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Structural Elucidation of the Mechanistic Basis of Degeneracy in the Primary Humoral Response

Tarique Khan* and Dinakar M. Salunke*†

The mechanistic basis for efficient combating of the infinite range of foreign Ags by the limited repertoire of naive Abs expressed on primary B cell surfaces during their first encounter was addressed through elegantly designed crystallographic analyses. Resolution of the discrepancy arising from the limited number of possible germline Ab receptors on primary B cells for recognizing the unlimited pool of possible Ags has been attempted by invoking the degenerate recognition potential of the germline Abs. Structural analyses of germline mAb BBE6.12H3 in an Ag-free state, as well as bound to four different peptide Ags, established the correlation of its degenerate specificity with conformational versatility of the paratope. Six distinct paratope topologies observed for a single germline mAb provided a quantitative description of the primary Ag recognition repertoire at the tertiary structural level. Each of the four different peptide Ags was bound specifically to a distinct conformation of the paratope, which was also different from that of the Ag-free states of the same germline mAb. A minimal conserved motif in the pristine Ag-combining site essential for multispecificity and Ag binding-mediated change in the elbow angle of Fab was also discernible. It is proposed that the generation of a primary Ab repertoire involves large, yet finite, germline Ab clones, each capable of adopting discrete conformations, which in turn exhibit diverse binding modes. The Journal of Immunology, 2012, 188: 000–000.

The antigenic repertoire is infinite. To generate an effective immune response against each of the possible Ags from this infinite pool, every Ag has to be specifically recognized in the initial encounter. Physicochemical principles of Ag recognition would suggest that Abs required to neutralize the infinite population of Ags have to be unlimited. This conundrum was elegantly answered many years ago by an expansion of the primary Ab repertoire through genetic recombination of VDJ gene segments and by resultant combinatorial diversity arising from rearranged heterodimers of light and heavy chains (1). Recent high-throughput sequencing data in zebra fish and humans have provided further insights into the extent of the expressed Ab repertoire, as envisaged by Tonegawa et al. (2, 3) However, quantitative assessment, even after considering other possible mechanisms, proves this diversity to be inadequate for recognition of infinite Ags (4).

It was suggested that a degree of polyreactivity within a germline Ab would provide a way by which a single Ab would be able to recognize a range of Ags and so obviate the requirement for a singular Ab for every potential non-self immunogen (5–9). Even the mutation-driven modulations of epitopes could be handled very efficiently by primary humoral immune defense through use of such polyreactive Abs (10). Thereafter, during secondary immune responses, mature Abs with higher affinity and specificity for a foreign molecule can be evolved when a rapid change in concentration or presentation of the foreign molecule triggers a mutagenic proliferation of the germline Ab in question (11). Nonetheless, crystallographic analysis of anti-p24 (HIV) mAb toward different analogs of a peptide epitope has demonstrated the polyspecific potential of even a mature Ab (12).

Indeed, the structural basis of Ag recognition has been addressed through a vast number of crystallographic data generated on mature Ab–Ag complexes during the past three decades. Together, these Ab–Ag complex structures have provided snapshots of the immune recognition of diverse epitopes and offer physiological as well as biophysical perspective to our understanding of Ag recognition. However, initial encounters of foreign Ags by naive primary Abs have not been adequately explored. We have sought to address the strategies needed for efficient recognition of the infinite range of foreign Ags by the limited repertoire of naive primary Abs during their first encounter.

To understand the mechanistic basis of this event, we have carried out structural investigations on a polyreactive germline mAb, BBE6.12H3 Fab. It is an immunologically well-characterized germline Ab derived from the major idiotypic response to the hapten (4-hydroxy-3-nitrophenyl)-acetate (NP) in C57BL/6 mice (13). The L and H chain V region of germline mAb BBE6.12H3 has been shown to be constructed from V{\text{H}} 186.2, D{\text{H}} Fl16.1, and J{\text{H}}2 germline gene segments, respectively (Supplemental Fig. 3). Its mature counterpart Bg 53-5 contains 11 point mutations, 1 in the L chain and the remaining 10 in the H chain (13). Through extensive kinetic and thermodynamic analyses, BBE6.12H3 was shown to bind to a series of independent dodecapeptide Ags (5, 6). In the current study, four among those peptide Ags—namely, B6-13, 3, BA7-09, and B6-07, which bind to the germline mAb BBE6.12H3 with $K_d$ values of 0.38 μM, 0.80 μM, 0.60 μM, and 0.26 μM, respectively (6)—were subjected to crystallographic analysis. The binding affinities of these peptides were 30- to 100-fold higher than that of the cognate Ag ($K_d = 23.0$ μM). Our data illustrate the importance of paratope adaptability...

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The online version of this article contains supplemental material.

Abbreviations used in this article: NP, (4-hydroxy-3-nitrophenyl)-acetate; PEG, polyethylene glycol.

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for facilitating polyreactivity of germline mAb through systematically designed crystallographic experiments. The present analysis, in light of earlier studies (1, 5, 8, 9, 14–19), suggests the possibility that generation of diversity in the primary Ab repertoire may involve multiple mechanisms in a hierarchical order. The proposed model may account for enhanced diversity of primary immune receptors without increasing the size of the germline gene repertoire.

Materials and Methods

Peptides

Peptides were essentially derived using the Phage Display Peptide Library Kit (New England Biolabs, Cambridge, MA), as previously reported (6). Interactions between Ab and peptide and their thermodynamic analysis by surface plasmon resonance have also been previously reported (6). Independent peptides selected from a panel of a random phage library binding to germline mAb (6) were used for cocystalization experiments. The 12-mer peptides were synthesized by the solid-phase method on an automated peptide synthesizer (431A; Applied Biosystems, Foster City, CA). Cleavage was performed using trifluoroacetic acid (Sigma-Aldrich, St. Louis, MO). Crude peptides were purified on a Delta Pak C18 column (Waters, Milford, MA), using a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid. The purity of peptides was characterized by mass spectrometry.

Abs

The hybridomas used in this study were previously generated by following a standard method (13). Hybridoma cells secreting IgG BBE6.12H3 were suspended in 500 ml Dulbecco’s PBS and were injected into the peritoneal cavity of male BALB/c mice gamma irradiated (400 rads) and primed with Freund’s incomplete adjuvant 72 h prior to injecting the hybridoma cells. A total of 5 × 10^4–5 × 10^5 hybridoma cells were injected into each mouse. Ascitic fluid could be tapped from the peritoneal cavity of mice corresponding models were subsequently refined.

The structures of Fab–peptide complexes were determined by molecular replacement using PHASER (23), and the variable and constant domains of native germline Ab Fab were used as separate ensembles. The corresponding models were subsequently refined.

Refinement and model building

Refinement was conducted with CNS (24). Both conventional Rwork (crystallographic R-factor) and Rfree (free R-factor) (24) values were monitored during the refinement. In each case, 10% of the total reflections were set aside for calculation of Rfree values. Initially, rigid body refinement was carried out for the complete Fab molecule, and subsequently, VH, VL, Cα and Cβ domains were treated as discrete units. The models were further refined using the positional refinement protocol of CNS. Electron density maps were displayed with the help of the program COOT (25). As the refinement progressed, the peptide models could be built into electron densities within the corresponding Ag-combining sites. Water molecules were added using the water pick program in CNS. The quality of the model was checked with MolProbity (26). Structural models have been generated with PyMOL (http://www.pymol.org), and the structural superimpositions were accomplished using the program SUPERPOSE from the CCP4 suite (27). The epitope–paratope interactions and buried surface areas were analyzed using PISA (28).

Accession codes

The coordinates and structure factors have been deposited in the Research Collaboratory for Structural Bioinformatics Protein Data Bank (http://www.pdb.org) under the accession codes 4szy, 2y07, 2y06, 2y36, and 2x2q.

Results

Crystal structures of germline mAb BBE6.12H3

The diffraction-quality crystals of BBE6.12H3 Fab, as well as its complexes with the peptide Ags B6-13, 3, BAg7-09, and B6-07, were crystallized using 21% polyethylene glycol (PEG; 3.3 kDa), 21% PEG (8.0 kDa), 19% PEG (6.0 kDa), 17% PEG (6.0 kDa), and 23% PEG (3.3 kDa) in the presence of ZnCl2, respectively. In the current study, these peptides are referred to as Dlw, Gdp, Pyy, and Yqy, and their complexes with Fab as BBE6.12H3-Dlw, BBE6.12H3-Gdp, BBE6.12H3-Pyy, and BBE6.12H3-Yqy, respectively. The crystal structures were determined at the 2.4–2.9 Å resolution range. The coordinates and structure factors for all five structures are shown in Table I. The L and H chains of all BBE6.12H3 structures contained 211 and 220 residues, respectively. Electron density for Ser136H–Asn140H was ambiguous in all Fab–peptide complexes, as in many other reported Fab structures (29).

The asymmetric unit of the native Fab contained two molecules (BBE6.12H3-Native-AB and BBE6.12H3-Native-LH). The four complexes with the dodecapeptides Dlw, Gdp, Pyy, and Yqy belonged to the same space group, and each contained one molecule per asymmetric unit (Table I). Therefore, five crystals generated a database of six Fab molecules. Models were built for the first 10, 6, and 9 residues of the peptide Ags Dlw, Gdp, Pyy, and Yqy, respectively. Nine residues of Gdp peptide, starting at the second residue from the N terminus, were evident in the electron density (see Table II). Electron densities for the remaining amino acids were not visible even when the corresponding electron density maps were rendered at low σ values. The electron density maps of the peptides observed in the structures of the Fab–peptide complexes are shown in Supplemental Fig. 1.

Ag-free Fab structures

Evaluating the structural flexibility of the germline Ab paratope by superimposition of the variable domains of Ag-free BBE6.12H3 Fab structures revealed two distinct CDR3 loop conformations (Fig. 1A). The remaining CDR loops of both H and L chains were identical. The pairwise RMSD values for backbone and all atoms of CDRs, shown in Supplemental Table I, also indicated major backbone movement in CDR3.

The surface features of CDRs of the two Ag-free Fab molecules enhanced with hydrophathy features are shown in Fig. 1B and 1C. Paratopes of these molecules exhibit dissimilar topologies and charge distribution. Both backbone conformations and side chain orientations contribute to these differences. The movement of
CDRH3 is manifested by displacement of the backbone by ≤5.71 Å and by the rotation of several side chains (Fig. 1A). The RMSD of the superimposed native structures showed that Tyr95B and Tyr95H differ in position by 6 Å (measured between the side chain atoms).

Comparative analysis of peptide Ag-bound Fab structures

Crystal structures of the BBE6.12H3 in complex with dodecapeptide Ags provided insights into the mechanism by which germline mAbs can recognize and bind independent ligands. The CDRH3 loop of mAb exists in different conformations in the absence of the Ag. We sought to investigate whether this conformational diversity was indeed exploited while binding to different Ags. Indeed, the Cα superimposition illustrated distinct conformations of CDRH3 in the four Ag-bound structures (Fig. 2A). This dissimilarity was also perceptible from the diverse surface topologies of the Fab molecules in the Ag-bound form, as shown in Fig. 3.

Comparison of the CDRH3 loops in four complexes and in the native state (Figs. 1A, 2A) indeed shows that CDRH3 backbone and side chain orientations construct a lid-like structure that covers the Ag-binding pocket in native Fab molecules. In contrast, CDRH3 bends away from the hapten-binding cavity in all Ag-bound structures. In all the peptide-bound Fab molecules, the binding site is exposed owing to this outward movement of CDRH3 (Fig. 2A). The loop conformations start to diverge at Tyr95H and do not realign until Phe100cH. This movement is accompanied by displacement of the CDRH3 main chain by 7.68 Å and by the rotation of several side chains (Fig. 2A). The CDRH2 also displayed variation in conformation, although to a lesser extent (Fig. 2A). The pairwise superimposition of the CDRs showed that the backbone of the CDRH3 in complexed isomers deviates significantly (Supplemental Table I).

The variable and constant domains of two native BBE6.12H3 Ab structures are not in line with each other, as their elbow angles are 201.2° and 200.1°, respectively. Elbow angles >180° have been reported for the murine λ-chain–bearing Fabs, even though elbow angles range mostly from 130° to 180° (30). In peptide Ag-bound structures, the two domains are in line with each other, and the elbow angles of Dlw-, Gdp-, Ppy-, and Yql-bound Fab structures are 175.5°, 175.9°, 176.6°, and 174.5°, respectively (see Table II) (31). Thus, native Fab molecules, compared with Ag-bound Fab structures, show differences in the relative orientations of the variable and constant domains. The variable domain reorientations during complex formation with the peptides Dlw, Gdp, Ppy, and Yql induce movement of the VL and VH against each other, in comparison with native Fab molecules. Consequently, major changes occur in the elbow angles of complexed Fab fragments.

### Table I. Data collection and refinement statistics

<table>
<thead>
<tr>
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<td>C2</td>
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<td>86.3, 81.4, 128.9</td>
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<td>55.5, 61.9, 112.5</td>
<td>54.4, 60.8, 111.7</td>
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<td>6.0 (23.0)</td>
<td>10.0 (34.0)</td>
<td>6.0 (17.0)</td>
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<td>100 (99.8)</td>
<td>99.9 (99.0)</td>
<td>95.5 (93.6)</td>
<td>99.3 (98.1)</td>
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<td>23.3/26.6</td>
<td>23.4/25.7</td>
<td>24.0/25.0</td>
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<td>3217</td>
<td>3223</td>
<td>3204</td>
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<td></td>
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<td>77</td>
<td>65</td>
<td>53</td>
</tr>
<tr>
<td>B-factors (Å²)</td>
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<td>41.9</td>
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<td>67.9</td>
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<tr>
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<td>Bond lengths (Å)</td>
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<td></td>
<td>Bond angles (°)</td>
<td>1.7</td>
<td>1.9</td>
<td>1.7</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Parameters in parentheses represent last resolution shell statistics. The single-letter amino acid names of three initial residues at the N terminus of the peptide have been used for naming the peptide in each case.

### Table II. Buried surface area and elbow angle analysis

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<tr>
<th>Complexed Peptides</th>
<th>Peptide (Å²)</th>
<th>VL (Å²)</th>
<th>VH (Å²)</th>
<th>VL–VH Interface (Å²)</th>
<th>Elbow Angle (°)</th>
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</thead>
<tbody>
<tr>
<td>DLWTTAIPTPS (BBE6.12H3-Dlw)</td>
<td>505</td>
<td>992</td>
<td>1147</td>
<td>2650</td>
<td>175.5</td>
</tr>
<tr>
<td>GDRPRSYSYISHLL (BBE6.12H3-Gdp)</td>
<td>492</td>
<td>1141</td>
<td>1213</td>
<td>2850</td>
<td>175.9</td>
</tr>
<tr>
<td>PPYPAPAIAPGNI (BBE6.12H3-Ppy)</td>
<td>431</td>
<td>1049</td>
<td>1030</td>
<td>2509</td>
<td>176.6</td>
</tr>
<tr>
<td>YQLRPNAETLRF (BBE6.12H3-Yql)</td>
<td>536</td>
<td>1286</td>
<td>1043</td>
<td>3049</td>
<td>174.5</td>
</tr>
<tr>
<td>BBE6.12H3-Native-AB</td>
<td>1770.0</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>BBE6.12H3-Native-LH</td>
<td>1750.0</td>
<td></td>
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</table>

Buried surface areas were calculated with the program PISA (22), using a probe radius of 1.4 Å and van der Waals radii. Elbow angles were calculated using a Web server (26). All values for buried surface area were calculated for the modeled peptide structures (shown in bold letters).
compared with Ag-free Fab. The largest change was found in the case of BBE6.12H3-Yql. Comparing the different BBE6.12H3/peptide complexes with an Ag-free Fab fragment, a clear-cut correlation between the buried surface area at the VL–VH interface and the extent of domain disposition was observed (Table II).

**Interactions and conformations of the peptide Ags bound to BBE6.12H3**

All the peptides are located in and around a deep, hydrophobic pocket in different conformations, but in the same orientation (Fig. 2B). Their binding sites overlap. Three common residues—Trp33 (CDRH1), Arg50 (CDRH2), and Trp91 (CDRL3)—form the shared paratope (Fig 2A). For the conformation and relative orientations of the different peptides, see Fig. 2B. These diverse conformations of the peptides suggest topological nonequivalence between the bound epitopes and further confirm degeneracy of the germline Ab toward the Ags that were remarkably different (Fig. 2B). A comparison of the mode of binding of the four peptide Ags to BBE6.12H3 is shown in Fig. 3. All the four Ags were seen interacting with an independent set of CDR residues (Fig. 3, Table IV).

**FIGURE 1.** Comparative analysis of conformations of the CDR loop of Ag-free Fab molecules. A, Stereo view of superimposed CDRs of Ag-free molecules highlighting divergence in the CDRH3 conformation are shown as thin ribbons, and side chains of only CDRH3 loops are shown as sticks; BBE6.12H3-Native-AB, magenta; BBE6.12H3-Native-LH, cyan. Connolly surfaces of the Ag-free mAb BBE6.12H3 Fab molecules enhanced with the hydropathy feature are shown, in which the red to blue color spectrum represents hydrophobicity to hydrophilicity, respectively. B, BBE6.12H3-Native-AB. C, BBE6.12H3-Native-LH. A box in B and C highlights the inward orientation of CDRH3 residues compared with outward movement of the same residues in Ag-bound structures, as shown in Fig. 3.

**FIGURE 2.** Stereoscopic representation of superimposition of Ag-bound Fab molecules. A, CDRs of Ag-bound Fab molecules and side chains of all interacting residues are shown as thin sticks. Three common interacting residues—W33H (CDRH1), R50H (CDRH2), and W91L (CDRL3)—are shown as thick sticks. BBE6.12H3-Dlw, red; BBE6.12H3-Gdp, green; BBE6.12H3-Ppy, blue; BBE6.12H3-Yql, yellow. B, Only peptide epitopes from the superimposed complexes are displayed as sticks for clear depiction of conformational distinctions between them. Dlw, red; Gdp, green; Ppy, blue; and Yql, yellow.
The buried surface areas for the different peptides, as well as for Fab, VL, VH, and the VL–VH interface, given in Table II depicted variations in the paratope topologies of BBE6.12H3 while binding to independent peptides. In all complexes, the interactions were specific, as many hydrogen bonds and salt bridge contacts were observed (Table III). In all, 22 residues of the Fab CDRs were involved in interactions with the peptides in one case or another (Table IV). The side chain orientations of all the interacting residues in the different structures were identical, except those of the residues from CDRH3 and CDRH2 (Fig. 2A).

The peptide Dlw lies in a twisted conformation at the interface of the variable domains of the H and L chains, and it predominantly interacts with CDRL3 and CDRH2 residues (Fig. 3A, Tables III and IV). Trp makes the most of the hydrophobic interactions by snuggly fitting into the hydrophobic pocket (for residue-wise interactions, see Table III and Fig. 3A). Details of the atoms in-
volved in polar interactions are given in Table III. The CDRH2 residues form most of the polar interactions; Thr57H makes three hydrogen bonds with three different peptide residues, namely, Ile7, Pro8, and Pro11; Tyr59H makes a polar contact with Ile10. Another CDRH2 residue, Lys64H, makes a salt bridge with Ile10 (Table III). Only one residue of the L chain, Ser93L (CDRL3), interacts with Asp1 through a hydrogen bond. In Table IV, all the van der Waals contacts between peptide and the Ab residues have been outlined. Dlw interactions include 135 van der Waals contacts with the Ab residues. The common interacting residue Trp91L makes strong hydrophobic interactions with Leu2 and Trp3, whereas Trp33H contacts only Trp3. In addition to this, all three common interacting residues make several van der Waals contacts with different peptide residues, as shown in Table IV.

Table III. Polar and side chain hydrophobic interactions between BBE6.12H3 and different peptides

<table>
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<tr>
<th>CDR L</th>
<th>Ser50b O</th>
<th>Tyr52b O</th>
<th>Thr57b O</th>
<th>Tyr59b N</th>
<th>Ser60b N</th>
<th>Thr67b N</th>
<th>Thr67b O</th>
<th>Thr70b N</th>
<th>Ser72b N</th>
<th>Thr87b N</th>
<th>Ile87b O</th>
<th>Tyr92b O</th>
<th>Ser93b O</th>
<th>Lys95b O</th>
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<tbody>
<tr>
<td>Pro3 O</td>
<td>Pro5 O</td>
<td>Pro3 O</td>
<td>Arg4 N</td>
<td>Ser6 O</td>
<td>Pro1 O</td>
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<td>Arg4 N</td>
<td>Glu O</td>
<td>Leu3 N</td>
<td>Arg4 N</td>
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</tbody>
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Side chain hydrophobic interactions were calculated using van der Waals radii in the contact program of CCP4 (15). Polar contacts were calculated using PISA (22). Salt bridges are indicated by bold text.

Table IV. Interactions of peptides with BBE6.12H3 Fab

<table>
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<tr>
<th>Ab Residues</th>
<th>Dlw residues</th>
<th>Gdp residues</th>
<th>Ppy residues</th>
<th>Yql residues</th>
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<td>Thr26L</td>
<td>Leu2 (2), Trp3 (20)</td>
<td>Pro3 (5)</td>
<td>Pro1 (11)</td>
<td>Tyr7 (5)</td>
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<td>Thr30aL</td>
<td>Asp3 (1), Pro5 (8), Arg4 (4), Pro6 (6), Ser6 (13)</td>
<td>Pro2 (2), Ser6 (2)</td>
<td>Arg4 (5), Leu3 (9), Pro5 (2), Pro (4)</td>
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<tr>
<td>Ser30bL</td>
<td>Pro1 (3), Leu1 (9)</td>
<td>Pro1 (13), Pro2 (4)</td>
<td>Leu (13), Arg4 (9), Pro5 (1)</td>
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<tr>
<td>Tyr32L</td>
<td>Trp3 (2)</td>
<td>Pro1 (7), Pro2 (2)</td>
<td>Pro3 (3), Glu (3)</td>
<td></td>
</tr>
<tr>
<td>Trp91L</td>
<td>Leu2 (2), Trp3 (3)</td>
<td>Pro3 (3), Leu4 (1)</td>
<td>Leu (13), Arg4 (9), Pro5 (1)</td>
<td></td>
</tr>
<tr>
<td>Tyr92L</td>
<td>Asp3 (1), Leu1 (9)</td>
<td>Pro1 (11), His10 (3)</td>
<td>Tyr3 (8), Pro4 (1), Glu (3)</td>
<td></td>
</tr>
<tr>
<td>Arg50N</td>
<td>Leu2 (2), Trp3 (5)</td>
<td>Ser6 (1), His10 (3)</td>
<td>Tyr3 (8), Pro4 (1), Glu (3)</td>
<td></td>
</tr>
<tr>
<td>Ala52H</td>
<td>His10 (5)</td>
<td>His10 (1)</td>
<td>Glu (5)</td>
<td></td>
</tr>
<tr>
<td>Ser54H</td>
<td>His10 (5)</td>
<td>His10 (1)</td>
<td>Glu (5)</td>
<td></td>
</tr>
<tr>
<td>Gly56H</td>
<td>His10 (5)</td>
<td>His10 (1)</td>
<td>Glu (5)</td>
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</tr>
<tr>
<td>Thr57H</td>
<td>His10 (5)</td>
<td>His10 (1)</td>
<td>Glu (5)</td>
<td></td>
</tr>
<tr>
<td>Ala58H</td>
<td>Leu2 (3), Pro6 (6), Ile10 (6)</td>
<td>Tyr3 (2)</td>
<td>Glu (5)</td>
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<tr>
<td>Gly59H</td>
<td>Leu2 (3), Pro6 (6), Ile10 (6)</td>
<td>Tyr3 (2)</td>
<td>Glu (5)</td>
<td></td>
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<tr>
<td>Lys62H</td>
<td>Trp3 (4)</td>
<td>Trp3 (4)</td>
<td>Trp6 (3)</td>
<td></td>
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<tr>
<td>Tyr97H</td>
<td>Trp7 (1)</td>
<td>Trp6 (2)</td>
<td>Thr9 (1)</td>
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</table>

Residues corresponding to those involved in NP binding in N1G9 Fab are italicized. The interacting paratope residues common in four peptide complexes are indicated by underlined text. The number of interactions of each Ab residue is shown in parentheses.
The peptide Gdp binds in extended conformation comprising three bends that form a wave-like stretch. The residues are predominantly in β conformation and show interactions with both H and L chain CDRs (Fig. 3B, Table IV). Pro3, Pro7, and Tyr5 contribute to hydrophobic interactions (Table III). Atomic details of the polar contacts are presented in Table III. Whereas Ser5, Ser8, and His10 of Gdp interact with H chain residues, Tyr50G and Arg50G via four hydrogen bonds, the side chains of residues Tyr32L and Trp91L make polar contacts with Arg5 and Ser5, respectively (Fig. 3B, Table III). As illustrated in Table IV, 81 van der Waals contacts are formed with CDR residues. From the common interacting residues, side chain atoms of only Arg5 make polar contacts with Ser5 and His10. Trp33H and Trp91L are involved in hydrophobic contacts with Tyr7 and Pro5, respectively. As described in Table IV, the common residues play a dominant role by contacting a variety of peptide residues.

Only six residues starting from the N terminus of the Ppy peptide could be traced, as half of the peptide moved into the solvent and could not be mapped and the residues were presumably disordered. The entire peptide is seen bound around the periphery of the Ag-binding cavity by inserting a hydrophobic side chain into it. All the peptide residues, which were seen in electron density, are hydrophobic in nature; therefore, most of the binding energy comes from hydrophobic interactions. These hydrophobic interactions were observed mainly with the residues of CDRL1, CDRL3, CDRH1, and CDRH3 (Fig. 3C, Table III). Nonetheless, two peptide residues, Pro1 and Tyr3, make polar contacts with the side atoms of Ser93L, Trp91L, and Arg50G, respectively. The details of the atoms involved in the polar contacts are illustrated in Table III. Trp91L and Trp33H have hydrophobic interactions with the three initial residues and the last three residues of the Ppy peptide, respectively. Trp33H and Arg50G also contribute to most of the van der Waals contacts. Details of these interactions are presented in Table IV. The peptide Ppy makes 78 van der Waals contacts.

The Ag YqL lies in the gully at the interface of CDRL1 and CDRL3. It forms a hook-like structure by turning at residue Ala7, which anchors it into the Ag-binding cavity. Residue-wise interactions of YqL are depicted in Fig. 3D and Table IV. As shown in Table III, the main chain oxygen atoms of peptide residues Gin3, Arg4, Pro5, Glu6, and Thr6 are hydrogen bonded to the side chains of Ser93L, Trp91L, Arg50G, and Tyr95L respectively. The oxygen atoms of the peptide residue Leu7 make two polar contacts with the amide nitrogen of Tyr92L and Ser93L. Besides this, Leu3 and Arg4 form two more polar contacts with the hydroxyl of Ser93L. In this complex, unlike other three peptides, the majority of interactions are with the L chain and are electrostatic in nature. From the common interacting residues, Trp91L makes hydrophobic interactions with peptide residues. Trp33H is involved only in van der Waals interactions. Besides making van der Waals contacts, Arg50G also contributes to binding energy by making a hydrogen bond with the carboxyl oxygen atom of Glu6 (Table III). Residuewise van der Waals interactions are shown in Table IV.

Analysis of the structures of BBE6.12H3 in complex with the four synthetic peptide Ags revealed that the naive Ag-combining site could tolerate significant variability in the epitope (Fig. 2B). The residues of peptides, which bind at common paratope sites, were LWT (BBE6.12H3-Dlw), PSYIS (BBE6.12H3-Gdp), YPAW (BBE6.12H3-Pyy), and PNAET (BBE6.12H3-YqL), and these residues have been depicted as dots in Supplemental Fig. 2. Three common interacting residues, Trp33H (CDRH1), Arg50G (CDRH2), and Trp33H (CDRL3), form a minimal conserved motif essential for recognition of multiple Ags (Fig. 2A). Interestingly, the same residues were also shown to interact with the hapten (NP) (32). Therefore, it can be said that germline Abs have a predesigned binding site containing both conserved and nonconserved interacting amino acids. Despite the extraordinary differences between cognate hapten Ag and the selected peptides, the Ag-binding pocket of the germline Ab could accommodate diverse peptide residues and also formed new hydrogen bonds (Table III). A schematic of the proposed model for the generation of primary Ab diversity based on the present data and previous observations (1, 15) is shown in Fig. 4.

**Discussion**

Structural analyses involving a germline Ab that has not seen an Ag a priori demonstrated distinct conformational rearrangements to recognize several structurally independent Ags. Six structures of the germline mAb BBE6.12H3, in different chemical environments, quantitatively define the conformational repertoire of the CDRs, providing a rich data set for examining paratope topologies, although thermodynamic and computational studies have provided interesting clues regarding the generation of diversity in primary Ab response (5, 19, 33, 34). Similarly, crystallographic and kinetic analyses of mature Abs SPE7 and NQ22/61.1, respectively, have also suggested conformational isomerism (7, 19). Our data consisting of six BBE6.12H3 Fab structures—two Ag free and four Ag bound—provided a rich source of information reflecting correlation of paratope conformational flexibility and polyreactivity. In the case of germline mAbs 28B4, 48C7, and AZ28, conformational rearrangement of CDRH3 has been shown to facilitate hapten binding. However, none of these studies have associated conformational flexibility of CDRs with degenerate specificity (14, 35–37).

The observed conformational variations in germline Ab-combining sites are consistent with the predictions based on thermodynamic studies and molecular dynamics simulations (5, 33). On the basis of energy considerations, the conformations adopted by CDRH3 cannot represent a continuum of conformational space but, rather, correspond to a finite number of discrete states. The six different observed conformations of CDRH3 and four slightly different conformations of CDRH2 suggest the possibility of correlation between the flexible nature of these loops and the paratope recognition potential.

A correlation of the observed variability in CDR conformations with the polyspecificity of germline Ab has attractive physiological implications. The pluripotency at a mature level can lead to serious implications. The pluripotency at a mature level can lead to serious

![FIGURE 4](http://www.jimmunol.org/) Multiple mechanisms for enhancing primary Ab repertoire. Germline Ab repertoire is generated at three levels in an amplified manner. In this schematic, the inverted pyramid represents the hierarchical contribution to the diversity of these stages. At the first level, the BCR repertoire is generated during the development of B cells by somatic recombination of V, D, and J gene segments. The second level operates at the tertiary structure wherein each rearranged receptor exhibits diverse conformational isomers. At the final level, each conformer can recognize independent epitopes through alternative juxtapositions. The different colors represent distinct Ab sequences.
immunological consequences. However, the pluripotency of germline Ab can contribute substantially to the enhancement of the primary immune repertoire. All six conformational states of BBE6.12H3 represent an ensemble reflecting the conformational diversity extant in the primary humoral response. Thus, the Ab–Ag complex structures provided snapshots of how the immune system could generate multiple specificities that allow for recognition of diverse epitopes. Although these insights have arisen from studies involving a germline Ab that binds to diverse 12-mer peptides, it would be interesting to explore protein Ags as well.

The germline mAb BBE6.12H3 undergoes conformational changes in the CDRs while binding to independent Ags. In contrast, the germline mAb 36–65 was seen to bind different Ags in a similar conformation, essentially indicating that a rigid receptor binding site can have different interactions with structurally distinct Ag surfaces, without any conformational changes in the receptor (15). In the unbound state, however, both the Abs display conformational flexibility in CDRIH3. Two different germline Abs exhibit distinct strategies to achieve polyspecificity. Collective analysis suggests that these two mechanisms are not mutually exclusive. In fact, their coexistence is essential by virtue of the requirement of an infinite receptor repertoire.

Previously, genetic recombination of V, D, and J gene segments—alone or in conjunction with accessory processes, such as nontemplated nucleotide addition and somatic hypermutation (1, 38–40)—had been accepted as the core mechanism for repertoire diversity. However, the plastid-encoded mAb 36–65 (15) and the BM14 (1, 38–40)—had been accepted as the core mechanism for recombination activity analyzed by immunochemical diversity. These models have been shown to require of an infinite receptor repertoire.


