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V_H-RELATED IDIOTOPES DETECTED BY SITE-DIRECTED MUTAGENESIS

A Study Induced by the Failure to Find CD4 Anti-Idiotypic Antibodies Mimicking the Cellular Receptor of HIV[1]

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The function of the CD4 cell surface protein as coreceptor on T helper lymphocytes and as receptor for HIV makes this glycoprotein a prime target for an immune intervention with mAb. A detailed understanding of the structural determinants on the therapeutic CD4 mAb that are involved in Ag binding or are recognized by anti-idiotypic mAb (anti-Id) may be important for designing antibodies with optimal therapeutic efficacy. Seven anti-Id raised against the CD4 mAb M-T310 were selected from a large panel with the intention to obtain CD4 mimicking structures with specificity for HIV gp120. The selected anti-Id did not react with other CD4-specific mAb cross-blocking M-T310. Among these, mAb M-T404, although having the same L chain as M-T310 and a V_H region sequence differing only at 14 amino acid positions, was not recognized by the anti-Id. M-T310 H chain complexed with the J558L L chain reacted with all anti-Id, thus demonstrating that the recognized idiotopes are located within the V_H region. To identify the idiotopes of M-T310 seen by the anti-Id, variants of M-T404 containing one or more of the M-T310-derived substitutions were generated by oligonucleotide-directed mutagenesis. The reactivity pattern of the mutant proteins with the anti-Id demonstrated that the idiotopes reside within the complementarity determining region (CDR) 2 and CDR3 loops of the V_H region. A major idiotope was defined by a single amino acid in CDR2 that was recognized by three anti-Id, whereas the four other anti-Id reacted with determinants of CDR3. Although the performed amino acid substitutions did influence the Id recognition, Ag binding was not significantly affected, suggesting that none of the anti-Id can be considered as a mimicry of the CD4 Ag.

Different CD4-specific mAb, including M-T310, are used either as original mouse mAb or as chimeric mAb for the treatment of various autoimmune diseases. It is not yet clear what characteristics will distinguish the most efficient CD4 mAb. Furthermore, it is not known whether and which antibodies induced in the treated patients will neutralize the injected CD4 antibody. The V- or Ag-binding region of an antibody itself can act as Ag (Id). The determinants unique to an antibody or a group of antibodies referred to as idiotopes represent a three-dimensional assemblage of all residues, usually confined to the CDR.[3] The experimental induction of Id antibody is well established (1, 2) and it has been directly associated with the Fv fragment (3). Theoretically, anti-Id raised against CD4 mAb, competing with HIV/gp120 for the binding site on the CD4 molecule, should mimic the CD4 receptor site (4) and, therefore, might be used as anti-idiotypic vaccine. mAb M-T310, although blocking the HIV-1/rgp120-CD4 interaction and inhibiting infection of susceptible cells by HIV-1,[4] as well as other similar CD4 mAb (5, 6) did not induce anti-Id suited for anti-idiotype vaccine. This apparent failure warranted a more detailed analysis of the structural determinants, recognized by these anti-Id.

The structural basis of idiotypic determinants has been analyzed for a number of mAb. Id located within V_H region have been described for mAb directed against arsonate (7) and 3-fucosyllactosamine (8). The CDR3 of V_H that includes the D segment, contributes to the Id determinants in a number of antibodies including those to $\alpha(1-3)\alpha(1-6)$dextrans (9, 10), $\beta(1-6)$galactan (11), phosphorylcholine (12), nitroiodophenyl (13, 14), and *p*-azophenylarsonate (15, 16), whereas in antibodies directed to $\alpha(1-6)$dextran, the Id has been associated with the V_H CDR2 (17). In addition, both V and C regions of mAb to phosphorylcholine were required to form a certain Id determinant (18).

The present study shows that the V_H region of the CD4 mAb M-T310 determines the Id reaction patterns of seven anti-Id. M-T404, another CD4-specific mAb possessing an identical V_L region and a V_H region differing only at 14 amino acid positions, is not recognized by these anti-Id. Several variants of M-T404 containing one or more M-T310-derived substitutions were generated by mutagenesis and expressed together with the M-T310 L chain. The binding pattern indicates that a major Id is located in CDR2, recognized by three anti-Id, whereas the remaining four anti-Id predominantly react with determinants located in CDR3. Furthermore, none of the amino

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[3] Abbreviations used in this paper: CDR, complementarity determining region; anti-Id, anti-idiotypic mAb.

[4] **W. Weissenhorn, C. Reiter, C. Federle, Y-H. Chen, G. Riethmüller, E. H. Weiss, and E. P. Rieber. 1992.** Structural diversity of monoclonal CD4 antibodies and their capacity of blocking HIV gp120/CD4–interaction. *Submitted for publication.*

acid substitutions abolishes Ag binding and the anti-Id reaction pattern is not influenced by the C region.

MATERIALS AND METHODS

Production of antibodies. mAb M-T310 and M-T404 were raised against T lymphocytes as described (19). To induce anti-Id against mAb M-T310, purified mAb was conjugated with keyhole limpet hemocyanin and mice were immunized three times with 100 μg over a period of 2 mo. Fusions were made and Id specific for the paratopic site of mAb M-T310 were selected.

Immunofluorescence. PBL were incubated with CD4 mAb and detection of stained cells by a FITC-conjugated antiserum was analyzed using a FACScan (EPICS IV, Coulter Electronics, Hialah, FL). Histograms represent the log of fluorescence intensity versus cell number.

Analysis of blocking capacity of anti-Id. Aliquots of 0.1 μg/ml biotinylated mAb M-T310, preincubated with limiting dilutions of anti-Id (100 μg to 0.03 μg/ml) for 20 min at room temperature, were incubated with PBL at 4°C for an additional 20 min. Binding of M-T310bio and M-T404bio was detected by FACScan analysis after FITC-conjugation with FITC-conjugated avidin.

ELISA. To detect binding of recombinant and mutant Ig to the anti-Id, wells of a 96-well polystyrene plate were coated with 10 μg/ml rabbit anti-human IgG (Dakopatts, Glostrup, Denmark) and placed at 4°C overnight. The plates were washed three times with PBS 0.05% Tween 20, and blocked with 1% BSA in PBS for 1 h at room temperature. The wells were rinsed three times with Tween 20-saline solution and incubated for 2 to 3 h at room temperature with concentrated cell culture supernatants, containing 1 μg/ml of recombinant chimeric IgG1, followed by three washes with Tween 20-saline solution. In the next step, wells were incubated with anti-Id for an additional 2 to 3 h at room temperature, washed three times with Tween 20-saline solution, and binding of anti-Id to the recombinant IgG was detected by incubation with Fc-specific peroxidase coupled rabbit anti-mouse IgG serum.

Cloning of the functional rearranged V_H and V_L genes coding for mAb M-T310 and M-T404. Phage libraries with vector EMBL3 (Promega, Madison, WI) were generated according to Maniatis et al. (20). Functional rearranged V region genes on *Eco*RI fragments (2.6 kb) for both H chains and on a *Hin*dIII fragment (4.3 kb) for the L chain were obtained by screening the libraries with the probes pJ11 (V_H-specific (21)) and $J_{\kappa 1\text{-}5}$ (V_L-specific (22)). The functional rearrangement of the obtained clones was confirmed by sequence analysis. dsDNA was directly sequenced using oligonucleotides matching the J_H and J_κ region by the dideoxy chain termination method using the T7 polymerase sequencing kit supplied by Pharmacia/LKB (Piscataway, NJ).

Site-directed mutagenesis. Mutagenesis of the M-T404 V_H region was performed by polymerase chain reaction, using a modified procedure as described by Nelson et al. (23). Briefly, DNA containing the M-T404 V_H region was amplified in two cycles: In the first cycle, two fragments were produced by either using a 5′-specific oligo (A: located in the 5′ untranslated region, 5′GGTCGACGAATTCCTGAA CACACACTGAC 3′ with an *Eco*RI restriction site) and the oligo containing the mutation as the 3′ primer (B), or an oligonucleotide with the mutation as the 5′ primer (C) and a J_H/intron specific 3′ oligonucleotide with a *Not*I restriction site (D, 5′ CCAGCGGCCGCG GACTCACCTGCCAGCGAC 3′). The two fragments containing an overlap of 15 to 20 nucleotides were purified from agarose gel, fused, and further amplified by the A and D oligonucleotides. The obtained V_H fragments were digested with *Eco*RI and *Not*I, and subcloned into the expression vector. The introduced mutations were verified by DNA sequence analysis.

Oligonucleotides B and C for site-directed mutagenesis in 5′- 3′ orientation:

h1–1: B: ACTTACAACATAATCAGTGAATGTG; C: ACTGAC-TATGTTGTAAGTTGGATG;

h2–1: B: CATTGTAGTACGAACTACCACTTC; C: GCTTACTACAAT-GAGAAAT;

h3–1: B: GTGAGCAAACCCGAGGGAACCCTGTCCTGACCTTGCAC; C: GGTTCCCTCGGGTTTGCTCACTGG;

h3–2: B: CCCGAGGGAACCATCTCCTCGCCTTGCACAG; C: as h3–1 C-oligo;

h9–1: B: CTGCAGTCAGTGTGGCCTTGCCC; C: GGCCACACT-GACTGCAGACAAATC.

Expression vectors. The 7.0 kb-*Eco*RI/*Bam*HI fragment containing the exons coding for the human IgG1 C region, has been subcloned into the vector pSVgpt (24). After introduction of a *Not*I linker 5′ of the *Eco*RI site, the Ig enhancer on a 1.9-kb *Not*I/*Eco*RI fragment has been introduced. The *Eco*RI site between enhancer and C region was destroyed by trimming with T4 DNA polymerase and blunt end ligation. Then the 2.2-kb *Eco*RI/*Not*I fragment of the vector pcDNAI (British/Bio-technology, Oxford, UK) containing the sup F region, the CMV promoter, and its polylinker has been subcloned by replacing the *Pvu*I/*Not*I fragment of the pSVgpt vector. Thus this vector (CMVh-1) allowed subcloning of polymerase chain reaction fragments generated by the primers A and D, as described above, into the *Eco*RI and *Not*I site.

For the L chain, the 4.3-kb *Hin*dIII fragment, containing the functional rearranged V_L region, was blunt-ended as the *Sal*I cloning site of pSV2-neo vector and ligated. This vector contains the human C region gene on a 4.6-kb *Sal*I/*Bam*H I fragment.

Transfection assays. Transfection of actively growing P3.X63.-Ag8.653 and J558L mouse myeloma cells was carried out by electroporation using a Bio-Rad Laboratories (Richmond, CA) gene pulser following the recommendations of the supplier.

RESULTS

Inhibition of CD4 Ag binding by anti-Id. As part of the characterization of the interactions between anti-Id and mAb M-T310 and M-T404, the ability of anti-Id to inhibit binding of mAb to the Ag CD4 on PBL was determined by FACScan (Coulter) analysis. All anti-Id generated against mAb M-T310 blocked CD4-binding activity of mAb M-T310 (Fig. 1*A*) and they did not react with mAb M-T404 (Fig. 1*B*).

CD4 Ag binding of the recombinant Ig. The deduced amino acid sequences of the M-T310 (h310–1) and M-T404 (h404–1) V_H regions and their mutated variants are shown in Figure 2. Although generated from different mice, both mAb have identical L chains. Transfection of the constructs h310–1 and h404–1 containing the chimeric H chain genes together with the chimeric L chain gene resulted in the expression of recombinant Ig with CD4 specificity as shown in Figure 3. Transfection of the H chain construct h310–1 into the cell line J558L (expressing a lambda L chain, but no H chain) also led to the expression of a chimeric Ig Jλ310–1 with CD4 specificity. The mutated M-T404 V_H chains also showed a normal distribution of CD4 positive T lymphocytes by staining PBL (Fig. 3). As none of the introduced mutations abolished CD4 Ag binding, it seems that the exchanged amino acids are not decisive for Ag binding. Furthermore, Ag specificity is determined by the H chain, as the CD4 Ag binding specificity can be preserved by the combination of the M-T310 H chain and the J558 λ L chain.

Anti-Id binding pattern of the mutated Ig. mAb M-T404 did not react neither as murine mAb nor as chimeric mAb (h404–1) with the analyzed anti-Id, which recognized the mAb M-T310 used as immunogen as well as its chimeric counterpart h310–1 (Fig. 4). A virtually identical reactivity was observed with mAb Jλ310–1 (M-T310 V_H chain and J558 lambda L chain), thus demonstrating that all idiotopes recognized are determined by the V_H region with no obvious interference by the L chain. The exchange of amino acid 34 Ile-Val (h1–1), the only difference within CDR1 between M-T310 and M-T404, did not lead to a measurable binding of any anti-Id. In contrast, a major idiotope appears to be determined by amino acid 58, the only difference within CDR2 of the two antibodies. The exchange of Asn to Ala (h2–1) reconstituted binding reactivity with anti-Id 21–48, 20–02, and 20–26. Also the recombinant Ig h9–1, a hybrid between M-T310 and M-T404 H chains retaining CDR3 of M-T404, showed an identical reactivity pattern as h2–1. The results indicate that CDR1 appears not to be involved in the binding reactivity of the anti-Id. As M-T404 differs from M-T310 by four closely linked amino acids in CDR3, site-directed mutations within this region were of consid-

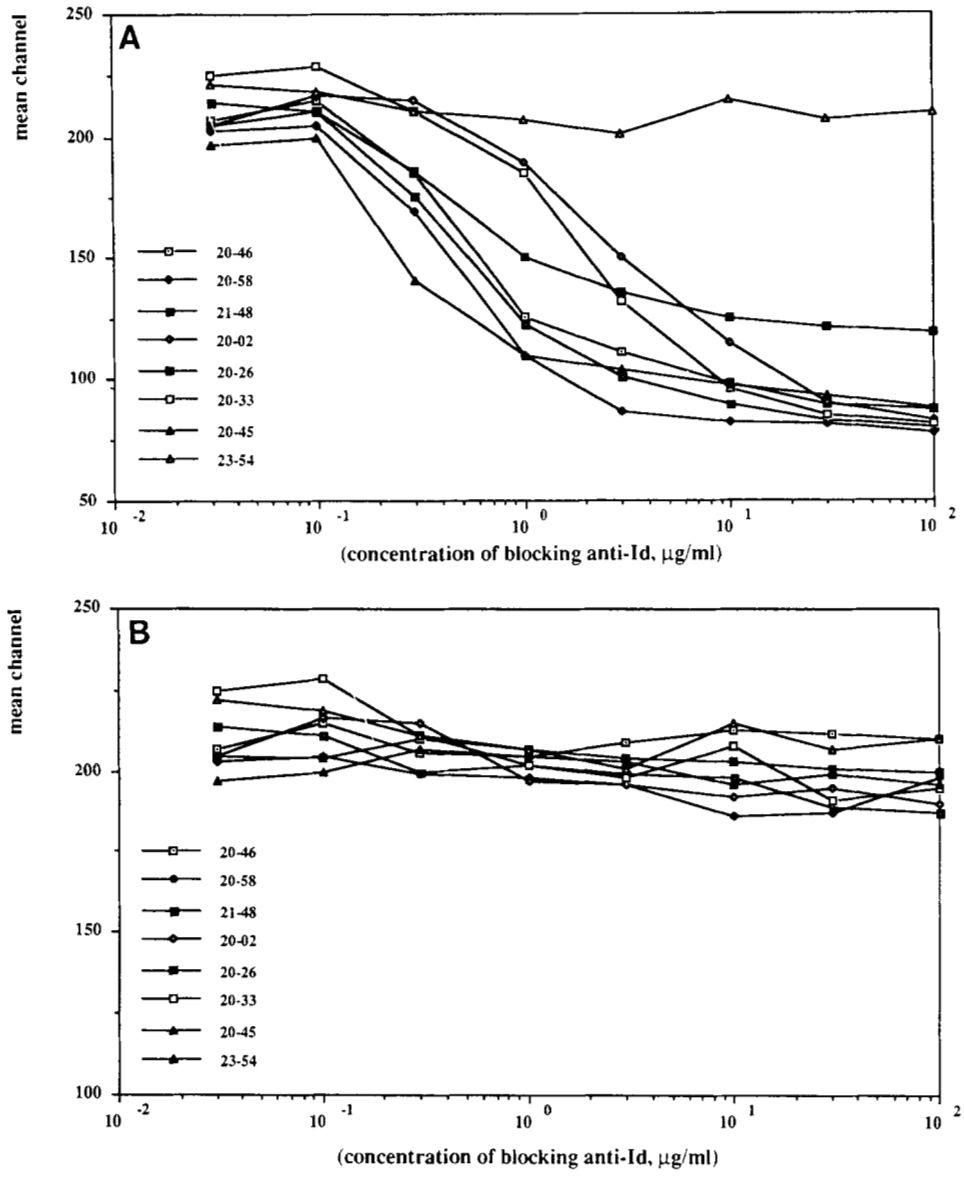

Figure 1. Capacity of anti-Id to block binding of M-T310bio (*A*) and M-T404bio (*B*) to Ag CD4 of PBL. The blocking capacity is shown as the reduction of mean channel as determined by FACScan (Coulter) analysis. The anti-Id 23–54 was raised against mAb M-T151 and serves as negative control.

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                                20                  40                  60
h310-1: QVHLQQSGPELVKPGPSVKMSCKASGYTFT DYVVS WMQQRTGQVLEWIG EIYPGSGSAYY
h1-1  : ------------------R---F------- -----  -VK-----G----- --------N--
h2-1  : ------------------R---F------- ---I-  -VK-----G----- ----------
h3-1  : ------------------R---F------- ---I-  -VK-----G----- --------N--
h3-2  : ------------------R---F------- ---I-  -VK-----G----- --------N--
h9-1  : ------------------------------ -----  ------------- ----------
h404-1: ------------------R---F------- ---I-  -VK-----G----- --------N--
                                CDR1                          CDR2

                                80                  100
h310-1: NEKFKG KAILTADKSSSTAYMEFSSLTSEDSAVFFCAR RGDGSLGFAH WGQGTLVTVAA
h1-1  : ------ --T------------Q-----------Y---- S-Q..----- ----------
h2-1  : ------ --T------------Q-----------Y---- S-Q..----- ----------
h3-1  : ------ --T------------Q-----------Y---- S-Q-------- ----------
h3-2  : ------ --T------------Q-----------Y---- ---------- ----------
h9-1  : ------ --T------------Q-----------Y---- S-Q..----- ----------
h404-1: ------ --T------------Q-----------Y---- S-Q..----- ----------
                                                CDR3
```

Figure 2. Deduced amino acid sequences of V_H regions of native and mutant mAb analyzed for anti-Id binding reactivity. The amino acid residues are numbered consecutively. The location of CDR are indicated in frames. The symbol (–) corresponds to the same amino acid as in sequence h310–1 and (.) marks a deletion.

erable interest. In mAb h3–1, we inserted amino acid Gly 102 and amino acid Ser 103 present in mAb M-T310, but no reactivity with any of the anti-Id was observed. The complete exchange of CDR3 between M-T310 and M-T404 (h3–2) led to the binding of anti-Id 20–46, 20–58, 20–33, and 20–45. Moreover, a distinct, albeit weak, binding reactivity was observed with anti-Id mAb 21–48, 20–02, and 20–26 recognizing the idiotope in CDR2. Thus, determinants within CDR3 may directly be involved as contact sites in the interaction with these anti-Id. Differences within the framework regions of M-T310 and M-T404 apparently have no effect on the binding of anti-Id.

DISCUSSION

The structural basis for the anti-Id binding ability of two closely related CD4 mAb M-T310 and M-T404 was investigated. Variants of M-T404 H chain that contain single or combined M-T310 V_H substitutions were generated by oligonucleotide-directed mutagenesis followed by expression of mutant proteins containing the M-T310/M-T404 (identical L chain) L chain. The mutated M-T404 V_H chains showed a normal distribution of $CD4^+$ T lymphocytes by staining PBL. As none of the introduced mutations abolished CD4 Ag binding, it seems that the exchanged amino acids are not decisive for Ag binding. Furthermore, the combination of the M-T310-specific H chain and the λ L chain of the J558L cell line resulted in

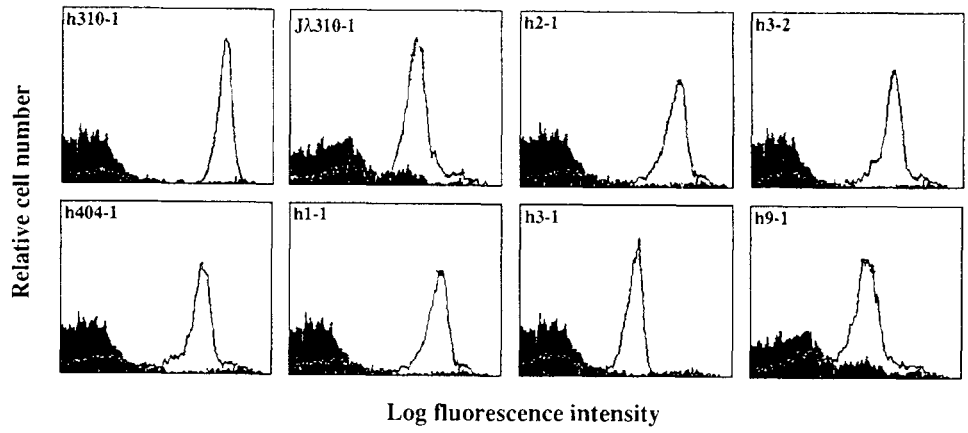

Figure 3. FACS analysis with chimeric Ig. PBL were incubated with the indicated Ig and stained with FITC-anti-human IgG serum. The distribution of negative and CD4 positive cells is shown as two peaks.

	23-54	20-46	20-58	21-48	20-02	20-26	20-33	20-45
h310-1		■	■	■	■	■	■	■
Jλ310-1		■	■	■	■	■	■	■
h404-1								
h1-1								
h2-1				■	■	■		
h3-1								
h3-2		■	■	▒	▒	▒	■	■
h9-1				■	■	■		

Figure 4. Comparative representation of anti-Id reactivity with the different V_H region constructs shown in Figure 1. The binding reactivity was measured by ELISA as described. The determined OD was valued as follows: OD < 0.3, no binding activity (*white boxes*: measured cross-reactivity of POX antiserum with any human IgG antibody); OD 0.4 to 0.6, weak binding activity (*gray boxes*); OD > 0.6, binding activity marked with *black boxes*; anti-Id 23–54 as negative control was raised against mAb M-T151 which recognizes a different epitope on the CD4 molecule.

an Ig with CD4 Ag-binding specificity. This result is consistent with earlier data demonstrating that V_H chains can be expressed together with any L chain (25) without losing their Ag specificity. Moreover, it has been shown that V_H domains alone are able to bind Ag (26). In all these cases, the V_H region predominantly contributed to Ag-binding specificity.

The combination of the M-T310 H chain and the λ L chain J558 clearly shows that the reactivity of all seven anti-Id is associated with the H chain. M-T310-derived substitutions in the M-T404 H chain by mutagenesis analysis show that the anti-Id react with idiotopes determined by CDR2 and CDR3. The conservative exchange in CDR1 (Ile-Val; h1-1) had no effect on anti-Id binding, whereas the single substitution in CDR2 (Asn -Ala; h2-1) restores binding of three anti-Id.

The observations that single substitutions in CDR2 and CDR3 can profoundly affect idiotypic specificity has been observed in several systems (13, 16, 17). As has also been described in these studies, the exchange of amino acids involved in recognition by anti-Id did not affect Ag binding. Thus, major idiotopes appear not to be involved in Ag binding or play no important role. As CDR3 of M-T310 and M-T404 H chains do not only differ in sequence but also in length, the substitution of Ser to Arg (position 99) and Gln to Asp (position 101) in the construct h3-1 does not restore anti-Id binding. Only the additional lengthening of CDR3 by insertion of Gly and Ser (position 102, 103) in the h3-2 construct reconstituted the idiotope recognized by four anti-Id. The involvement of all four exchanged residues does not necessarily mean direct binding between these residues and the anti-Id, but could, alternatively, mean that some of these residues indirectly affect binding by changing the conformation of the CDR3 loop. The differences in the length most certainly lead to different three-dimensional structures in the CDR3 loop regions of mAb M-T310 and M-T404.

The network theory of idiotope-anti-idiotope interactions (4, 27, 28) includes anti-Id that are the internal image of Ag. The binding of such anti-Id to their idiotopes is inhibited by Ag. A subdivision into two groups of Ag-inhibitable anti-Id has been proposed (29, 30): those that mimic an antigenic structure and those that do not. Although the binding of all anti-Id is Ag-inhibitable, we conclude from the presented data that the analyzed anti-Id do not react with the paratope of M-T310 and fail to mimic the internal image of the Ag CD4. They fall into the second group of anti-Id according to the above classification and do not bear conformational resemblance to the Ag CD4. This may be because of the fact that M-T310 and also M-T404 do not bind CD4 in the same way as gp120, although they efficiently block the HIV1/CD4-interaction. Our results are also confirmed by failure of these anti-Id to bind HIV/gp120. Although we did not analyze possible changes in affinity caused by the substitutions, staining of PBL by the mutated proteins indicates that residues involved in anti-Id binding and Ag binding differ from each other.

It has been suggested that receptors that share the Ig fold, may provide more favorable targets for anti-Id mimicry (31). M-T310 and M-T404 recognize the CDR2-like region in domain 1 of CD4 which resembles very closely the V domain of Ig (32, 33). The conformation of CDR2 analog of CD4 differs from that of Ig as it is extended by three amino acids in comparison to the Bence-Jones protein REI V_L domain. Particularly, the CDR2-like loop juts out at the tip of the D1 domain, creating a prominent ridge. This structure might hinder anti-Id antibody to provide exact images of the external CD4 Ag. A previously published study (34) described the detection of a cross-reactive idiotope on CD4 mAb Leu 3a and ascribed this reactivity to the L chain. As in our CD4 system, the anti-Id induced against mAb Leu 3a failed to bind gp120. But in contrast to the presented data, these anti-Id showed

cross-reactivity with different other CD4 mAb with a similar reactivity pattern. We could not find cross-reactivity of the analyzed anti-Id with other CD4 mAb that are supposed to recognize the same or an overlapping epitope on the CD4 molecule, although the used V regions of these antibodies are closely related (see footnote 4). The presence of L chain-associated idiotopes in our system is not excluded, but we selected for anti-Id that are able to inhibit binding of CD4 mAb to CD4. In our system, the CD4 Ag binding of M-T310, as well as of M-T404, is exclusively determined by the H chain. The Ag specificity of CD4 mAb Leu 3a and related mAb might be determined mainly by the L chain V region, thus leading to a selection of L chain-specific anti-Id. The failure to detect cross-reactive anti-Id upon immunization with mAb M-T310 cannot be explained by the presented data.

By using mouse/human chimeric Ig, we demonstrate that, at least in the presented system, anti-Id binding reactivity is independent of the isotype and not influenced by the C region as stated to be required to form a correct idiotypic determinant on phosphorylcholine-specific antibody (18). Furthermore, the finding that a single amino acid substitution renders an antibody essentially invisible for a variety of anti-Id might be of importance for the regulation of the antibody response, because an exchange induced by somatic mutation would easily allow escape from Id control (4). A practical application of this work has arisen from the current use of CD4 mAb as therapeutic agents in chronic autoimmune diseases, where repeated administrations are necessary, with the consequence that the treated patients produce neutralizing anti-Id (35). To circumvent this effect, the possibility of selecting non–cross-reactive idiotopes would be of great advantage.

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