A Role of the Third Complementarity-determining Region in the Affinity Maturation of an Antibody*

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We recently found that there are two distinct antibody maturation pathways for the immune response of C57BL/6 mice to (4-hydroxy-3-nitrophenyl) acetyl and that a junctional amino acid introduced at a point far in advance of somatic hypermutation determined which pathway of affinity maturation was used. We describe here the structural basis for this aspect of maturation using recently developed H3 rules, which allow for reliable identification of the conformation of the third complementarity-determining region of the heavy chain (CDR-H3) from the primary amino acid sequences only. By the application of these rules, the anti-(4-hydroxy-3-nitrophenyl) acetyl antibodies examined here were classified into two major groups on the basis of their CDR-H3 structure, and these groups were found to be consistent with the maturation pathways. In addition, circular dichroism measurements revealed that the versatile nature of the antigen binding of the antibodies was significantly influenced by the pathway employed. We postulated in this study that flexibility in the CDR-H3 structure in the antigen-combining site could facilitate efficient antibody maturation supported by a plurality of possible antigen binding modes.

The affinity of an Ab1 increases gradually in response to T cell-dependent Ags. This germinal center process is referred to as affinity maturation and involves both somatic mutations introduced into rearranged V-(D)-J genes and Ag-driven selection of B cells (1–4). Ab affinity maturation is therefore similar to Darwinian evolution in that both processes consist of mutation and selection. Recently, we described several findings in a report (5) on the immune response of C57BL/6 mice to NP. In brief, we found that 1) two distinct Ab maturation pathways are used in this immune response, 2) each pathway involves Abs having high affinity for the Ag with a slow maturation (high evolvability group; group H) or having medium affinity for the Ag with a rapid maturation (low evolvability group; group L), 3) the thermodynamic properties of the Ag-Ab interaction in group H indicate versatility in the conformation of the Ag-combining site, accounting for the enhanced evolvability of these Abs, and 4) junctional amino acids introduced in a process far in advance of somatic hypermutation determine the maturation pathway. Because junctional amino acids introduced by imprecise joining in the combinatorial rearrangement of VH, D, and JH genes (6) provide increased diversity of CDR-H3, CDR-H3 would likely play a significant role in determining the evolvability of Abs. We examined here the structural features of anti-NP Abs along the maturation pathway, “using H3 rules” to especially focus on the CDR-H3 conformation (7) in order to interpret the role of CDR-H3 in the conformational versatility of the Ag-combining site.

In exploring 55 Ab structures from Protein Data Bank entries, we uncovered novel rules for the structural classification of CDR-H3 from the primary amino acid sequence only (8) and then established these as H3 rules by confirmation using 45 new entries of antibody structures (7). This set of rules also served to predict the flexibility of CDR-H3. In the present study, we show that anti-NP Abs can be classified using the H3 rules and that this system coincided well with the maturation pathways. In addition, we also performed CD measurements of a series of anti-NP Abs and obtained evidence that the manner in which NP bound to the Ab depended on the maturation pathway employed.

EXPERIMENTAL PROCEDURES

CD Measurements—CD spectra were measured at 25 °C in 50 mM sodium phosphate buffer (pH 7.0) containing 200 mM NaCl with a Jasco (Tokyo) spectropolarimeter (model J-600) in the presence or absence of 53 mM NP-Cap. The protein concentration was 1 mg/ml. A 1-cm optical path length cell was used.

Amino Acid Sequence Data of Anti-NP mAbs—The 29 anti-NP mAbs used in this study reflect the maturation pathways employed in the immune response of C57BL/6 mice to NP derivatives (5). Their amino acid sequences had also been determined in that study, and their accession numbers in the DDBJ/EMBL/GenBank™ data bases are AB030755–AB030796.

Numbering of the Amino Acid Residues in CDR-H3—Cys around the end of framework region 3 and Trp around the beginning of FR4 were assigned as −2 and n + 1, respectively, and m represents the end position of the β-hairpin in CDR-H3.

Assignment of CDR-H3 Conformations—To determine the CDR-H3 conformations of the 29 anti-NP antibodies, we analyzed their amino acid sequences using the H3 rules that we had previously proposed (7). The rules are briefly as follows. CDR-H3 should first be divided into two regions, base and β-hairpin. The structure of the base region is classified as one of the following base-types: a normal kinked base (KB), a kinked base with additional bulges inserted just above the base (KB*), an extended-positive base (E+), or an extended-negative base (E−). The critical amino acid residues at this step are those at positions −1, 0, n − 3, n − 2,
and \( n = 1 \) and at L46 and L49 (H3 rules i and ii). In the second step, structural features of the \( \beta \)-hairpin region are predicted using rules iii and iv. The critical amino acid residues at this step are those at positions 2, 4, \( m = 2, m = 1, m/2 + 1, m - 1, m/2, \) and \( m + 3/2 \).

**Molecular Modeling of the Ag-combining Site—**Modeling of the Ag-combining site was performed with the program Insight II (Molecular Simulations Inc.). For the framework region, CDR-L1, L2, L3, H1, and H2, the tertiary structure of N1G9 (Protein Data Bank: 1NGP), the crystal structure of a germline anti-NP mAb was used as the template. For CDR-H3, the crystal structures of Abs corresponding to CDR-H3 conformations determined by the H3 rules were used as the template. When it was impossible to use the side chain coordinates in the template structure, the atomic coordinates were generated using the program PRESTO-V2 (9), and the side chain conformations were then optimized by the dead-end elimination method (10). The whole structure was optimized by minimizing conformational energy using the program PRESTO-V2 with the following three minimization steps. First, the positions of all heavy atoms were restrained; second, all atoms other than those of CDR-H3 were restrained; and third, all CA atoms were restrained.

**RESULTS**

**CD Measurements—**Wedemayer et al. (11) carried out a structural study of catalytic Abs before and after maturation and showed that amino acids introduced in somatic mutation were mainly involved in an indirect contribution to hapten binding although the manner of binding to the hapten was significantly modified. With an anti-NP Ab, the manner in which it binds to NP might also be altered during affinity maturation, which may or may not be dependent on its evolvability. The crystallographic structure of N1G9, a germline anti-NP mAb, revealed that several amino acids with aromatic side chains were involved in the Ag-combining site and that some of these directly participated in NP binding (12). As shown in Fig. 1, aromatic side chains exhibit a CD absorption at approximately the near UV (250–350 nm) region, and their spectra are hypersensitive to a change in circumstances around the aromatic ring although we cannot describe this precisely at the atomic level. Thus, analysis of the near UV CD spectrum should provide helpful information for exploring the manner in which NP binds to anti-NP mAbs.

In the absence of NP, all the mAbs had a CD profile characterized by a weak negative absorption at \( \sim 250–300 \) nm because of the side chains of Trp and Tyr. (An example is shown in Fig. 1A.) All the mAbs examined showed essentially the same profile as in Fig. 1A, although there were variations in their amino acid compositions originating from both junctional diversity and somatic mutation (data not shown). The addition of NP brought about a significant change in the profile, as shown in Fig. 1A. The mAbs of group L showed almost identical \( \Delta[\theta] \) spectra in NP binding (Fig. 1B), clearly showing that, on a structural basis, they bound to NP-Cap in almost the same manner. This was in good agreement with the previous prediction that the mAbs of group L might have a common Ag-combining site that would not permit NP binding with a \( K_a \) value higher than \( 3.3 \times 10^7 \), even with various kinds of amino acid substitutions, because of low conformational versatility (5). The mAbs of group H, however, did not show a convergence such as that of the mAbs of group L. The \( \Delta[\theta] \) spectra of NP binding diverged, especially in the region of 250–300 nm (Fig. 1C), and these spectra formed three patterns (Fig. 2, A-C). Because CD absorption in this region varies in response to the environment, aromatic side chains as described above, group H may consist of subgroups among which aromatic amino acids participate in NP binding in various ways. In our previous work, the mAbs in this group were divided into at least three minor groups with thermodynamically different properties in NP binding (Fig. 2D).

In the absence of NP, all the mAbs had a CD profile characterized by a weak negative absorption at \( \sim 250–300 \) nm because of the side chains of Trp and Tyr. The critical amino acid residues at this step are those at positions 2, 4, \( m = 2, m = 1, m/2 + 1, m - 1, m/2, \) and \( m + 3/2 \).
groups. In the case of A6 and 6T3, their base structures were considered to be the extended base type because the amino acid residue at position 0 was not basic and tended to form a hydrogen bond between position 0 and \( n \). The amino acid residue at position 0 in A6 is Thr, whereas that in 6T3 is Gly, which enabled us to define them as having the extended base subgroup structures of EP and EN, respectively. Although 6L4 also lacks a basic amino acid residue at position 0, the base structure of 6L4 was categorized as kinked base in type because of Arg at position \( n \), which could form a salt bridge with Asp at position \( n + 4 \). The base structure of CDR-H3 in 6L4 was not further classified into KB\(^1\) or KBG.

In the second step of the assignment, the presence of a \( \beta \)-hairpin structure in CDR-H3 was assessed. (The overview is shown in the upper flow chart of Fig. 5.) Among the mAbs having a kinked base structure in their CDR-H3, the following were classified as mAbs with a hydrogen bond ladder in the \( \beta \)-hairpin region: 6T10, 6L1, 6L2, B2, F4, 3L2, 9L2, 3L1, 6L4, and 9L18. In addition, 9L11 and F4 have amino acid residues specific to Abs forming a typical \( \beta \)-turn in the \( \beta \)-hairpin region of CDR-H3. In the previous study, we explored the relation between the backbone root mean square deviations upon Ag binding and the hydrogen bond ladder or \( \beta \)-turn in Ab crystal structures and found that significant structural changes of larger than 1 Å occur only in the CDR-H3 segments with \( \beta \)-hairpin amino acid sequences that lack the features for both hydrogen bond ladders and a typical \( \beta \)-turn (7). We could roughly predict the maturation process from the following: A mutation from Trp\(^{H33}\) to Leu\(^{H33}\) (a feature of Abs of group L) brought about a 10-fold increase in the “kinked base group” were classified as class B, and these lacked an apparent rigid CDR-H3 conformation (Table I). A6 and 6T3, mAbs with extended base structures in their CDR-H3, were classified as class C (Table I).

We have already shown that the anti-NP mAbs obtained previously could be divided into four groups, group H, group L, group G (a germline group), and group D (a group L intermediate), and that these groups were clearly separated on a phylogenetic tree created by the software program CLUSTAL W (15). Fig. 3 shows the relationship between classification by the phylogenetic tree and that by the CDR-H3 conformations analyzed above. A good correlation between the two methods of classification was observed. Group H consisted of class B mAbs without exception. Ten mAbs out of the 12 mAbs in group L were class A. The mAbs of group G, 6T10, F8, and A6 were class A, class B, and class C, respectively. The group D mAb was class C. It should be noted that the key residue for classification using the phylogenetic tree is at position 1 (H99) (5) and that the residue at this position was not critical for the assignment of the CDR-H3 conformation.

**DISCUSSION**

**Immune Response of C57BL/6 Mice to NP Hapten**—We previously hypothesized that junctional amino acids may determine the structural properties of the Ag-combining site, giving certain Ab structures a conformational advantage in increasing affinity (5). We could roughly predict the maturation process from the following: A mutation from Trp\(^{H33}\) to Leu\(^{H33}\) (a feature of Abs of group L) brought about a 10-fold increase in
affinity, and mutations at other locations contributed little to this increase (16, 17), whereas Abs of group H seemed to require multiple mutations into the V region of both light and heavy chains (5). Recently, Papavasiliou and Schatz (18) demonstrated that somatic hypermutation was closely linked to DNA double-strand breaks that occur during DNA replication and concluded that a repair process for these breaks resulted in the introduction of mutations coupled with the presence of an error-prone DNA polymerase. On the basis of these findings, only a few mutations could be introduced with each cell division, and hence a longer period may be required for the maturation (11).

In mAbs of class A, a rigid CDR-H3 conformation might not provide enough versatility to configure a finely tuned fitting of the Ag-combining site, but a rigid conformation might nevertheless bring about rapid Ab maturation. Class B mAbs, however, did not have an apparent rigid β-hairpin structure in CDR-H3 (Table I). The amino acid residue at position n – 3 in group H was His without exception. Because only aromatic amino acids at this position could enhance the formation of the H bond ladder stabilizing a β-hairpin structure (Fig. 5, upper flow chart), this residue would prevent CDR-H3 from having a rigid β-hairpin structure. We consider that this lack of an apparent rigid loop structure attributable to His at position n – 3 is an advantage in creating an Ag-combining site that is finely fitted to NP. The His residue at this position may be indispensable for the high evolvability of class B Abs.

In our previous study, analysis of precisely determined crystal structures of highly maturated Abs revealed that 58% of these had CDR-H3 that lacked rigid β-hairpin structures (7). Conformational versatility in Ag combining caused by a flexibility of the CDR-H3 was deemed necessary for efficient Ab maturation.

As representatives of group G, 6T10 and A6 were studied in terms of the molecular modeling of their Ag-combining sites (Fig. 4, a and b, respectively). Although both mAbs have rigid β-hairpin structures in their CDR-H3, A6 has a base structure with extended conformation (E3) in contrast to the kinked base CDR-H3 of 6T10 (Table I). CDR-H3 with an extended base tends to lean toward the L chain (Fig. 4a) as compared with CDR-H3 with a kinked base, which leans toward the Ag-recognition cavity (Fig. 4b), and this extended base results in a relatively wider and more rounded cavity. In A6, the relatively relaxed Ag-combining site caused by the extended base type of CDR-H3 could result in a 10-fold higher affinity to NP than that of 6T10. However, the wide cavity observed in Abs with an extended base structure is not suitable for the high affinity recognition of small haptens such as NP. It is possible that the reason why 6T3 (group D) could not achieve high affinity despite its many somatic mutations within the V region is because the cavity would be too wide for NP to bind tightly. Group D mAbs could be referred to as “dead-end” Abs, although we classified them as class L intermediate.

We previously described the importance of Gly at position 1 in Ab maturation (5). We considered that the high adaptability of group H is a reflection of the inherently flexible Gly at this position and that this Gly residue would facilitate a greater variety of CDR-H3 conformations than any other amino acid,
thus providing the potential for a high degree of maturation. Recently, our statistical analysis of Ab structures from Protein Data Bank entries revealed that Gly at position 1 caused a structural diversity in the N-terminal strand of CDR-H3 and afforded a variety of CDR-H3 conformations (20). In addition, Smith and Xue showed from their sequence profile analysis for

TABLE I

| Clone | H3 sequence | Base structure | H bond ladder | β-turn | Loop structure | Classification | Group |
|-------|-------------|----------------|---------------|--------|---------------|----------------|-------|
| 6T10  | CARYYGGSSY | FDYW           | Kinked        | Yes    | n.f.          | Rigid          | Class A |
| F8    | CARYRVYYA  | MDYW           | Kinked        | n.f.*  | n.f.          | Not rigid      | Class B G |
| A6    | CATTERYGSSF | DYW            | Extended      | Yes    | n.f.          | Rigid          | Class C G |
| 6T13  | CAGNYGGGTYF | DYW            | Extended      | Yes    | n.f.          | Rigid          | Class C D |
| 6L1   | CARRYGYYTF | FDYW           | Kinked        | Yes    | n.f.          | Rigid          | Class A L |
| 6L2   | CARRYGGRY  | FDYW           | Kinked        | Yes    | n.f.          | Rigid          | Class A L |
| A2    | CARAFYGSL  | FDYW           | Kinked        | n.f.   | n.f.          | Not rigid      | Class B L |
| 9L11  | CARYFYGGS  | FDYW           | Kinked        | n.f.   | Yes           | Rigid          | Class A L |
| G6    | CARYYGGSS  | FDYW           | Kinked        | n.f.   | n.f.          | Not rigid      | Class B L |
| B2    | CARRYGGAY  | FDYW           | Kinked        | Yes    | n.f.          | Rigid          | Class A L |
| F4    | CARYGGYSY  | FDYW           | Kinked        | Yes    | n.f.          | Rigid          | Class A L |
| 3L2   | CARRYGGSY  | FDYW           | Kinked        | Yes    | n.f.          | Rigid          | Class A L |
| 9L2   | CARRYGGRY  | FDYW           | Kinked        | Yes    | n.f.          | Rigid          | Class A L |
| 3L1   | CARYYGLY   | FDYW           | Kinked        | Yes    | n.f.          | Rigid          | Class A L |
| 6L4   | CATTERYARY | FDYW           | Kinked        | Yes    | n.f.          | Rigid          | Class A L |
| 9L18  | CRARYGGYE  | FDYW           | Kinked        | Yes    | n.f.          | Rigid          | Class A L |
| B10   | CARYYGGSH  | FDYW           | Kinked        | n.f.   | n.f.          | Not rigid      | Class B H |
| 9T1   | CARGYGGSH  | FDWF           | Kinked        | n.f.   | n.f.          | Not rigid      | Class B H |
| 20T2  | CARGRGYGH  | FDYW           | Kinked        | n.f.   | n.f.          | Not rigid      | Class B H |
| 9T8   | CARGRGYLG  | FDYW           | Kinked        | n.f.   | n.f.          | Not rigid      | Class B H |
| C6    | CARGKLGH   | FDYW           | Kinked        | n.f.   | n.f.          | Not rigid      | Class B H |
| 9T15  | CARGRGYLG  | FDYW           | Kinked        | n.f.   | n.f.          | Not rigid      | Class B H |
| 6T9   | CSGRGFYAH  | FDYW           | Kinked        | n.f.   | n.f.          | Not rigid      | Class B H |
| 20T1  | CVGRGLWH   | FDWF           | Kinked        | n.f.   | n.f.          | Not rigid      | Class B H |
| 9T7   | CARGRGYHL  | FDYW           | Kinked        | n.f.   | n.f.          | Not rigid      | Class B H |
| 9T10  | CARGRGYHL  | FDYW           | Kinked        | n.f.   | n.f.          | Not rigid      | Class B H |
| E11   | CARGCYGH   | FDWF           | Kinked        | n.f.   | n.f.          | Not rigid      | Class B H |
| 9T13  | CARGRGYHL  | FDWF           | Kinked        | n.f.   | n.f.          | Not rigid      | Class B H |
| E3    | CARGCYGCH  | FDWF           | Kinked        | n.f.   | n.f.          | Not rigid      | Class B H |

a Amino acid sequences of CDR-H3 segments from position 2 to n + 1 are shown with position m (bold).
b When either an H bond ladder or a typical β-turn was observed in the CDR-H3 structure, the loop structure was predicted as rigid.
c Class A, kinked base and rigid loop structure; class B, kinked base and nonrigid loop structure; and class C, extended base structure.
d Groups determined in our previous study; G, germline group; D, intermediate type; L, low evolvability group; and H, high evolvability group.
e n.f., no features.

Fig. 4. Shape of the Ag-combining site cavity dependent on the base structure of CDR-H3. Molecular modeling of the Ag-combining site of 6T10 (A, kinked base) and A6 (B, extended base) was performed. All amino acids other than those at position –1 (red letter) are identical between 6T10 and A6. The views of their Ag-combining sites are from the same angle. The van der Waals surfaces of all atoms other than those of CDR-H3 are shown in a Corey-Pauling-Koltun space-filling model (light blue, H chain; gray, L chain). The CA atoms and backbone structure of CDR-H3 are represented by balls and sticks, respectively, and are either red (kinked or extended base region) or yellow. Side chains determining the base structure are also shown with hydrogen bonds (white dotted lines). The following atoms in 6T10 appear transparent to afford a full view of the CDR-H3 structure: CA and HA of GL51, CB, HB3, CG, OD1, ND2, and HD21 of NL55, CD and HD3 of PL58, and CD2, HD2, CE2, HE2, CZ, and OH of YH32. Figures were prepared with the program MOLMOL (27).

thus providing the potential for a high degree of maturation. Recently, our statistical analysis of Ab structures from Protein Data Bank entries revealed that Gly at position 1 caused a structural diversity in the N-terminal strand of CDR-H3 and afforded a variety of CDR-H3 conformations (20). In addition, Smith and Xue showed from their sequence profile analysis for...
the Kabat data base that Abs having Gly at position 1 are abundant, although amino acids at this position are inherently varied because of junctional diversity (28). It may be that a Gly at position 1 provides conformational versatility to the CDR loop, resulting in the enhanced evolvability of Abs. It should be noted again that position 1 is not considered a key position for the assignment of CDR-H3 conformation (Fig. 5).

**Maturation Pathway and NP Binding Mode**—The manner of the binding of an anti-NP mAb to NP is thought to be altered during affinity maturation. In a structural study, Wedemayer et al. (11) described the binding of a catalytic Ab to a hapten as being significantly modified. In the present study, we were able to assess differences in NP binding using CD measurements, and we found a close relationship between the binding mode and the maturation pathway employed and found that this relationship was highly dependent on the CDR-H3 structure, as discussed above.

Fig. 2, A-C, shows clusters in the Δ[θ] profiles of group H. Because NP-Cap does not have a CD absorption of its own, the differential spectra mainly reflect changes attributable to the Abs engaged in NP binding. It was suggested that there are at least three subgroups in group H from the viewpoint of a structural change of the Ag-combining site. In our previous study, the mAbs of this group were divided into at least three smaller groups, each having thermodynamically different properties of NP binding (Fig. 2D). We believe that the mAbs of group H have a common Ag-combining site that allows for more than one type of NP binding, i.e., their Ag-combining site would have high conformational versatility (5). Clusters in the Δ[θ] profiles shown in Figs. 2, A-C almost completely coincided with those obtained with the thermodynamic properties shown in Fig. 2D.

We estimated the [θ] profiles of group H on the basis of CD absorption at ~250–270 nm and 270–310 nm as follows. The mAbs of cluster I showed no significant [θ] change on NP binding at ~270–310 nm (Fig. 2A, inset), the mAbs of cluster II had extremes of positive and negative CD absorption on NP binding at ~250–270 nm and 270–310 nm, respectively (Fig. 2B, inset), and the mAbs of cluster III had a high positive CD absorption of only ~250–270 nm (Fig. 2C, inset). The negative CD absorption at ~270–310 nm probably arose from a change in the environment surrounding the aromatic side chains. Because the amino acid residues that directly participated in NP binding were considered to be essentially the same in group H mAbs, as deduced both from the amino acid sequences and the crystallographic structure of N1G9, the mAbs of cluster II might have arisen from a conformational change in the Ag-combining site. On the other hand, we do not have a clear explanation for the positive CD absorption of around 250–270 nm observed in mAbs of clusters II and III.
however, mAbS having a positive CD absorption within the 250–270 nm range on NP binding (those of both clusters II and III) harbored Arg at H59 instead of Lys without exception. Because NP has no CD absorption in solution, the replacement of LysH59 with Arg would alter the environments accompanying the change in the CD profile. From the crystallographic structure of N1G9, it is apparent that LysH59 interacted directly with NP. (The distance between the ε-amino group of LysH59 and the 3-hydroxyl group was within 2 Å.) (12) Because the 4-hydroxyl group would be deprotonated, Arg as a strong base would be effective in raising the affinity to NP in group L as well. However, this replacement was never seen in group L. An NP binding mode that does not require a strong base at H59 may exist.

The mAbs of group L showed almost identical Δ[θ] profiles (Fig. 1B). Because these mAbs shared an amino acid replacement of TrpH33 → Leu, and because TrpH33 was directly involved as seen in NP binding in the crystallographic structure of N1G9 (12), the convergence might be mainly attributable to the absence of TrpH33 in the Ag-combining site. A6, however, which was judged to be a germline mAb of group L from the amino acid at position 1 and according to the H3 rules, was again classified as group L from the Δ[θ] profile clustering (Fig. 1B, red line), even though this mAb had Trp at H33. As described in a previous study, ΔH versus TΔS plots of group L yielded a straight line with a slope of nearly 1 (5). If the slope of this line reaches 1 (ΔH/TΔS = 1), there has been no change in affinity to NP, even if a large change in ΔH or TΔS is noted. The mAbs of group L appeared to show a strict balance between ΔH and TΔS in response to somatic hypermutation. Therefore, it is expected that the mAbs of this group could have a common Ag-combining site that does not allow for high affinity NP binding, even if there have been various kinds of amino acid substitutions. In other words, these mAbs have low adaptability. The finding presented in Fig. 1B was in good agreement with this in terms of a structural change in the NP binding site. The mAbs of group L bound NP-Cap in the same manner, although the precise structural basis remains unclear.

Significance of CDR-H3 Conformations in Ab Maturation—Functional Ab H chain genes are activated and expressed after the rearrangement of VH, D, and JH gene segments at the pro-B cell stage (21) and further modified during the recombination process by terminal deoxynucleotidyltransferase activity in a manner that gives rise to N region variation in CDR-H3 (6). Diversity in CDR-H3 is essentially determined at an early stage of B cell development. Using the H3 rules, we could determine the nature of the structural contribution made by junctional diversity. Among our anti-NP mAbs, the possible amino acids resulting from this diversity are those at positions 0, 1, 2, n = 3, n = 4, and n = 5 (Fig. 5). All amino acids at these positions except for position 1 play important roles in determining the CDR-H3 conformation, as shown in Fig. 5. The diversification of amino acids in key positions that determine the base and loop structures could arise at the VDJ-joining step.

We can summarize the maturation of anti-NP Abs as follows: i) germline type B cells are represented by three groups, group H, group L, and group D, and are generated at an early stage of development, ii) Gly at position 1 and a less than rigid β-hairpin with a kinked base structure in CDR-H3 are essential features of group H, which is divided into at least three clusters in terms of the NP binding mode, and iii) a rigid β-hairpin with a kinked base in CDR-H3 has the advantage of affording rapid maturation (group L has a single cluster in terms of NP binding mode).

Immunological Importance of Diversity in CDR-H3 Structures—Functional diversity has been regarded as one way in which Abs provide a defense against diverse immunogenic substances. By studying the effect of this diversity on the anti-NP immune response, we found that the maturation pathway was forked in a manner reflected in the CDR-H3 structure and that the differences in this structure were caused by junctional amino acids. Because the CDR-H3 loop is located in the center of the Ag-combining site facing CDR-L2, it is possible that this loop not only controls the angle between the VH and VL domains but also affects the flexibility as well as the cavity size of the combining site. In fact, Vargas-Madrazo et al. (22) reported that there is a correlation between the type of Ag and the combination of canonical structures of five CDRs and also described the importance of the CDR-H3 conformation. It is interesting that B cells in fetal and neonatal mice generally lack N regions because of a very low level of terminal deoxynucleotidyltransferase expression at this early age, and this has a significant impact on Ab repertoire expression and diversification (23, 24). Benedict and Kernel (25) demonstrated that the lack of junctional diversity in fetal mice was indispensable for shaping the B cell repertoire against Streptococcus pneumoniae and that the repertoire was maintained throughout their lifetime.

The immune system is thus so flexible that it maintains mechanisms to diversify the region important in determining the CDR-H3 conformation and to regulate this process developmentally. A close relationship between Ab maturation and the CDR-H3 structure may contribute to the fine-tuning at which the immune system excels.

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