particular antibody molecule, its V-region gene, and all B-cell clones that utilize the same V-region gene. The individual idiosyncratic antigenic determinants are called "idiotopes"; the term "idiotype" refers to the collection of idiotopes over the entire molecule. Jerne (5) proposed that the immune system is composed of a network of idiotypes that are intimately involved in immune response regulation. A kind of "missile/anti-missile" network of antibodies keeps the B cells from proliferating uncontrollably subsequent to antigenic stimulation; a similar network probably acts on T cells as well.

Because the members of a given set of homogeneous (i.e., monoclonal) antibodies are themselves immunogenic, one can immunize an animal from the same genetic background and derive "anti-idiotypic" antibodies that react with the idiotopes of the first set of antibodies. Several kinds of anti-idiotypic antibodies have been recognized (Figure 1). One type of antibody (AB2α) is able to bind external idiotopes but does not inhibit ligand binding. Another (AB2β) is able to inhibit ligand binding and is ligand inhibited; this antibody, termed an "anti-paratope," is a mimic of the original ligand. The anti-paratope antibodies complement an internal image of the binding site. Some antibodies may bind close to the ligand-binding site and inhibit ligand binding, but are not completely inhibited by ligand (AB2γ). The network can be expanded to include a third level of anti-anti-idiotypes, leading to a number of more complicated antibodies, but that is beyond the scope of this discussion.

If antibodies (A) are raised against a given ligand, B, some will form a particular complex (AB), which may mimic the binding of B to its receptor protein C (the normal biological receptor). Hence, the anti-idiotypic antibodies against idiotypic antibody A may have structures in common (related epitopes) with ligand B and should bind receptor C (molecular mimicry) (Figure 2). In support of this assumption, Sege and Peterson (6) raised anti-idiotypic antisera directed against antibodies to retinol-binding protein, and this antisera was found to react with prealbumin ( transhyretin), a normal receptor for retinol-binding protein. In the past several years many experimental models utilized the molecular mimicry concept to develop antibod-

1 Department of Pathology and Laboratory Medicine, The University of Texas Health Science Center at Houston, P.O. Box 20708, Houston, TX 77225.
2 The University of Southern California School of Pharmacy, Los Angeles, CA 90033.

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ies to receptors or serve as vaccines. Table 1 lists a few of the best-studied systems in this area, as reviewed by Linthicum and Farid (7).

The concept of the anti-ligand antibody binding site as a pocket or cleft (paratope) that represents a "negative molecular mold" or "internal image" of the ligand led to the idea that the anti-paratope antibodies to this region represent, in molecular terms, a positive mold or molecular analog of the ligand. The molecular basis for the mimicry of some ligands is not known at this time, but several investigators suggested that the mimicry may be based on the use of homologous residues, deposition of specific contact residues, or merely molecular shape based on analogous components.

An important drawback to many early studies involving antisera was that the precise molecular nature of the anti-ligand and anti-idiotype antibodies was not really known, given their polyclonal nature. The use of monoclonal antibodies in recent schemes provided a better understanding of the molecular characteristics of these antibodies and their interaction with each other and receptor. The two hypotheses—(a) that the binding site of an antibody to a ligand will resemble the receptor for the ligand with respect to specifictiy, i.e., anti-ligand = receptor for ligand, and (b) that antibodies to the anti-ligand will cross-react with receptor—i.e., anti-anti-ligand = anti-receptor—need to be substantiated. This can best be accomplished by careful structure-activity studies with monoclonal antibodies (mAbs).

Quantitative Structure-Activity Relationship of Monoclonal Antibodies to Neuroleptic Drugs

For the past four years we have investigated the molecular characteristics of monoclonal anti-neuroleptic idiotypes and their anti-idiotypes. We developed several techniques and some unique chemical techniques for immunogen synthesis. Our approach involves coupling dopaminergic ligands to carrier proteins in a way that maintains the important structural features of neurotransmitter binding, e.g., a cationic amino group and an aromatic ring that is substituted with only naturally occurring hydroxyl groups. We successfully produced both idiotypic and anti-idiotypic mAbs to several dopaminergic ligands. Examination of our existing "library" of anti-haloperidol (dopamine D-2 antagonist) mAbs, using the techniques of molecular biology and physical chemistry, allowed us to study resulting changes in hapten binding affinity and specificity. An extensive quantitative structure-activity relationship (qsar) study (5) was designed to elucidate the precise nature of the interactions between haloperidol and five mAbs (A, B, C, D, and E) that possess high but different affinities for the drug. By sequencing the cDNA derived from the mRNA of the hybridoma lines that secrete those mAbs, amino acid sequences of the Fv portions of these antibodies were derived (9). Combining sequence information with the qsar data and x-ray crystallographic studies from the literature, and applying three-dimensional computer graphics technology, we generated three-dimensional models of the combining site of anti-haloperidol antibodies (10). Sherman and Bolger (10) showed that mAbs A and B bind a folded conformer of haloperidol that is unrelated to the one that binds at the receptor, the primary antigenic determinants of haloperidol being located in the fluorophenyl ring. Sequence data suggest that these mAbs are constructed from identical V-region genes. The predicted geometry of the haloperidol combining site is a classic example of a deep pocket β-strand barrel formed by long hypervariable loops. In contrast, mAbs D and E are characterized as having groove-like binding sites, which recognize an extended conformer of haloperidol whose primary antigenic determinants lie at the opposite end of the molecule, the chlorophenyl piperidine ring moiety. Monoclonal antibody C, whose affinity for the related butyrophene moiety exceeds that for haloperidol, is
characterized as recognizing an extended conformer of haloperidol resembling the type of interaction predicted for the D-2 dopaminergic receptor. The combining site for mAb C is predicted as being a surface or shallow channel (10).

Nucleotide and Amino Acid Sequence of Monoclonal Antibodies

A quick method of sequencing immunoglobulin mRNA, discovered by Hamlyn et al. (11), has been very useful in determining the amino acid sequence of mAbs. Rather than isolating the full length of cDNA for cloning and sequencing, dideoxynucleotides are added directly to the primer extension reaction, such that a set of labeled fragments are produced with identical 5' ends as the mRNA is being copied. By separating these fragments on a polyacrylamide sequencing gel, the nucleotide sequence (and thus the amino acid sequence) can be obtained. Hamlyn et al. (11) designed two oligonucleotide primers, a 17-mer and a 15-mer, that specifically bind to IgG light and heavy chains, respectively. These primers hybridize to a region near the variable–constant region junction of immunoglobulin mRNA. In the presence of reverse transcriptase, the 3' end of the primer is extended, producing a copy of a small portion of the constant region sequence and the entire variable region sequence. This method (11) has therefore been the method of choice for rapidly generating amino acid sequences of mAbs.

Each chain may need to be sequenced at least two times with a different radiolabeled nucleotide so that the true identity of any nucleotide obscured by a band running across all four lanes can be ascertained. Such artifacts sometimes arise because the dideoxynucleotide used as the radiolabel is present in very low concentration as compared with the other three nucleotides (e.g., 2 vs 50 μmol/L); this can cause the reverse transcriptase to pause or disassociate from the template when this low-concentration base is required for chain elongation. To alleviate the problem, we added a high concentration of all four nucleotides, as a "chaser," to extend all chains that terminated sequencing for reasons other than incorporation of a dideoxynucleotide.

This method frequently permits rapid determination of the entire variable region, which requires reading 350 bases (very close to the practical limit for the number of bases that can be read accurately from priming at a single site). Fortunately, the last 75 to 100 bases of each chain code for highly conserved framework regions, for which the sequences are largely known, make it possible to assign the correct base to bands in this region, the identity of which would otherwise remain doubtful. In general, the ability to read to the end of the variable region is controlled by factors such as purity of the RNA preparation (presence of DNA or protein), use of fresh radiolabeled nucleotide stocks (less than two weeks old), and the stability of the gel under the high-temperature conditions of electrophoresis. All of these factors help to eliminate a high background level of radioactivity, which tends to obscure faint bands in the upper regions of the sequencing ladder.

Another problem, unique to light chains, often makes difficult the correct assignment of a given nucleotide. This problem consists of two bands appearing at a single position in the sequencing ladder, and it arises because of the presence of one or more nonfunctionally rearranged light-chain gene segments that are contributed by the myeloma fusion partner. Fortunately, however, this sort of contamination is present in less than 20% of the bands, resulting in the darker of the two bands being the correct band. Other investigators chose to circumvent this problem by preparing full-length cDNA copies of both RNAs, excising the resulting bands from the gel, and sequencing each by chemical cleavage reactions.

The mRNA nucleotide sequences and the deduced amino acid sequences of the variable regions of the light and heavy chains of our anti-haloperidol antibodies were reported (9). As anticipated from our binding specificity study, two entirely different sets of sequences are selected for in response to the two different haloperidol–protein conjugates used for immunization. The light chains produced in response to a given conjugate are highly homologous, if not identical. On the other hand, the heavy chains selected in response to a given conjugate can belong to entirely different families. This illustrates how combinatorial association of different heavy and light chains contributes to the generation of different specificities. Perhaps the most interesting feature of the sequence data is the pattern of insertions and deletions found within the hypervariable loops. These result in the production of loops which differ from one antibody to another, and are directly responsible for establishing the gross architecture of the binding site (groove vs pocket) (Figure 3).

We compared the $V_H$ and $V_L$ anti-haloperidol sequences with previously published closely related sequences. Most of the reference sequences are germline in nature, representing sequences that have not been subjected to somatic mutation. This allows a direct evaluation of point mutations, which most likely arose during "fine tuning" events as the immune response to haloperidol matured. This will most likely be the case for the sequences of two antibodies that appear not to differ at all in their variable-region sequences. The fact that these antibodies were isolated from separate mice is a strong argument favoring the existence of an unreported germline gene that codes directly for these sequences. By examining the various gene segments encoding the selected anti-haloperidol mAbs, a sort of ancestral tree can be constructed depicting the maturation of the immune response to haloperidol. Our analysis indicates that the sequences of mAbs A and B are probably germline-

![Fig. 3. Computer-generated model of the F(ab) fragment of an antibody, highlighting the positions of the hypervariable loops (dark-shaded spheres) on the amino terminal (right-hand side) of the molecule.](image)

The position of these hypervariable loops determines, in part, the overall shape of the binding pocket of the antibody molecule. The model shown is a side view of the molecule.
related, and represent an initial response to haloperidol. The shortened H3 region of mAb C in comparison to A or B clearly demonstrates that this mAb is produced from a B-cell that is not derived somatically from a B-cell producing mAb A or B; the shortened H3 region is formed by gene rearrangements that occur before exposure to antigen. However, it is unclear whether or not the germline VH gene expressed by mAb C is a somatic variant of the mAb A/B germline gene. Finally, although mAb D and E were isolated from the same mouse, they clearly derive from clonally unrelated B-cells, as evidenced by their totally different heavy chains. The selection of nearly identical light chains therefore implies that the light chain is intimately involved in the binding of haloperidol, or that this specific chain is required to provide a unique structural architecture near the combining site.

Computer-Assisted Modeling

Recent advances in amino acid and nucleotide sequencing have resulted in an exponential growth in the number of immunoglobulins for which variable-region sequences have been studied in three dimensions by x-ray crystallography. Crystallization and structure refinement is the rate-limiting step in understanding the exact nature of the interaction between antigen and antibody at the molecular level. However, comparative studies performed on the crystal structures solved thus far have revealed that, despite the multitude of antigen specificities and affinities displayed by the immunoglobulins, the fundamental structure of the variable fragment (Fv) is highly conserved, especially the regions not directly involved in the actual binding interaction, the so-called framework regions. This observation had made it increasingly attractive to use these frameworks as a sort of scaffolding on which the nonconserved antigen-binding hypervariable loops can be assembled (4). Although this approach has provided some informative hypothetical binding-site models in the past, concrete evidence that this approach is indeed valid was only recently provided by the antilysozyme antibody, D1.3, whose predicted structure was largely confirmed by x-ray crystallographic data at 0.26 nm resolution (4). The high degree of sequence and structural homology that exists among the framework regions of the immunoglobulins can be summarized as follows: (a) The sequence homology shared by the frameworks of the six published light-chain structures ranges from 65 to 84%. The comparison includes both kappa light chains and lambda light chains, both of which share even greater intra-subtype homology. (b) The heavy-chain framework regions of the three studied heavy chains share approximately 60% sequence identity, despite their differing origins (mouse vs. human) and their differing subclasses (\(\gamma\) vs. \(\alpha\)). (c) When framework regions from all studied structures are superimposed, the percentage of alpha carbon atoms that can be superimposed within 0.15 nm ranges from 50 to 75%, with a root mean square error ranging from 0.061 to 0.085 nm. This extraordinary complementarity, particularly among the framework amino acids, is not surprising in light of the fact that without this common foundation, the relative positioning of the hypervariable loops would be lost along with antigen binding ability. (d) If one compares the above sequences after taking into account similarities in physicochemical properties shared by many amino acid residues (Leu is often substituted for Met, on the basis of similar size and hydrophobicity), one obtains values for framework sequence variability equivalent to those obtained for the highly conserved constant regions. This demonstrates that in many cases a specific type of amino acid residue at each position (e.g., hydrophobic, hydrophilic, charged, bulky, flexible, etc.) is responsible for maintaining the framework structure rather than a unique amino acid. As long as this pattern is maintained, the three-dimensional structures are likely to be superimposable.

Orientation of Ligands in the Antibody Binding Site or Receptor Site

Inspection of antibody binding sites, whether derived from crystallographic data or from modeling studies, indicates that most of the hypervariable loop amino acid side chains within the central core of the \(\beta\)-barrel structure point upward toward the mouth of the central cavity and are closely packed together, thus restricting mobility. This is also true of the binding-sites models proposed for our antibodies to haloperidol. Neuroepitopes possess a great deal of flexibility as a result of the alkyl chains. Thus, it is expected that, within certain energy-defined limits, the ligand molecules will be able to conform to the contours of the binding cavity. Structurally rigid antigens would be expected to have a much more limited set of complementary binding sites within the immune repertoire as compared with molecules for which many conformations are possible.

As an example of the validity of using antibodies as models of neurotransmitter receptors, we compared each of the five monoclonal anti-haloperidol antibodies with the dopamine receptor (10). This type of study was accomplished by determining the pharmacologic potency of structurally rigid neuroepitopes and their analogs. There exists a region, equivalent in size to two adjacent aromatic rings, that binds drug moieties with extended \(\pi\)-systems, probably through a ring-stacking interaction. This region accepts one of the aromatic rings of the phenothiazine neuroepitopes such as chlorpromazine, an aromatic ring of the rigid neuroepitopes butaclamol and dexclamol, and one of the aromatic rings of agonists such as apomorphine. The \(\pi\)-binding region was also proposed to be involved in antagonist binding only. The moieties accepted by this region are a bit more flexible and include \(\pi\)-rich substituents such as the keto group of butyrophenones. Replacement of the keto group with a phenyl ring provides the active diphenylbutyl derivatives, thus confirming the theory.

Conclusions

Examination of the molecular epitopes of anti-ligand antibodies and their respective anti-idiotypes antibodies by computer-assisted modeling enables us to further our understanding of antibody interactions as well as those of ligand receptors in general. The comparison of anti-ligand and receptor-binding properties allows us to discern features that may be required for constructing an artificial receptor. Similarly, comparison of anti-idiotypes that mimic ligands provides us with some salient features critical to both, and these data may be very useful in the design of new and more specific drugs. Careful studies of the QBAR, amino acid sequence, and three-dimensional models of such antibodies are all critical steps required to accomplish these goals.

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