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A common ancestry for multiple catalytic antibodies generated against a single transition-state analog

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ABSTRACT **Immunization with a single haptenic transition-state analog generates a few catalytic antibodies among the dozens of antibodies capable of binding the hapten. The diversity of the immune response has raised some fundamental issues, such as How do catalytic and noncatalytic antibodies differ on a structural basis? To address this issue, the variable region primary sequences of 11 antibodies (including 6 catalytic and 5 noncatalytic antibodies) elicited against a single haptenic transition-state analog were deduced from cDNA sequences. Cluster analyses using phylogenetic trees constructed by the neighbor-joining method have revealed that the amino acid sequences of noncatalytic antibodies bear no relationship to one another, while the catalytic antibodies share significant structural identity. Furthermore, no catalytic antibodies possessing amino acid sequences with high homology to those of noncatalytic antibodies were detected. Five catalytic antibodies examined showed 89–95% and 74–84% sequence homologies in the complete light- and heavy-chain variable regions, respectively. Thus, it seems likely that the catalytic antibodies elicited against a single hapten use the canonical set of variable region genes. Interestingly, one catalytic antibody showed only limited sequence similarity to the other catalytic antibodies and was found to exhibit a distinctly different substrate specificity. From the broad range of their binding constants to the hapten, it is unlikely that highly homologous catalytic antibodies are generated as a result of simple high-affinity choices. These results emphasize the utility of rationally designed transition-state analogs for the induction of antibody molecules with catalytic activity.**

Since the first example of antibody catalysis involving relatively simple acyl transfer reactions was reported in 1986 (1, 2), antibodies that catalyze a wide array of chemical reactions ranging from pericyclic reactions to peptide bond cleavage have been generated (3). For inducing catalytic functions into antibody molecules, the steric and electronic complementarity of an antibody to its corresponding hapten has been exploited to generate combining sites, which are either complementary to the rate-determining transition state or contain an appropriately positioned catalytic amino acid chain. The development of catalytic antibodies will not only provide insight into the nature of molecular recognition and catalysis but may also afford tailor-made catalysts for applications in chemistry, biology, and medicine (4, 5).

Although numerous strategies for the generation of catalytic antibodies have been developed, investigations of the expressed immunoglobulin proteins have resulted in only a limited understanding of antigen-combining site structures and catalytic function. When mice are immunized with a hapten conjugated to a carrier protein, a few, and occasionally several, of the dozens of antibodies that bind the hapten are characterized to be catalytic. Thus, the diversity of the immune response can provide a panel of catalytic antibodies that possesses various degrees of catalytic activity and substrate specificity. However, it is unclear how structural differences in the antigen-combining sites of these antibodies correlate with catalytic activity. For example, how do catalytic and noncatalytic (affinity) antibodies differ on a structural basis? Another aspect of antibody diversity concerns the structural refinement of a predetermined binding site through somatic mutation and how this is coupled with the diversity of the immune response to gain higher binding and catalytic activities. Are catalytic antibodies derived from noncatalytic antibodies by mechanisms involved in antibody diversification or are they encoded in the germ-line repertoire? The investigation of the correlation between the antigen-combining site structures and function, within a panel of catalytic antibodies elicited against a single hapten, can potentially provide a more global understanding of the molecular mechanisms by which catalytic antibodies are generated in immune responses.

Recently, we have reported prodrug activation via catalytic antibodies, which catalyze the hydrolysis of a nonbioactive chloramphenicol monoester derivative **1** to generate chloramphenicol **2**, as shown in Fig. 1 (6). Immunization with hapten **3**, designed on the basis of the transition-state stabilization concept, yielded 12 IgG proteins binding to the hapten, 6 of which were found to catalyze the hydrolysis with various degrees of activity. Thus, this antibody system seems to be a suitable model for the study of catalytic antibody structural diversity. To begin to understand the relationship between active site structure and function, heavy- and light-chain variable region (V_H and V_L) primary structures of antibody repertoires (involving catalytic and noncatalytic antibodies) elicited against haptenic phosphonate **3** were deduced from cDNA generation, cloning, and sequencing.† Comparison of the amino acid sequences of catalytic and noncatalytic antibodies has provided insight into the immune responses that create catalytic antibodies.

MATERIALS AND METHODS

Hybridoma Cell Lines and Production. Hybridoma cell lines were generated by fusion of immunized BALB/c splenocytes with the X63/Ag 8653 myeloma cell line according to standard protocols (7). Monoclonal antibodies were amplified in ascitic fluids and purified as described (6).

Competitive ELISAs. Antibodies were mixed with hapten **3** and incubated in microtiter plates coated by hapten–bovine serum albumin conjugate at 37°C for 1 h. The plates were

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Abbreviations: V, variable; C, constant; J, joining; D, diversity; H chain, heavy chain; L chain, light chain; CPP, chloramphenicol phosphonate; DMSO, dimethyl sulfoxide; FR, frame region; CDR, complementarity-determining region; GAT, $(Glu^{60}Ala^{30}Tyr^{10})_n$ random terpolymer.

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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. D29921–D29942).

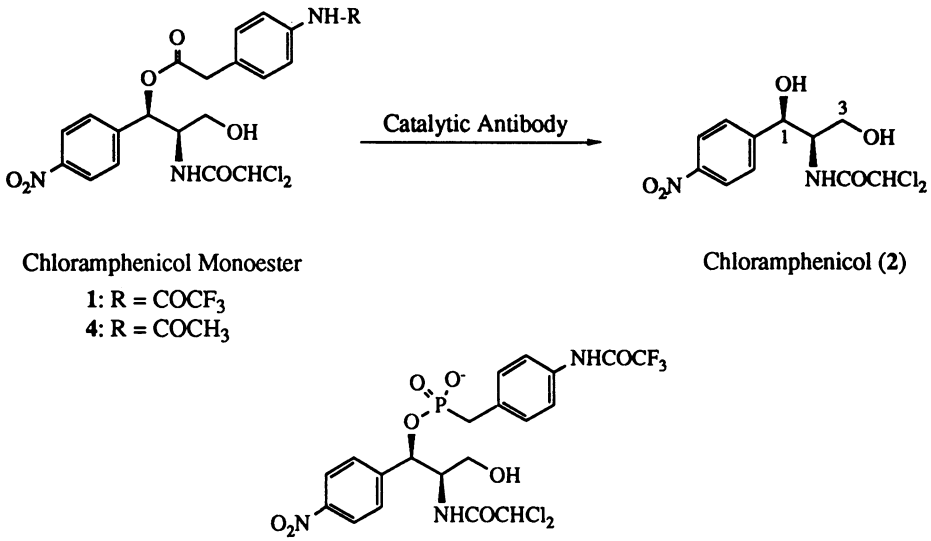

FIG. 1. Simplified model for prodrug activation via the antibody-catalyzed reaction.

washed with PBS, and then ELISA was performed using the Vectastain Elite ABC kit (Vector Laboratories) and the peroxidase substrate kit (Bio-Rad). Apparent affinities were determined as the reciprocal of the hapten concentration required to inhibit 50% maximal binding in a competitive ELISA (8).

Antibody Assays. Relative velocities were determined as follows. The reactions were initiated by adding 10 μl of a 2.5 mM stock solution of substrate 1 in dimethyl sulfoxide (DMSO) to 90 μl of antibody solution in Tris buffer. The reaction mixture consisted of 20 μM highly purified antibody and monoesters in 10% DMSO/50 mM Tris·HCl, pH 8.0, and was incubated at 30°C. Hydrolysis rates were measured by monitoring the production of chloramphenicol 2 via reversed-phase HPLC on a C_{18} column eluted with water (0.1% trifluoroacetic acid)/acetonitrile (50:50) at a flow rate of 1 ml/min, with UV detection at 278 nm. Initial rates were determined from the first 5–10% of the reaction for a given range of substrate concentrations.

cDNA Synthesis and Cloning. Total RNA was isolated from 10^8 hybridoma cells using a guanidium thiocyanate/phenol procedure (9). Poly(A)$^+$ RNA was used in a 50-μl reverse transcription reaction mixture containing 50 mM Tris·HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 1.0 mM dNTP, 2 μg of oligo$(dT)_{15}$ per ml, and 200 units of Molony murine leukemia virus RNase H reverse transcriptase (BRL), which was incubated at 37°C for 1 h. The reverse transcription reaction mixture (10 μl) was then used in 100-μl PCR mixtures containing PCR buffer (Perkin–Elmer), 2.5 mM all dNTPs, 2.5 units of *Taq* polymerase (Perkin–Elmer), and 30 pmol of Fd (V_H-C_H1) (C, constant) or κ sequence-specific 5′ and 3′ oligonucleotide primers (10). The PCR was run for 40 cycles at 91°C for 1 min, 52°C for 2 min, and 72°C for 0.5 min, followed by a final incubation at 72°C for 10 min (Perkin–Elmer/Cetus thermal cycler). Fd and κ amplified fragments were digested with *Spe* I/*Xho* I and *Xba* I/*Sac* I, respectively. The DNA was ligated into the vector arms λHc2 or λLc1 (10) and was packaged with Gigapack II Gold (Stratagene). Phage DNA was prepared using LambdaSorb phage adsorbent (Promega). The H- and L-chain libraries were digested with *Hin*dIII or *Mlu* I, respectively, and after treatment with phosphatase, both DNAs were digested with *Eco*RI, religated, and packaged. Phage plaques were picked, and the plasmids containing H- and L-chain encoding sequences were excised by *in vivo* excision (11). Overnight cultures of each clone were inoculated 1:100 in L broth, and after incubation for 2 h at 37°C Fab synthesis was induced by the addition of 1 mM isopropyl β-D-thiogalactoside overnight at 25°C. Culture supernatants were assayed by an ELISA.

Nucleotide Sequence Analysis. To determine nucleotide sequences, the H- and L-chain cDNA fragments were inserted into pBLUESCRIPT (Stratagene). Nucleotide sequencing was carried out using the T7 sequencing kit (Pharmacia) with the M13 universal and reverse primers, the C_H1 primer (5′-GCCAGTGGATAGACAGA-3′), and the $C_κ$ primer (5′-AACTGCTCACTGGATGG-3′).

RESULTS

In this study, we address several issues pertinent to the functional and structural diversity of a panel of catalytic antibodies induced against a single hapten. Six catalytic (6D9, 4B5, 8D11, 3G6, 9C10, and 7C8) and five noncatalytic (1G6, 7A11, 6A6, 9B7, and 3A7) monoclonal antibodies specific to chloramphenicol phosphonate (CPP; 3) were isolated and characterized in terms of their binding and catalytic activities. The V_H and V_L primary structures of these antibodies (for both catalytic and noncatalytic antibodies) were deduced from cDNA generation, cloning, and DNA sequencing.

Catalytic Activity and Binding Affinity. Catalytic activities of purified monoclonal antibodies were examined for hydrolysis of chloramphenicol monoester 1. In the assay, six antibodies were found to catalyze the hydrolysis at a rate above the uncatalyzed background reaction. The most active antibody, 6D9, was characterized in detail and was shown to have a k_{cat} of 0.133 min^{-1} and a K_m of 64 μM (6). The relative velocities of hydrolytic activity of the remaining five catalytic antibodies were compared to that of antibody 6D9 as shown in Table 1 and revealed the order of activity (6D9 > 8D11 > 9C10 > 3G6 > 7C8 > 4B5). In addition, the binding affinities of 11 antibodies to the phosphonate transition state analog 3 were determined by competitive ELISA (8). The aK (apparent binding constant) values determined by this method probably underestimate the true binding constant but are useful in allowing the variation in affinity to be understood (12). The aK determination illustrates the diversity of the affinity molecules, with aK values in the range 10^3–10^8 M^{-1} (Table 1).

Table 1. aK values (means ± SD) determined for the monoclonal antibodies by hapten inhibition ELISA and their relative velocities in hydrolysis of chloramphenicol monoester 1

Clone	I_{50} (SD), M	aK (SD), M^{-1}	V_{rel}, %
1G6	$1.4 (\pm 0.1) \times 10^{-8}$	$7.2 (\pm 0.5) \times 10^{7}$	ND
4B5	$8.5 (\pm 2.5) \times 10^{-8}$	$1.2 (\pm 0.4) \times 10^{7}$	25
7A11	$1.1 (\pm 0.1) \times 10^{-7}$	$9.2 (\pm 0.9) \times 10^{6}$	ND
3A7	$6.8 (\pm 1.2) \times 10^{-7}$	$1.5 (\pm 0.3) \times 10^{6}$	ND
8D11	$3.0 (\pm 0.6) \times 10^{-6}$	$3.5 (\pm 0.7) \times 10^{5}$	65
9C10	$5.0 (\pm 1.0) \times 10^{-6}$	$2.1 (\pm 0.4) \times 10^{5}$	58
6D9	$5.9 (\pm 0.5) \times 10^{-6}$	$1.7 (\pm 0.2) \times 10^{5}$	100
9B7	$8.2 (\pm 1.1) \times 10^{-5}$	$1.3 (\pm 0.2) \times 10^{5}$	ND
3G6	$2.2 (\pm 1.2) \times 10^{-5}$	$6.5 (\pm 3.5) \times 10^{4}$	40
6A6	$2.7 (\pm 0.7) \times 10^{-5}$	$4.0 (\pm 1.1) \times 10^{4}$	ND
7C8	$2.5 (\pm 0.3) \times 10^{-4}$	$4.1 (\pm 0.5) \times 10^{3}$	38

I_{50}, mean hapten concentration required for 50% inhibition of maximal binding in a competitive ELISA; aK = $1/I_{50}$. Relative velocities (V_{rel}) were compared to monoclonal antibody 6D9 at concentrations of 20 μM antibody and 250 μM substrate. ND, not determined.

In practice, the catalytic antibodies are identified among a panel of antibodies based on high binding constants for transition-state analogs (13). However, the results from comparisons of the affinities and the catalytic activities within the panel of antibodies indicate that antibodies with high binding affinity are not always highly catalytic. In the present study, increased catalytic activity of the six antibodies does not correlate with increased binding affinity to the transition-state analog. For example, the most active catalytic antibody, 6D9, has only moderate affinity for the transition-state analog, while the highest-affinity antibody, 4B5, has the lowest activity of the six catalytic antibodies. Antibody 1G6 shows the highest binding activity of the 11 antibodies but has no catalytic activity. It is likely that antibody–hapten contacts in each antibody involve specific interactions that are not present in the reaction transition state, such as interactions with dichloroacetylamide or with the linker chain that was presented with the hapten during immunization.

V-Region Sequence Determination. The H and L chains of monoclonal anti-CPP antibodies (6D9, 4B5, 8D11, 3G6, 9C10, 7C8, 1G6, 7A11, 6A6, 9B7, and 3A7) were subjected to amino acid sequence analysis in an attempt to correlate V-region usage with binding affinity and catalytic characteristics. V_H and V_L cDNAs were generated from hybridoma mRNA by reverse transcription PCR and then cloned and sequenced. The deduced amino acid sequences were aligned and classified into V_H and V_L subgroups on the basis of homologies between frame regions (FRs) 1 and 3, according to Kabat *et al.* (14).

Fig. 2 shows the complete V_H deduced primary amino acid sequences of monoclonal anti-CPP antibodies aligned to antibody 6D9. With the exception of 7C8, the V_H regions of the group of antibodies with catalytic activity, 6D9, 9C10, 8D11, 3G6, and 4B5, expressed high amino acid sequence homology (89–95%) in FR1 and -3. Each clone appeared to be derived from the V_H IIID subgroup. The amino acid sequence of 7C8 was distinctly different from the others (43% homology to that of 6D9) and was classified into the V_H IIA subgroup. In the group of antibodies possessing only affinity, without catalytic activity, antibodies 3A7 and 9B7 were >85% homologous to the V_H IIC subgroup common amino acid sequence. Antibodies 6A6 and 7A11 were classified into the V_H VA subgroup with >76% homology. Antibody 1G6 appeared to be derived from the V_H IIID subgroup, which is the same subgroup as the antibodies possessing catalytic activity. In contrast to the catalytic antibodies, the V_H amino acid sequences in the noncatalytic antibodies show low homologies to one another (43–65%). H-chain complementarity-determining region 3 (CDR3), in which positions 95–102 are encoded by diversity and joining (D and J_H) minigenes, exhibited extensive heterogeneity. The CDR3 primary structures for catalytic antibodies 6D9, 8D11, and 4B5 were of the same length and seem to be relatively longer than those for antibodies without catalytic activity.

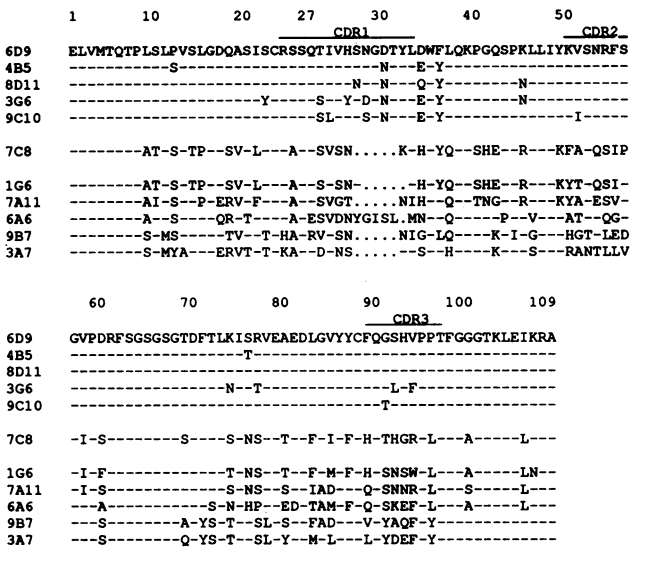

FIG. 2. Deduced amino acid sequence comparison of V_H cDNA generated from 11 anti-CPP monoclonal antibodies. Dashes denote identity to antibody 6D9 and dots, used to align sequences, represent no amino acid residue at a position. CDR designations and amino acid residue numbering are as described by Kabat *et al.* (14).

V_L deduced amino acid sequences are shown in Fig. 3. The V_L primary structures of the anti-CPP monoclonal antibodies (except antibody 6A6) were divided into two groups, the V_L II and V_L V subgroups (14). The monoclonal catalytic antibodies 6D9, 9C10, 8D11, 3G6, and 4B5 expressed highly homologous amino acid sequences relative to each other in the V_L complete regions, including CDR3 and FR4, and were assigned to the V_L II subgroup, with >92% homology to the consensus sequence. The nucleic acid sequence comparison with V_κ germ-line genes revealed these antibodies to be highly homologous to the K1A5 germ-line gene, which accounts for the expression of most anti-GAT [$(Glu^{60}Ala^{30}Tyr^{10})_n$ random terpolymer] anti-

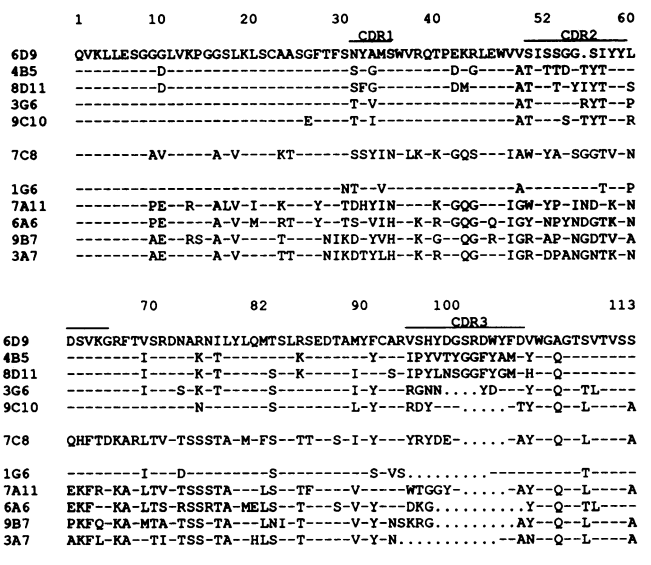

FIG. 3. Comparison of the deduced amino acid sequences of V_L for 11 anti-CPP monoclonal antibodies. See legend to Fig. 2 for symbol designation.

bodies (15). The numbers of different nucleotides for each of the V_κ genes, as compared with the K1A5 gene, are as follows: 6D9, 4B5, 8D11, and 9C10, 5 nucleotides; 3G6, 10 nucleotides. Most nucleotide sequence differences were observed in CDR1, as shown in Fig. 4. These antibodies either rearranged similar V_κ gene(s) or the same V_κ gene as K1A5, and somatic mutation occurred after rearrangement. However, considering the heterogeneity of V_H CDR3 observed in these antibodies, it is unlikely that any of them are clonally related. If they are clonally related to each other, the antibodies must use the same V_κ, J_κ, V_H, D, and J_H gene segments, and the recombination events that brought them together during B-cell development must have occurred in the same position. Unfortunately, it is therefore not possible to assess the contribution of somatic mutation relative to the affinity maturation by a comparison of these amino acid sequences. Although antibody 7C8 was found to be catalytic, the V_L amino acid sequence was rather different from those of the catalytic antibodies.

The noncatalytic antibodies 1G6, 3A6, 7A11, and 9B7 were classified into the V_L V subgroup (14), but they exhibited low sequence homologies to one another (50–65%). Antibody 6A6 showed 78% homology to the consensus sequence of the V_L III subgroup (14). In contrast to the catalytic antibodies, antibodies possessing only binding affinity appeared to be derived from various V_L germ-line genes.

DISCUSSION

In general, simple and complex antigens induce an immune response to provide a variety of antibodies resulting from several genetic processes (16). The most definitive method for studying antibody diversity is to determine the variability of amino acid sequences. Most often, however, antibodies that share a common idiotype are selected for sequencing (17–20). Thus, the antibodies are selected not only for binding activity to the antigen but also for the presence of a related structure in the region of the antigen-combining site (i.e., a related idiotype). Studies of this kind have demonstrated that antibodies within an idiotype family are highly homogeneous in the amino acid sequences of their V regions. At the beginning of our study, we expected that the V-region primary structures of anti-CPP antibodies (both catalytic and noncatalytic antibodies) would be heterogeneous, since these antibodies were generated by an unrestricted immune response that revealed the broad range of their binding constants, rather than by idiotypic selection.

On the basis of a phylogenetic tree reconstructed by the neighbor-joining method (21), the cluster analyses of anti-CPP antibodies revealed the noncatalytic antibodies to express unrelated amino acid sequences, as shown in Fig. 5. The noncatalytic antibodies 1G6, 3A7, 6A6, 7A11, and 9B7 have completely different sequences and show approximately 50% and 45% homology in the V_L and V_H regions, respectively. Although the hapten is relatively small and possesses a limited number of epitopes, the heterogeneity of the amino acid sequences in these antibodies is within an acceptable range, given the diversity of the immune response. In accordance with the diversity of their primary structures, the noncatalytic antibodies may have heterogeneous molecular interactions of the combining sites with the epitopes.

On the other hand, the catalytic antibodies 6D9, 9C10, 8D11, 3G6, and 4G5 shared significant structural identity to one another and had 89–95% and 74–84% sequence homologies in the complete V_L and V_H regions, respectively (Fig. 5). Some published data regarding nucleotide sequence comparisons of genes from antibodies elicited against several defined epitopes have revealed that immune responses to small haptens, phosphorylcholine (22) and phenyloxazolone (23), use a single set or only a few sets of V-region genes. The simplest explanation for this type of response is that the available B-cell repertoires offer few high-affinity choices for some epitopes. However, it is unlikely that the catalytic antibodies described here are generated as a result of high-affinity choices, as they exhibited a broad range in their binding constants. Furthermore, the similarity of the primary structures suggests that these antibodies could belong to a shared idiotype family, in terms of combining site structures and binding properties. The various degrees of catalytic activity observed in the hydrolysis of chloramphenicol monoester 1 seem to be due to limited differences in the amino acids in both the V_L and V_H chains. It was expected that a comparison of catalytic antibodies with structurally related

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                                              FR1                              CDR1
            4                 10                             20
germline  ATGACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCCATCTCTTGC AGATCTAGT
6D9       --------G--------------------------------------------------- ---------
4B5       --------G---------------T----------------------------------- ---------
8D11      --------G--------------------------------------------------- ---------
3G6       --------G----------------------------------------------A---T ---------
9C10      --------G--------------------------------------------------- ---------

                                                              FR2
          27  a  b  c  d  e        30                             40
germline  CAGAGCATTGTACATAGTAATGGAAACACCTATTTAGAA TGGTACCTGCAGAAACCAGGCCAGTCTCCA
6D9       ----C-------------------G----G--------T ----T-------------------------
4B5       ----C---------------------------------- ------------------------------
8D11      ----C-----------A-------------------C-- ------------------------------
3G6       -----------GT-----G-------------------- -----TT-----------------------
9C10      ------C------------G------------------- -----------------------------C

                                   CDR2                        FR3
                         50                          60
germline  AAGCTCCTGATCTAC AAAGTTTCCAACCGATTTTCT GGGGTCCCAGACAGGTTCAGTGGCAGTGGATCA
6D9       --------------- --------------------- ---------------------------------
4B5       --------------- --------------------- ---------------------------------
8D11      --C------------ --------------------- ---------------------------------
3G6       --T------------ --------------------- ---------------------------------
9C10      --------------- ---A----------------- --------------------------------T

                  70                            80                       88
germline  GGGACAGATTTCACACTCAAGATCAGCAGAGTGGAGGCTGAGGATCTGGGAGTTTATTACTGC
6D9       ---------------------------------------------------------------
4B5       -----G-------------------C---G---------------------------------
8D11      -----------------------------------------A---------------------
3G6       --------------------T-------C----------------------------------
9C10      ---------------------------------------------------------------
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FIG. 4. Comparison of V_L nucleotide sequences of five catalytic monoclonal antibodies with the K1A5 germ-line gene, accounting for the expression of anti-GAT antibody (15).

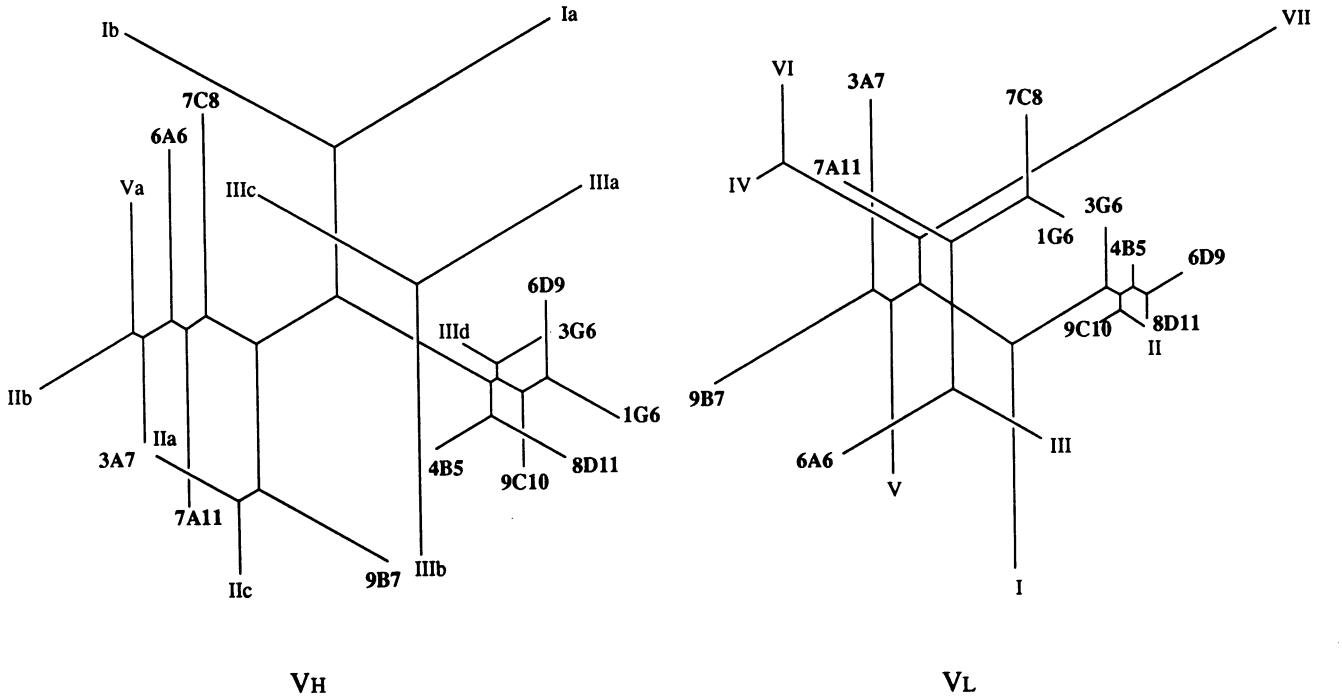

FIG. 5. Cluster analyses of V_H and V_L chains of anti-CPP antibodies and Kabat's subgroups (refer to consensus sequence) on the basis of an unrooted phylogenetic tree constructed by the neighbor-joining method (21).

noncatalytic antibodies is central to an understanding of the catalytic mechanisms. However, unfortunately, we did not obtain a noncatalytic antibody possessing an amino acid sequence with high homology to the catalytic antibodies.

Antibody 7C8 was found to be exceptionally catalytic, but its structure was different from the other catalytic antibodies, with 55% and 44% amino acid sequence homologies in V_L and V_H regions, respectively, as compared to the prototype 6D9. This analysis suggests that 7C8 binds to the substrate or hapten in a mode of molecular interaction different from those of the other catalytic antibodies. In fact, examination of the antibody-catalyzed reactions using various substrates demonstrated the substrate specificity of 7C8 to be different from that of the others. The catalytic antibodies hydrolyze *N*-trifluoroacetyl 4-aminophenylacetyl derivative **1** corresponding to the hapten, but *N*-acetyl derivative **4** with significant decreased rates, while antibody 7C8 was able to catalyze the hydrolyses of both trifluoroacetyl **1** and acetyl derivatives **4**. Thus, the *N*-trifluoroacetyl group is a crucial epitope for all of the catalytic antibodies, with the exception of 7C8. The detailed results of substrate specificity, including the kinetic parameters, will be reported separately.

The diversity of the immune response permits antibodies to be generated against virtually any substance and has the potential to provide catalysts for many chemical transformations. The fact that such catalysts could be tailored by immunization with rationally designed transition-state analogs is the main attraction of the catalytic antibody research field. Therefore, a central question in catalytic antibody research concerns the extent to which the rationally designed hapten dictates paratopes for catalytic function in the antigen-combining site. At least in the case of the anti-CPP antibodies described here, the fact that the group of catalytic antibodies has highly homogeneous V-region sequences, despite the antibody diversity, suggests a limited number of modes of molecular interaction (antigen binding specificity) of paratopes with epitopes for inducing catalytic activity in the antigen-combining sites. Thus, these results emphasize the utility of rationally designed transition-state analogs as antigens for the creation of catalytic antibodies.

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