Molecular Evolution of Catalytic Antibodies in Autoimmune Mice

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Catalytic Abs (catAbs) preferentially evolved in autoimmune MRL/MPJ-lpr/lpr (MRL/lpr) mice upon immunization with the phosphonate transition-state analogue (TSA), but this did not happen in normal BALB/c mice. The majority of the catAbs from MRL/lpr mice were from several independent clones of the same family. Most of them had a lysine at position 95 in the heavy chain (H95), which is at the junctional region. This residue, which interacts with the phosphonate moiety of the TSA and presumably is involved in the catalytic activity, was not changed even after extensive evolution following multiple mutations. By contrast, the majority that arose from BALB/c mice were the non-catAbs, which were quite different in the sequence from the catAbs from MRL/lpr mice, but they were clonally related to one another, so most of them were originated from a single clone. In the MRL/lpr mice, the catalytic subsets that existed in the initial repertoire were effectively captured by the phosphonyl oxygens in the TSA by interacting with the lysine at H95. In the BALB/c mice, however, another noncatalytic subset with only the binding capability directed to a moiety other than the phosphonate moiety was alternatively evolved, because of the lowest abundance or elimination of the catalytic subsets. The Journal of Immunology, 2001, 167: 5775–5785.

Directed in vivo selection based on the complementarity to the transition-state analogue (TSA) for a given reaction (1, 2) has made it possible to efficiently produce catalytic Abs (catAbs). Phosphonate derivatives were the first and most successful haptens to elicit tailor-made esterolytic catAbs. They show good approximation of the transition state of substrates for ester hydrolysis because of their tetrahedral geometry and anionic character (3, 4). Since then, a variety of catAbs has been generated based on haptenic TSAs (5).

CatAbs are potentially selected from an enormously diverse repertoire that continuously evolves and comprises the limitless potential of a given individual to produce different Abs following antigenic stimuli (6, 7). Nevertheless, only a small portion of repertoire subsets that bind haptenic TSAs expresses catalytic activity. One of the reasons of lower recovery could be that the virtual limit of shape space, whose diversity is represented by the complementarity-determining regions (CDRs), was within the existing repertoire of $5 \times 10^{10}$ mature B cells in the peripheral lymph nodes. Expanding the shape space beyond the existing repertoire, in which the possible combinations of Ab repertoire are in the range of $10^{11}$–$10^{12}$ (8), could allow us to recruit more efficiently potential candidates for catalysts with desired rate enhancements and/or unique reactions. One of the most promising ways of expanding the shape space could be to select candidate Abs by a phage-displayed system, in which heavy and light chain gene shuffling with subsequent randomized mutagenesis is possible. It is underway and has reached some success, although it was possible only within the existing repertoire (9, 10). Finding a way to expand the repertoire beyond the existing repertoire is of critical importance.

Autoimmunity caused by the breakage of self-tolerance, which resulted in a high incidence of abnormal autoreactive Abs, gives us a chance to investigate an unusual Ab repertoire (11, 12). Such a trial has been conducted by testing the ability of several autoimmune-prone mouse strains to elicit esterolytic catalysts (13). The occurrence of catAbs is dramatically higher in autoimmune mouse strains, such as MRL/MPJ-lpr/lpr (MRL/lpr) and SJL, than it is in the conventionally used normal mouse strains (13). This study was motivated by previous findings of naturally occurring catAbs in patients with autoimmune diseases, such as asthma (14) or systemic lupus erythematosus (SLE) (15). Using a different phosphonate hapten, we have confirmed that catAbs could be recovered at higher incidence from the MRL/lpr mice than from the conventionally used normal mouse strains (16, 17).

Taken together, these findings strongly suggest that catAbs exist at a high incidence in the autoimmune repertoire, and that these catAbs are not included in the repertoire of conventionally used normal strains. In our effort to explore the immunological evolution of Ab catalysts in an expanded shape space, we have tried to elucidate the evolution of catAbs in the autoimmune repertoire based on the sequence analysis. In the present work, we have shown that catAbs were uniquely obtained from a subset of the repertoire of an autoimmune mouse strain, but not from the repertoire of a normal mouse strain.

Materials and Methods

Preparations of chemicals and cloning of mAbs

TSA1 (I, $R = \text{OH}$) and the haptenic TSA1, or a protein conjugate form of TSA1 (I, $R = \text{NH-keyhole limpet hemocyanin (NH-KLH)}$ or NH-BSA),
TSA2 (2), dapsylated substrate (4), and its cleaved product (5) were synthesized, as previously described (18) (Fig. 1). The compound 3, resembling the structure of the cleaved product 5, or product analogue, was also synthesized. The sequences of the Ig genes are double underlined. The enzymes used for cloning are shown in parentheses, and their sites are directly sequenced. DNA sequences were determined by a DNA sequencer.

Measurement of catalytic activities and kinetic parameters
We classified all the purified mAbs as catalytic or noncatalytic, as described previously (16–18). Reaction rates were determined using HPLC by measuring the cleaved product 4 (18). The kinetic parameters, $k_{cat}$ and $K_{m}$, were determined by fitting the data to a Michaelis-Menten equation with the program KALEIDAGRAPH (Synergy Software, Reading, PA), and $K_{m}$ was determined by an initial rate analysis and by extrapolating to a zero buffer concentration (16–18).

Measurement of binding parameters
Dissociation constants were measured for two inhibitors (TSA1 and TSA2) that were used for an ELISA assay and for the compound 3, which resembles the structure of the cleaved product 4, as previously described (16–18).

Results
To define the 3′ primer sequences of V regions in conjunction with the C regions, we determined the isotypes of the H and L chains. In the vast majority of the catAbs derived from MRL/lpr mice, the isotypes were IgG2a, and the remaining were IgG1 (Table I). In the group of IgG2a, the L chains were all $\kappa$. In the non-catAbs from MRL/lpr mice, the isotypes were either IgG1 or IgG2a for the H chain, and $\kappa$ or $\lambda$ for the L chain (Table I). In BALB/c, the isotypes of the H chain were all IgG1, the L chain of the catAbs was $\kappa$, and the L chain of the non-catAbs was either $\lambda$ or $\kappa$ (Table I).

Utilization of the V gene segments of the H and L chain genes in the catAb and non-catAb repertoire
Based on the isotyping as described above, we used the 5′ and 3′ heavy chain primers in conjunction with a Cγ2 or a Cγ1 sequence, and the 5′ and 3′ L chain primers in conjunction with a Cκ or a C$\lambda$ sequence for PCR amplification of cDNAs. With the sequence information, we defined each Ig gene segment for the MRL/lpr mice (Fig. 2) and the BALB/c mice (Fig. 3). The results are also summarized in Table I.

In the sequence study, we found two distinct features in the utilization of the DNA segments, specifically both in the group of the catAbs derived from MRL/lpr mice, and of the non-catAbs derived from BALB/c mice. In the group of the catAbs from MRL/lpr mice, there was a major subgroup, in which the utilization of the Ig gene segments was homologously conserved (subgroup M). In this subgroup, which included MS6-164, MS6-191, MS6-192, MS6-126, MS5-255, MS5-346, MS5-393, MS5-233, MS5-200, MS5-9, and MS6-12, the $V_{H}$ genes belonged to V186.2 of the J558 family with 93% homology, and all the $V_{L}$ belonged to Vx24 with

\[
\begin{align*}
\text{acyl serine} & \quad \text{acyl serine} \\
\text{acyl serine} & \quad \text{acyl serine} \\
\text{acyl serine} & \quad \text{acyl serine} \\
\text{acyl serine} & \quad \text{acyl serine} \\
\text{acyl serine} & \quad \text{acyl serine} \\
\text{acyl serine} & \quad \text{acyl serine} \\
\end{align*}
\]

\[\text{FIGURE 1.} \quad \text{Chemical structures of TSAs, substrate, and cleaved products. 1, A hapten used for immunization (R = NH-KLH), ELISA screening (R = NH-BSA), and measuring the affinities of the Abs (R = OH or TSA1); 2 (TSA2), used for ELISA screening and measuring the affinities of the Abs; 3, the analogue of the cleaved product 5 used for measuring the affinities of the Abs; 4, the substrate; 5, used for the assay of catalytic activities; and 6, another cleaved product.}\]
98% identity. In this group, most of the D segments were DSP2.3-4, and the rest were DQ52 (MS6-12) and DFL16.1 (MS5-9), and all the JH were JH3, although the JL were Jκ4 or Jκ5. Two of the catAbs, MS6-51 and MS5-298, were different from the subgroup M in the utilization of the gene segments. The VH gene of MS6-51 belonged to MOPC104E of the J558 family, and that of MS5-298 belonged to VHOx2 of the Q52 family, both of which had Vκ9 as the VL. In these cases, the D segments were DQ52 and DFL16.1, the JL was Jκ4, and the JL was either Jκ4 or Jκ5.

In the non-catAbs derived from MRL/lpr mice, four V genes, including V186.2, MOPC104E, VHOx2, and vhsm7-13 of the SM7 family, were utilized for the VH, and Vκ1, Vκ9, and Vκ24 for the VL. In this group, the D segment was DFL16.1, DSP2.7, or DSP2.9 with JL3 for the JL and Jκ4 or Jκ5 for the JL.

In both the catAbs and non-catAbs that were derived from BALB/c mice, the utilization of the VH gene segments was completely different from the utilization of the Abs derived from MRL/lpr mice. In more than half of the non-catAbs from BALB/c, both the heavy and light chain gene segments were completely identical to one another. In this subgroup (subgroup B), which included BS6-29, BS6-16, BS6-17, BS6-35, BS6-40, BS6-37, BS6-14, and BS5-18, the VH genes belonged to M315 of the 36-60 family, the VL were all Vκ2, the D segments were all DQ52, the JL were all JL3, and the JL were all Jκ2.

**Somatic hypermutations and recombinations with N addition in the catAb repertoire**

Detailed analysis of recombination and mutation events by comparing the genomic consensus sequences of each segment can reveal the clonal relations of the Abs and the evolving process of the catAbs in the original Ab repertoire. Accordingly, we compared the sequences of the heavy chain V regions (H1 to H94) with the reported genomic sequences. The D and JH and the sequences of the N addition showed that the subgroup M was divided into three subsets (MS5-255 and MS5-346) and the third subset (MS5-393).

---

**Table 1. Utilization of gene segments of Abs derived from MRL/lpr and BALB/c mice**

<table>
<thead>
<tr>
<th>Ab</th>
<th>Injection</th>
<th>Isotype</th>
<th>VH</th>
<th>Family</th>
<th>D’</th>
<th>RF^d</th>
<th>JL</th>
<th>VL</th>
<th>JL</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS6-164*</td>
<td>3</td>
<td>IgG2a</td>
<td>V186.2</td>
<td>(J558)</td>
<td>DSP2.3-4</td>
<td>3</td>
<td>JH3</td>
<td>Vκ24</td>
<td>Jκ5</td>
</tr>
<tr>
<td>MS6-191*</td>
<td>3</td>
<td>IgG2a</td>
<td>V186.2</td>
<td>(J558)</td>
<td>DSP2.3-4</td>
<td>3</td>
<td>JH3</td>
<td>Vκ24</td>
<td>Jκ5</td>
</tr>
<tr>
<td>MS6-192*</td>
<td>3</td>
<td>IgG2a</td>
<td>V186.2</td>
<td>(J558)</td>
<td>DSP2.3-4</td>
<td>3</td>
<td>JH3</td>
<td>Vκ24</td>
<td>Jκ5</td>
</tr>
<tr>
<td>MS6-126*</td>
<td>3</td>
<td>IgG2a</td>
<td>V186.2</td>
<td>(J558)</td>
<td>DSP2.3-4</td>
<td>3</td>
<td>JH3</td>
<td>Vκ24</td>
<td>Jκ5</td>
</tr>
<tr>
<td>MS5-255*</td>
<td>2</td>
<td>IgG2a</td>
<td>V186.2</td>
<td>(J558)</td>
<td>DSP2.2-4</td>
<td>3</td>
<td>JH3</td>
<td>Vκ24</td>
<td>Jκ4</td>
</tr>
<tr>
<td>MS5-346*</td>
<td>2</td>
<td>IgG2a</td>
<td>V186.2</td>
<td>(J558)</td>
<td>DSP2.2-4</td>
<td>3</td>
<td>JH3</td>
<td>Vκ24</td>
<td>Jκ4</td>
</tr>
<tr>
<td>MS5-393*</td>
<td>2</td>
<td>IgG2a</td>
<td>V186.2</td>
<td>(J558)</td>
<td>DSP2.2-4</td>
<td>3</td>
<td>JH3</td>
<td>Vκ24</td>
<td>Jκ4</td>
</tr>
<tr>
<td>MS5-233*</td>
<td>2</td>
<td>IgG1</td>
<td>V186.2</td>
<td>(J558)</td>
<td>DSP2.2-4</td>
<td>3</td>
<td>JH3</td>
<td>Vκ24</td>
<td>Jκ4</td>
</tr>
<tr>
<td>MS5-200*</td>
<td>2</td>
<td>IgG2a</td>
<td>V186.2</td>
<td>(J558)</td>
<td>DSP2.2-4</td>
<td>3</td>
<td>JH3</td>
<td>Vκ24</td>
<td>Jκ4</td>
</tr>
<tr>
<td>MS5-4*</td>
<td>2</td>
<td>IgG1</td>
<td>V186.2</td>
<td>(J558)</td>
<td>DFL16.1</td>
<td>3</td>
<td>JH3</td>
<td>Vκ24</td>
<td>Jκ5</td>
</tr>
<tr>
<td>MS6-12*</td>
<td>3</td>
<td>IgG2a</td>
<td>V186.2</td>
<td>(J558)</td>
<td>DFL16.1</td>
<td>3</td>
<td>JH3</td>
<td>Vκ24</td>
<td>Jκ5</td>
</tr>
<tr>
<td>MS5-389</td>
<td>2</td>
<td>IgG2a</td>
<td>V186.2</td>
<td>(J558)</td>
<td>DFL16.1</td>
<td>3</td>
<td>JH3</td>
<td>Vκ24</td>
<td>Jκ5</td>
</tr>
<tr>
<td>MS5-290</td>
<td>2</td>
<td>IgG2a</td>
<td>V186.2</td>
<td>(J558)</td>
<td>DFL16.1</td>
<td>3</td>
<td>JH3</td>
<td>Vκ9</td>
<td>Jκ4</td>
</tr>
<tr>
<td>MS6-51*</td>
<td>3</td>
<td>IgG1</td>
<td>MOPC104E</td>
<td>(J558)</td>
<td>DSP2.7</td>
<td>3</td>
<td>JH4</td>
<td>Vκ1</td>
<td>Jκ4</td>
</tr>
<tr>
<td>MS6-153</td>
<td>3</td>
<td>IgG2a</td>
<td>MOPC104E</td>
<td>(J558)</td>
<td>DSP2.7</td>
<td>3</td>
<td>JH4</td>
<td>Vκ1</td>
<td>Jκ4</td>
</tr>
<tr>
<td>MS6-298*</td>
<td>2</td>
<td>IgG1</td>
<td>VHOx2</td>
<td>(Q52)</td>
<td>DFL16.1</td>
<td>3</td>
<td>JH3</td>
<td>Vκ9</td>
<td>Jκ4</td>
</tr>
<tr>
<td>MS6-171</td>
<td>3</td>
<td>IgG1</td>
<td>vhsm7-13</td>
<td>(SM7)</td>
<td>DSP2.9</td>
<td>2</td>
<td>JH3</td>
<td>Vκ1</td>
<td>Jκ1</td>
</tr>
</tbody>
</table>

| BS6-29   | 3         | IgG1    | M315   | (36-60) | DFL16.1 | 1    | JH3    | Vκ2  | Jκ2  |
| BS6-16   | 3         | IgG1    | M315   | (36-60) | DFL16.1 | 1    | JH3    | Vκ2  | Jκ2  |
| BS6-17   | 3         | IgG1    | M315   | (36-60) | DFL16.1 | 1    | JH3    | Vκ2  | Jκ2  |
| BS6-35   | 3         | IgG1    | M315   | (36-60) | DFL16.1 | 1    | JH3    | Vκ2  | Jκ2  |
| BS6-40   | 3         | IgG1    | M315   | (36-60) | DFL16.1 | 1    | JH3    | Vκ2  | Jκ2  |
| BS6-37   | 3         | IgG1    | M315   | (36-60) | DFL16.1 | 1    | JH3    | Vκ2  | Jκ2  |
| BS6-14   | 3         | IgG1    | M315   | (36-60) | DFL16.1 | 1    | JH3    | Vκ2  | Jκ2  |
| BS5-18   | 2         | IgG1    | M315   | (36-60) | DFL16.1 | 1    | JH3    | Vκ2  | Jκ2  |
| BS6-4*   | 3         | IgG1    | VH6    | (3606)  | DSP2.3-4 | 3    | JH3    | Vκ9  | Jκ2  |

| BS6-12   | 3         | IgG1    | VH6    | (3606)  | DFL16.1 | 1    | JH3    | Vκ9  | Jκ1  |
| BS5-17   | 2         | IgG1    | VH6    | (3606)  | DFL16.1 | 1    | JH3    | Vκ9  | Jκ2  |
| BS8-8*   | 2         | IgG1    | —      | (J558)  | DFL16.1 | 2    | JH2    | Vκ21 | Jκ2  |
| BS6-38   | 3         | IgG1    | VH4    | (X-24)  | DFL16.1 | 3    | JH4    | Vκ24 | Jκ5  |

a,*, catAbs
b, Number of injections with haptenic TSAI.
c, Classification of the D and JL, JL regions were determined according to Kabat et al. (20).
d, RF, reading frame of the D region according to Raapoh et al. (21).
e, Not determined.
and MSS-233) had completely identical sequences of the N addition at the VH-D, and the same JH segments. In addition, the second subset had similar D segments, and the third subset had the same D segments.

In the light chain genes, no germline sequences completely matched with the consensus sequence of the subgroup M. Subsequently, we compared the sequences with H926024A, the genomic sequence that is the most homologous to the consensus sequence of the subgroup M (Fig. 2B). The sequences of the subgroup M all had Pro at L9, Val at L11, Pro at L15, Glu at L17, Val at L19, Asn at L28, Arg at L39, Arg at L50, Met at L51, and His at L91. We tentatively considered these conserved residues to be nonmutated (polymorphism). They are probably new germline residues that have not yet been reported. Leu at L89 in the CDR3 is one of consensus residues, but it seems to be a mutation, rather than a new germline residue, because its conservation was incomplete in the subgroup M. Other residues scattered in the sequences were counted as mutational events.

The overall results showed that the catAbs in the subgroup M utilized the H and L chain V gene segments of the same family. The subgroup M could be further separated into at least three subsets, each of which was distinguished by the different utilizations of the D and JH segments and by the sequence variability of the N addition in the H-CDR3 region.

### Somatic hypermutations and recombinations with N addition in the non-catAb repertoire

In the heavy chain genes, none of the germline sequences completely matched the consensus sequence in the subgroup B (BS6-29, BS6-16, BS6-17, BS6-35, BS6-40, BS6-37, BS6-14, and BS6-18). Subsequently, we compared the sequences that were most homologous with the sequences of subgroup B, 8-1-12-B (23), which is the M315-related gene derived from a BALB/c Ig null immature B cell line (Fig. 3A). Of all the residues that were different from the 8-1-12-B sequence, only one residue (Arg at H52) was shared by all the sequences of the subgroup B. We tentatively excluded these residues to be a mutational event, but consider a polymorphism (new germline sequences) that has not yet been reported. The other consensus residues (Phe at H53, Thr at H56, Ser at H79, Leu at H81) found in the subgroup B could be derived from mutational events, because they were not completely conserved among Abs in the subgroup B. Phe at H53, Thr at H56, and Ser at H79 could be derived from single point mutations, whereas Leu at H81 could be derived from double mutational events. Ala (GCT), Gly (GGT), and Asn (AAT) at H32, which were observed in several Abs, could be derived from Asp (GAT) at H32 in the germline sequence, if one point mutation occurred. Ser (AGT) at H32 of BS6-14 might be due to a rare double-mutational event.

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**FIGURE 2.** Comparison of amino acid sequences of a H chain V region and a L chain V region of the catAbs and non-catAbs derived from MRL/lpr mice. A. VHGN2-M is a VH germinal sequence of MRL/lpr mice (22) belonging to the V186.2 gene of the J558 family. MS con. denotes the MRL/lpr H chain V consensus sequence commonly conserved in the group of Abs (MS6-164 to MS5-290). B, k24A is a VH germinal sequence of BALB/c mice (GenBank database, accession AAA39052) belonging to the k24 family. MS con. denotes the MRL/lpr L chain V consensus sequence commonly conserved in the group of Abs from MS6-164 to MS5-290. Asterisks denote a catAb. The numbering of amino acid residues and the alignments of the D, JH, and JL segments follow the criteria of Kabat et al. (20).
Even after neglecting these consensus residues, the same mutations were found in the same positions of the CDRs and the frames in a subset of the subgroup B of the non-catAbs (BS6-29, BS6-16, BS6-17, and BS6-35), and were also found in another subset in the subgroup B (BS6-37 and BS6-40). For example, changes from Asp to Gly at H32 in the CDR1, from Asn to Asp at H43 in the frame 2, and from Tyr to Phe at H91 in the frame 3 were commonly found in BS6-16, BS6-17, and BS6-35. In BS6-40 and BS6-37, a change from Thr to Pro at H73 in the frame 3 was commonly observed.

As shown in Fig. 3A, the H-CDR3 regions, which were characteristic in length, very short, with only three amino acid residues, were almost completely identical among the subgroup B, with the exception of BS5-18, which had no N addition events found at either the VH-D junction or the D-J H junction.

In the light chains, we compared the sequences of the V regions with the genomic sequence of the /H9261-L chain (V/H92612) (Fig. 3B). We found two consensus residues of Ser at H32 and Ile at L85. We defined these changes as point mutations, as they were not completely conserved in the subgroup B. Three differences in the codon usage for the same amino acid, which were found at L32, L51, and L90, were derived from a mutational event. The codon for Ser at L32 was TCA in BS6-17, and TCT in the consensus sequence. The codon for Thr at L51 was ACT in BS6-17 and ACC in the consensus sequence. The codon for Leu at L90 was CTG in BS6-16 and CTA in the consensus sequence.

The overall results showed that, in most of the non-catAbs recovered from BALB/c mice, utilization of the heavy and light chain V gene segments was completely different from that in most of the catAbs recovered from MRL/lpr mice. In the subgroup B, the recovered non-catAbs utilized the same H and L chain V gene segments. In the subgroup B, there were at least two subsets with sequence similarity. These similarities indicated that the non-catAbs in these subsets were clonally related to one another. This was not found to be the case with the catAbs derived from MRL/lpr mice. This means that these Abs were derived from one clone.

**Binding and catalytic characters of the catAb and non-catAb repertoire**

The hapten used in this experiment is composed of three chemical moieties: a phosphonate, a benzyl group, and a phenetyl group (Fig. 1). TSA1, which was used for measuring affinity toward the hapten, has all components. Of these components, the phosphonate moiety was introduced to elicit a catalytic pocket for ester hydrolysis. A phenetyl group was introduced to create a binding pocket for the substrate by a hydrophobic interaction. TSA2, which does not have this phenetyl group, was used for the binding assay to estimate the contribution of the affinity between the phenyl group and the surrounding residues in the hydrophobic binding pocket. The product analogue (3), which has a side chain benzyl group, was also used for the binding assay to estimate how a side chain benzyl group contributes to affinity for the surrounding residues in the binding pocket.

The catAbs in the subgroup M showed a strong affinity toward TSA1 ($K_d = 10^{-10.7}$ M) (Table II). The affinity of these
catAbs for TSA2 was lower, but proportional to their affinity toward TSA1 (\(K_d = 10^{-9}–10^{-2} \text{ M}\)) (Table II). All of the catAbs in the subgroup M showed affinities for product analogue 3 (3), but the affinities were much lower than those of the other Abs (\(K_a > 2 \text{ mM}\)) (Table II). In the subgroup M, the catalytic activity expressed as a rate enhancement, \(k_{cat}/k_{uncat}\), were within the range of \(10^4–10^6\) (Table II). In general, the higher the catalytic activity, the stronger the affinity toward TSA1 and TSA2.

The non-catAbs in the subgroup B showed even a stronger affinity toward TSA1 than the catAbs in the subgroup M (\(K_d = 10^{-11}–10^{-9} \text{ M}\)) (Table II). The affinity of these non-catAbs for TSA2 was lower than, but proportional to their affinity for TSA1 (\(K_a = 10^{-5}–10^{-3} \text{ M}\)) (Table II). So, these values were again stronger than the values of the catAbs in the subgroup M. The affinities of all the subgroup B for product analogue 3 (3) (10^{-5}–10^{-3} \text{ M}) were stronger than those of the catAbs in the subgroup M.

In contrast to the MRL/lpr mice, the BALB/c mice had only two catAbs, which showed the catalytic activities of 100–200 \(k_{cat}/k_{uncat}\) and no sequence similarities to those of the catAbs from MRL/lpr mice or the non-catAbs from BALB/c mice.

In summary, the cat Abs in the subgroup M bound both TSA1 and TSA2, whereas they failed to bind the product (3). However, it was not the case with the non-catAbs in the subgroup B. These Abs showed positive binding toward all three compounds, TSA1, TSA2, and the product (3), with different levels of affinity.

**Key residues in the catAb repertoire**

In the amino acid sequences, we surveyed the consensus residues, especially in the CDRs commonly found in the catAbs, but not found in the non-catAbs. Such residues should play an important role in catalysis. If the catAbs stabilize the tetrahedral conformation of the transition state of the substrate, such residues could be basic and/or hydrophilic amino acids. In the H chain V regions, a Lys might be involved in a hydrogen bonding with a phosphonate moiety of the hapten was almost completely conserved at H95 in the H-CDR3 of the catAbs (Fig. 2A). But it was not conserved in the non-catAbs, such as MS5-389 and MS5-290, even though they were derived from MRL/lpr mice and utilized the same V and D segments as the catAb, such as MS5-9 in the subgroup M. In the subgroup M, there was only one exception (MS5-200), in which a hydrophilic Ser residue instead of a Lys residue was found at H95 (Fig. 2A). Of the subgroup M, only two catAbs, MS6-51 and MS5-298, did not utilize Lys at H95. MS6-51 had a Ser residue at this position, and MS5-298 had another basic amino acid, a His. Asp at H100 and Gly at H100a were also conserved among the catAbs in the subgroup M that had relatively higher catalytic activities.

In the survey of the L chain CDRs, four candidates of basic amino acid residues were identified that could form a hydrogen bond with a phosphonate moiety of the hapten (Fig. 2B). They were Arg at L24 and His at L27d in the CDR1, Arg at L50 in the CDR2, and His at L91 in the CDR3. They were completely conserved among the catAbs of the subgroup M and two other catAbs from MRL/lpr. All these residues were conserved in only one of the non-catAbs (MS5-389). Of the subgroup M, Arg at L24 was conserved among the non-catAbs from MRL/lpr. The other three residues, His at L27d in the CDR1, Arg at L50 in the CDR2, and His at L91 in the CDR3, are candidates that might be involved in a hydrogen bond with a phosphonate moiety of the hapten.

**Key residues in the non-catAb repertoire**

Neither basic nor the hydrophilic amino acids were placed at H95 in the Abs derived from BALB/c mice, with the exception of Tyr at H95 in the non-catAbs, such as BS6-12 and BS6-38 (Fig. 3A). The striking feature found in the non-catAbs from BALB/c mice was that all the non-catAbs in the subgroup B had a common short hydrophobic motif in the H-CDR3, Leu-Gly-Pro. This was quite different from the case of the H-CDR3 of the catAbs in the subgroup M, which had relatively long and variable lengths with an abundance of hydrophilic amino acid residues. The reading frame (RF) of the D_{\alpha} of the catAbs of the subgroup M utilized a very common RF3, whereas that of the D_{\alpha}, Leu-Gly-Pro, found in the non-catAbs of the subgroup B, was a rare RF1.

**Discussion**

The preceding results show that, even when identical haptens, immunization regimes, and screening protocols are used, the Ab repertoire selected from autoimmune disease-prone MRL/lpr mice was quite different from that selected from the normal counterpart, BALB/c mice. In the Abs selected from MRL/lpr mice, catAbs were selectively recovered, whereas in the Abs selected from BALB/c mice, the recovery of catAbs was much lower than the MRL/lpr, and non-catAbs were predominantly recovered. Based on the recovery of the catAbs, we roughly estimated that \sim3% of the hybridoma supernatants from the MRL/lpr mice could be catalytic, whereas it was one-fifth of the recovery from the MRL/lpr mice in the supernatants from the BALB/c mice.

The difference in the immune response might be related to the difference in such type of clonal selections. A phylogenetic diagram can demonstrate the clonal relations within the two subgroups, subgroup M and subgroup B, respectively.

Before doing so, the detailed nucleotide sequence alignments of the H-CDR region of the catAbs of the subgroup M were required.
to know the utilization of the N additions, D regions, and J regions (Fig. 4). Based on these sequences, we developed a phylogenetic diagram for the clonal relations found in the catAbs in the subgroup M (Fig. 5). This phylogenetic diagram suggests that the obtained catAbs were derived from separate clones (oligoclonal), as they utilized different D segments and/or different N-additional sequences, even though they utilized the same heavy chain (V186.2 of J558) and light chain gene (V_H24) families. This diagram shows that there could be at least five independent clonal lines, each of which utilized the same D segment and the N-additional sequences. This is what we thought at first. Even though, however, the junctional sequences at the N addition corresponding

**FIGURE 4.** Comparison of nucleotide sequences of H-CDR3 regions covering the V-D-J junctional sequences of the catAbs and non-catAbs derived from MRL/lpr mice. The alignments of the V_H, D, and J_H segments follow the criteria of Kabat et al. (20). RSS, recombination signal sequence. Asterisks denote a catAb.

**FIGURE 5.** Clonal relations of the catAbs of the subgroup M from MRL/lpr mice. Squares and circles on the offshoot arrows show amino acid substitutions due to mutations with their positions in the H and L chain genes. In the number X/Y in the Ab code, X represents the affinity ($K_d$, nM) to 1 (TSA1, R = OH), and Y represents the rate enhancement in $k_{cat}/k_{uncat}$. 

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to the sequences at H95 were completely conserved with respect to a Lys residue. The subsequent somatic hypermutations, which seem to be directed for the affinity maturation toward the haptenic TSA, can be seen in each offshoot of the phylogenetic tree. Even after the somatic hypermutations, which are given in each offshoot of the phylogenetic tree, the lysine at H95 was completely conserved among various clones.

In contrast, most of the non-catAbs in the subgroup B seemed to come out from a single clone that could be a germline and utilized the M315 (8-1-12-B) for the heavy chain gene and V32 for the light chain gene (Fig. 6). This means that the clonally related Abs, which originated from a single clone, were preferentially adapted to dominate the population upon immunization of the hapten. This speculation is supported by the finding that the recovered Abs were derived from a repertoire in the different stages of the normal process of the affinity maturation. The greater the affinity toward the haptenic TSA, the greater was the number of mutations that accumulated in the sequences.

The difference in the Ab evolution observed between these mouse strains is also reflected in their three-dimensional structures, both with/without TSA haptons. There are several precedent literatures of the three-dimensional structure of the catAbs useful for better understanding the interacting residues for catalysis. For example, in the structure of the CNJ206 complexed with the TSA ligand (25), the ligand is oriented with a p-nitrophenyl group at the bottom of the apolar pocket, where hydrophobic and van der Waals interactions predominate, and its phosphonate group is located at the mouth of the cavity with specific hydrogen bonds (26). In the structure of 17E8, an aryl leaving group buried deeply at the bottom of an apolar binding pocket and the hydrogen bonds from the backbone NH and the side chains of two cationic residues provided complementary electrostatic/hydrogen-bonding interactions with the phosphonyl oxygens (27). In the case of 48G7, the apolar binding pocket for the aryl moiety of the hapten was buried, and the phosphonate group was held in place near the mouth of the pocket through specific electrostatic and hydrogen-bonding interactions with the backbone or the side chain of the residues (28). In this case, a second pocket for the alkyl side chain of the hapten was also present. In the case of 43C9, the crystal structure obtained after three-dimensional modeling revealed an unexpected extended β-sheet that created a deep Ag binding site with a hydrophobic pocket that enclosed the p-nitrophenyl group (29, 30). It differed from structures in other reports in that it has amidase activity with two-step reactions, formation of a covalent acyl-Ab intermediate, followed by decylation.

In essence, the overall themes commonly seen in these cases are: 1) the aryl moiety of the hapten is deeply buried in the pocket through hydrophobic interactions, and 2) the phosphonate group is held in place near the mouth of the pocket through specific electrostatic and hydrogen-bonding interactions. In the residues, which interact with the phosphonate oxygens, the basic amino acids, such as a His (H35) for CNJ206, 17E8, 48G7, and 43C9, and an Arg (L96) for 17E8, 48G7, and 43C9, were commonly seen. In fact, when the former three Abs were overlaid, the phosphonate groups occupied similar locations (31). The most important variations for catalysis are the residues that form stabilizing contacts with the phosphonyl oxygens of the TSA, because esterolytic catalysis by the catAbs proceeds by facilitating a direct hydroxide attack on the scissile carbonyl of the substrate by stabilizing the oxyanion intermediate and flanking transition state through specific hydrogen bonds and/or electrostatic interactions.

The structure and orientation of the moieties used in this study, the alkyl ester (4) and its haptenic TSA1, are essentially similar to the reported aryl esters and their TSAs (31). Therefore, the fundamental theme of the catAbs in these reports could be applied to our catAbs. Based on the above assumption and the results on the affinities toward TSAs and the product analogue, we proposed a model structure of the Abs (Fig. 7). The structure suggests that the Abs essentially accommodate the three major pockets (or interacting residues), which interact with the phenyl moiety, the side chain benzyl moiety, and the phosphonate group, respectively. The affinity studies suggested that in the catAbs of the subgroup M, there could be two major pockets for binding: a hydrophobic pocket for the phenetyl group, which could be buried deeply in the cleft, and interacting residues for the phosphonate group, which is placed at the mouth of the hydrophobic pocket and which is a key for catalysis. A Lys at H95, which could be a key residue for interaction with the phosphonate group to form a hydrogen bond, which creates a catalytic pocket for the substrate, because it is a basic amino acid and is almost commonly conserved among the catAbs from MRL/lpr mice, is not conserved in the non-catAbs from BALB/c mice. This speculation is supported by the site-directed mutagenesis data of the catAb (manuscript in preparation). Taken together, the fundamental theme of the catAbs could be applied to our catAbs in the subgroup M, but the interacting residues (Lys at H95 and His at L91) that constitute the catalytic pocket are quite different from the reported esterase catAbs (His at H35 and Arg at L96).

In the non-catAbs obtained from BALB/c mice, however, there could be two hydrophobic pockets: one for the phenyl group and another that accommodates the benzyl side chain. The presence of a rare reading frame RF1 for the H-CDR3 and the rare λ2 light

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**FIGURE 6.** Clonal relations of the non-catAbs of the subgroup B from BALB/c mice. The circles on the offshoot arrows show amino acid substitutions due to mutations with their positions in the heavy and light chain genes. The number on the Ab code represents the affinity (Kd, nM) to 1 (TSA1, R = OH).
chain genes utilized in these Abs, which were reported to facilitate the hydrophobic interactions (21, 32), may constitute the second hydrophobic pocket for the benzyl moiety. The residues resulting from these changes could result in a stronger binding to the TSA, but they might also result in no residue(s) that interacts with the phosphonyl group. In these models, the catalytic activities of the Abs may be exerted after the surrounding residues interact with the phosphonyl group. But this interaction may force the phosphonyl group to have an inappropriate orientation, so that it cannot interact with the surrounding residues. As a result, the Abs would no longer be catalytic.

What is the source of the catAbs? Could the catAbs be derived from an abnormal repertoire that specifically exists in or is enriched in MRL/lpr mice? Autoreactive Abs, such as anti-nuclear Abs, Abs to ribonucleoproteins, ssDNAs, or dsDNAs, which are normally eliminated, but have a high incidence in autoimmune strains such as MLR/lpr mice (33, 34), seem like the most likely source of the catAbs. As a consequence, such mouse strains develop multisystem autoimmunity, including a lupus-like glomerulonephritis and arthritis (35, 36).

Thus, the amino acid sequences support the assumption that catAbs are derived from a repertoire of Abs to DNAs. However, this may not be necessarily the case in this study for the following reasons. First, the autoimmune strain NZB × NZW, which develops SLE, also produces Abs to DNAs that play a demonstrable role in the pathogenesis of disease in this strain (37–39), but this strain did not produce a higher incidence of catAbs as compared with the normal counterpart (13). Second, the catAbs from MRL/lpr mice obtained in this study, and those reported by Tawfik et al. (13) did not show a positive cross-react with either the bovine ssDNAs or dsDNAs (data not shown). Alternatively, the catAbs derived from MRL/lpr mice could be derived from the repertoire of the other autoreactive Abs to the molecules. Such molecules could be ones like flexible phosphodiester polymers such as RNA, teichoic acid, phosphotyrosine, phosphocholine, or other molecules whose distribution of phosphates (or equivalent negatively charged epitopes) conforms with the available contact with the combining sites of the Abs (40–44). The thorough survey on the data base did not show any identical sequences to our catAbs, but we found only one Ab that showed the highest similarity to one of our catAbs in the heavy chain gene sequence (GenBank accession no. L48662). It was an anti-DNA Ab, which was derived from C3H lpr, and had a Lys at H95, although the amino acid sequence of the H-CDR3 was quite different from our catAbs.

The lpr gene encodes the cell surface receptor molecule Fas, and its ligand FasL is encoded by the gld gene (45–48). MRL/lpr mice are characterized by a defect in Fas expression. As a result, they develop an autoimmune syndrome associated with massive lymphoaccumulation and excessive production of many of the autoreactive Ab specificities associated with the human disease SLE. Although Fas-FasL has an essential role in the tolerance to self Ag, its exact function is not clear. Immunization of prediseased lpr mice with conventional hapten-carrier conjugates results in germinal center reactions that are essentially normal (49, 50). Experiments with transgene-encoded B cell receptors have shown that the
central tolerance mechanisms are intact in lpr mice (51, 52). However, Fas-Fasl-mediated apoptosis were forced to nonspecific activation of such anergic (or indifferent) autoimmune B cells that normally circulate in the periphery (54, 55).

If the above story is the case, MRL/lpr mice defective in Fas-Fasl-mediated apoptosis were forced to nonspecifically accumulate the abnormal B cells that were normally inactivated after it evolved with somatic mutations, but without changing the key residue LyS at the junctional H95, which interacted with the phosphonoyl oxygen. It is noteworthy that the Abs with the key residue LyS at the junctional H95 already existed in the initial repertoire of MRL/lpr mice. In normal BALB/c mice, however, that would not happen, because of the lowest abundance (~1% in the data base: Ref. 20) or elimination of the autoactive B cell repertoire, in which a subset of B cells with the residue in the binding site of the Abs complementing to the phosphonoyl oxygen was included. Instead, another subset with hydrophobic residues in the binding site of the Abs was selectively captured to specifically bind to the haptenic TSA.

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