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Limiting CDR-H3 Diversity Abrogates the Antibody Response to the Bacterial Polysaccharide α 1→3 Dextran

Tamer I. Mahmoud,*† Harry W. Schroeder, Jr.,*‡§∥ and John F. Kearney*

Anti-polysaccharide Ab responses in mice are often oligoclonal, and the mechanisms involved in Ag-specific clone production and selection remain poorly understood. We evaluated the relative contribution of D H germline content versus N nucleotide addition in a classic oligoclonal, T-independent Ab response (α 1→3 dextran [DEX]) by challenging adult TdT-sufficient (TdT+/+) and TdT-deficient (TdT−/−) gene-targeted mice, limited to the use of a single D H gene segment (D-limited mice), with Enterobacter cloacae. D-limited mice achieved anti-DEX–specific levels of Abs that were broadly comparable to those of wild-type (WT) BALB/c mice. Sequence analysis of the third CDR of the H chain intervals obtained by PCR amplification of V H domain DNA from DEX-specific plasmablasts revealed the near universal presence of an aspartic acid residue (D99) at the V–D junction, irrespective of the composition of the D H locus. Although WT mice were able to use germline D H (DQ52, DSP, or DST) gene segment sequence, TdT activity, or both to produce D99, all three D-limited mouse strains relied exclusively on N addition. Additionally, in the absence of TdT, D-limited mice failed to produce a DEX response. Coupled with previous studies demonstrating a reduced response to DEX in TdT−/− mice with a WT D H locus, we concluded that in the case of the anti-DEX repertoire, which uses a short third CDR of the H chain, the anti-DEX response relies more intensely on sequences created by postnatal N nucleotide addition than on the germline sequence of the D H.


The ability of vaccines to elicit an effective defense against pathogens, such as Haemophilus influenzae type b, Streptococcus pneumoniae, and Neisseria meningitidis, depends largely on the induction of protective Abs to capsular polysaccharides or T-independent Ags associated with these microorganisms (1–3). This protection can depend on the ontogenetically regulated production of Abs with Ag binding sites of specific sequence or structure that bind to critical epitopes on the eliciting Ag. For example, S. pneumoniae protection is best offered by Abs produced by N region-deficient B-1a cells arising just after birth that bind to phosphorylcholine and bear the T15 idiotype (4). Thus, a better understanding of the mechanisms involved in the generation, ontogeny, production, and selection of B cells bearing Abs with Ag binding sites specific to polysaccharides and T-independent Ags is needed to optimize the design and ontogenetic timing of vaccines directed against pathogens bearing these types of immunodominant Ags.

Mice respond to many polysaccharides in a T cell-independent manner, with the rapid production of an oligoclonal Ab response consisting primarily of IgM and IgG3 Abs (5–7). The oligoclonal nature of this type of response facilitates the examination of factors that are important for the generation of polysaccharide-specific Ab diversity.

The Ag binding site of an Ab, as classically defined, is created by the juxtaposition of three hypervariable CDRs from the H chain and three CDRs from the L chain (8). In V H-restricted mice, the variability introduced by the third CDR of the H chain (CDR-H3) was shown to be sufficient for the generation of the primary Ab specificities to proteins but, surprisingly, not to selected polysaccharides (9). CDR-H3 is created by the combinatorial rearrangement of variable (V H), diversity (D H), and joining (J H) segments in conjunction with the variable insertion of random N nucleotides and the variable loss or P nucleotide gain of terminal nucleotide sequence (10). The D H gene segment in its entirety is a major component of CDR-H3, and both ends of the D H can undergo the extensive loss or gain of sequence. Coupled with its enormous potential for sequence and, thus, structural variation, its central position at the core of the classic Ag binding site permits the amino acids contributed by CDR-H3 to often play a critical role in the recognition and binding of the Ag to the Ab (9, 11).

α 1→3 Dextran (DEX) is a branched polymer of α 1→3 glucose sugar moieties displaying epitopes that are expressed by a variety of organisms, such as Enterobacter cloacae (12), Histoplasma capsulatum yeast cell wall (13), and Aspergillus fumigatus (B.L. Dizon and J.F. Kearney, unpublished observations). The Ab response of adult normal BALB/c mice to DEX is T cell independent, oligoclonal, and consists entirely of Abs bearing the λ1 L chain (14). The majority of anti-DEX Abs express either J558 or M104E Ids (14–16). Amino acid sequence analysis of DEX-binding hybridoma proteins showed V H region homology, with diversity clustered in that portion of CDR-H3 contributed by the D H and N addition. Unsurprisingly, this region contributes heavily to the individual idiotype expression identified by distinct B cell clones (17). The H chains of the prototypic J558 and M104E clones use identical J558.3 V H and J H gene segments but differ by two amino acids located within CDR-H3 (R100 and Y101 for J558 and Y100 and D101 for M104E) (18). Ontogenetic studies of
the BALB/c anti-DEX response showed that although the M104E idiotype predominates in newborn mice, almost 70% of adult anti-DEX Abs express the J558 idiotype, which requires N addition for its production (18, 19). Despite this dependence on TdT, the J558 clone has the same short-length CDR-H3 as all other anti-DEX clones reported (18). This is not surprising, because the length of CDR-H3 in Abs to a number of polysaccharide Ags and other T-independent Ags is often strictly maintained (20, 21).

To assess the role of DlH sequence and content on the Ab response to DEX, we challenged mice limited to the usage of one DlH gene segment (referred to as D-limited mice in this article) with E. cloacae. One strain of D-limited mice uses only the DFL1.6.1 gene segment that preferentially produces Abs enriched for tyrosine, serine, and glycine in CDR-H3 (ΔD-DFL) (22). A second strain (ΔD-DµFS) (23) preferentially produces Abs enriched for valine and threonine, which are encoded by DFL1.6.1 reading frame 2; however, it can also use reading frame 1 to produce Abs enriched with DlH-encoded tyrosine, serine, and glycine, albeit with CDR-H3s that tend to be shorter than those from ΔD-DFL. The third strain (ΔD-DiD) (24) preferentially introduces arginine, serine, asparagine, and histidine into CDR-H3. In these mice, the inclusion of a normal range of amino acids requires N addition or rearrangement by inversion, which is a rare event.

We found that the anti-DEX Ab response in the two strains of mice most capable of producing CDR-H3s of wild-type (WT) amino acid composition was robust. The loss of amino acids normally contributed by the DlH gene segments that had been deleted in the D-limited mice seemed to be compensated for by the activity of TdT, which was required for D-limited mice to encode an aspartic acid residue at position 99 (D99) that is conserved in the CDR-H3 regions of anti-DEX Abs. However, when challenged with a strain E. cloacae, ΔD-DµFS mice can elicit a superior J558 Id-expressing anti-DEX response compared with other D-limited and WT mice. Taken together, these results showed the extreme selectivity exerted by this classic T-independent Ag on the Ab repertoire, with specific amino acids in CDR-H3 proven to be not only sufficient, but necessary, to permit a normal response.

Materials and Methods

Mice

BALB/c mice homozygous for the ΔD-DFL, ΔD-DiD, and ΔD-DµFS DlH loci were generated as previously described (22–24). WT BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were bred and housed within the specific pathogen-free facility at The University of Alabama at Birmingham and used at 8–12 wk of age, according to protocols approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Immunization and serum analysis

Mice were immunized i.v. via the tail vein with 10⁸ DEX-expressing paraformaldehyde-fixed E. cloacae (strain MK7). ELISA was used to quantify serum Abs, as previously described (12). Plates were coated with either DEX (2 µg/ml) (Dr. M. Slodki, U.S. Department of Agriculture, Peoria, IL) or the anti-J558 idiotype EB3-7 (100 µg/ml) (25). Plates were washed three times with PBS and blocked with 1% BSA solution in PBS throughout the sort. The sorted cells were then centrifuged, and digestion buffer (50 mM Tris-HCl, 100 mM EDTA, 100 mM NaCl, 1% SDS [pH 8.0]) and protease K (0.5 mg/ml) was added to the cell pellet and incubated overnight at 50˚C. DNA was precipitated using isopropanol, and the DNA pellet was washed with 70% ethanol, air-dried, and resuspended in molecular grade water. All samples were analyzed using a FACSARia cell sorter (BD Biosciences, San Jose, CA). All Abs were purchased from BD Biosciences, with the exception of Ab 93 and JC5-1, which were developed by J.F. Kearney.

PCR

V(D)J rearrangements were amplified by PCR from genomic DNA extracted from sorted plasmablasts using primers specific for gene segments J558.3 Vµ3 5’-AGCTGCAACAATCTGGACCT-3’ and JH1 5’-CCCCA-GACATCGAAGTACCA-3’. PCR conditions were 95˚C for 3 min; 95˚C for 1 min, 58˚C for 1 min, and 72˚C for 1 min for 35 cycles; and then 72˚C for 7 min.

Cloning and sequencing

PCR products were cloned into TOPO-TA vector and transformed into TOP10-competent bacteria (Invitrogen, Carlsbad, CA). Templipli Amplification Kits (Amersham Biosciences, Piscataway, NJ) were used to isolate plasmid DNA from colonies. Plasmid DNA was sequenced at the Center for AIDS Research sequencing facility at the University of Alabama at Birmingham.

Sequence analysis

Any nucleotides that could not have been derived from a coding sequence or P addition were considered N nucleotides. To assign DlH gene segments used by CDR-H3, at least five nucleotides had to match germline DlH gene sequence. Sequences that did not meet this criterion were labeled as CDR-3Hs with “no DlH,” and their numbers were divided by the total number of nucleotides, and DNA was precipitated using isopropanol, and the DNA pellet was washed with 70% ethanol, air-dried, and resuspended in molecular grade water. All samples were analyzed using a FACSARia cell sorter (BD Biosciences, San Jose, CA). All Abs were purchased from BD Biosciences, with the exception of Ab 93 and JC5-1, which were developed by J.F. Kearney.

FIGURE 1. Anti-DEX Abs responses elicited by D-limited mice. Adult WT, ΔD-DµFS, ΔD-DiD, and ΔD-DFL mice were immunized i.v. with DEX-expressing E. cloacae, and serum was collected 7 d postimmunization. ELISA was used to determine DEX-specific IgM Abs (A) and J558 Id-expressing Abs (B). Horizontal lines represent the mean. *p < 0.05.
sequences to obtain the percentage of total sequences with “no D H” CDR-H3s. In the case of D-H-JH homology joining, where nucleotides could be assigned to either D H or JH, nucleotides were assigned to D H. CDR-H3 was identified as the region between the 3' VH-encoded conserved cysteine (TGT) at Kabat position 92 (IMGT 104) and the 5' JH-encoded conserved tryptophan (TGG) at Kabat position 103 (IMGT 118). Two types of repeated sequences were found. The first were completely identical duplicates (identical VH, DH, and JH segments, as well as junctional sequences). These were counted only once. The second were nearly identical, differing by one or more nucleotides in the VH. These were attributed to somatic mutation and were counted as unique. The numbers of both types of duplicates are reported in parentheses next to each sequence. The number of identical duplicates is highlighted in gray.

**FIGURE 2.** CDR-H3 sequences from DEX+ plasmablasts. DEX+ plasmablasts were sorted 7 d postimmunization with E. cloacae, and V(D)J rearrangements were PCR amplified from genomic DNA. A. The amino acid sequence of a typical DEX-specific H chain V(D)J rearrangement. The amino acids shown are canonical in all rearrangements reported in this study, with the exception of the two amino acids (labeled XX) at positions 100 and 101. B, CDR-H3 sequences are compared with those from 3' end of VH J558.3, the frame-shifted DFL16.1 gene (for ΔD-D-FS), the inverted DSP2.2 gene (for ΔD-D-D), the DFL16.1 gene (for ΔD-D-D), and the 5' end of JH1. In the case of sequence overlap, nucleotides were arbitrarily assigned to the DH gene segment. P-nucleotides are underlined. AA, the two amino acids at positions 100 and 101 that differentiate each DEX-specific H chain sequence from the others. Sequences from D-limited mice are compared with our database of WT sequences. In WT sequences, RY amino acids can have two assignments (indicated by a bracket). To assign D H gene segments used by CDR-H3, at least five nucleotides had to match germline D H gene sequence. Sequences that did not meet this criterion were labeled as CDR-3Hs with “no D H”. PCR amplification was done from at least three independent FACS sorts for each mouse strain. Two types of repeated sequences were found. The first were completely identical duplicates (identical VH, DH, and JH segments, as well as junctional sequences). These were counted only once. The second were nearly identical, differing by one or more nucleotides in the VH. These were attributed to somatic mutation and were counted as unique. The numbers of both types of duplicates are reported in parentheses next to each sequence. The number of identical duplicates is highlighted in gray.
mutation and were counted as unique. Somatic mutation occurs in T-independent responses, albeit at lower levels than T-dependent responses (27). The numbers of both types of duplicates are reported.

Statistics

Data comparing three or more groups were analyzed by a one-way ANOVA test, followed by the Tukey post test for data with normal distribution and the Kruskal–Wallis test, followed by the Dunn post test for data that did not distribute normally. Statistical significance was determined by a p value <0.05.

Results

Limiting D<sub>H</sub> diversity does not impair the Ab response to DEX

To test the role of limiting D<sub>H</sub> usage and, hence, CDR-H3 diversity in the Ab response to DEX, we used <i>E. cloacae</i> to immunize mice of three D-limited strains, as well as mice with WT D<sub>H</sub> loci. At all three D-limited strains produced a response to DEX (Fig. 1A). The highest level was observed in ∆D-D<sub>Q52</sub>FS mice, which was slightly greater than WT mice and significantly greater than D-iD and ∆D-DFL mice. We next determined whether the anti-DEX Abs from D-limited mice express the J558 idiotype that normally predominates in the adult WT BALB/c response. The level of anti-DEX Abs with the J558 idiotype that normally predominates in the adult WT BALB/c response. The level of anti-DEX Abs with the J558 idiotype that was equivalent among ∆D-iD, ∆D-DFL, and WT BALB/c mice but, again, was highest in the ∆D-D<sub>Q52</sub>FS strain (Fig. 1B). Thus, limiting CDR-H3 diversity by limiting D<sub>H</sub> segment availability still permitted robust production of anti-DEX Abs upon bacterial challenge. However, restricting the repertoire to a single D<sub>H</sub> gene segment that enriches for the use of hydrophobic reading frame 2, in place of hydrophilic reading frame 1, paradoxically generated Ab responses with enhanced levels of expression of the hydrophobic J558 idiotype.

Extensive nucleotide modifications at both ends of the D<sub>H</sub> gene segment of the anti-DEX repertoire of D-limited mice

To determine the molecular characteristics of the anti-DEX response elicited by D-limited mice, we PCR amplified and sequenced H chain V(D)J rearrangements from genomic DNA of DEX<sup>+</sup> λ<sup>+</sup> plasmablasts sorted from the spleens of <i>E. cloacae</i>-immunized mice (Fig. 2). Among the WT sequences, 28 (58%) used an identical V(D)J join that could either be attributed to DQ52 with seven nucleotides of N addition or to no D<sub>H</sub> with four nucleotides of N addition and one P nucleotide. The remaining 42% of sequences could be attributed to DQ52, DSP, or DST D<sub>H</sub> gene segments in conjunction with two to eight nucleotides of N addition. Thus, a D<sub>H</sub> gene could potentially be assigned to all of the WT sequences (Fig. 2, Supplemental Fig. 1).

All members of the DQ52, DSP, and DST families were deleted in the D-limited mice. In those mice, limited to use of DFL16.1 (ΔD-DFL), approximately three in four of the sequences could be attributed to this gene segment. In contrast, among the mice limited to either the ∆D-D<sub>Q52</sub>FS frame-shifted DFL16.1 gene segment or the inverted DSP2.2 ΔD-iD gene segment, only one in three of the anti-DEX sequences could be assigned a progenitor D<sub>H</sub> (Fig. 2, Supplemental Fig. 1). All of the D<sub>H</sub> gene segments in joins assigned to DSP or DFL D<sub>H</sub> gene segments were in reading frame 1, including those that, by our criterion, could be assigned to the D<sub>Q52</sub>FS gene segment.

The length of CDR-H3 among all of the sequences was the same. Additionally, among those sequences without an identifiable D<sub>H</sub> gene segment, the needed sequence was derived from either N addition or from an increased contribution from J<sub>H</sub>1 (Supplemental Fig. 1). Collectively, therefore, the single D<sub>H</sub> gene available for each D-limited mouse seemed to require more extensive nucleotide deletions with compensatory terminal modifications to generate an anti-DEX response than did WT mice.

The amino acid composition of CDR-H3 regions of anti-DEX Abs from D-limited mice is heterogeneous and shows conservation for an aspartic acid residue

The CDR-H3 sequence of WT anti-DEX V(D)J rearrangements exhibited variation in sequence only at positions 100 and 101 (Fig. 2, far right column). These differences reflected the use of alternative D<sub>H</sub> gene segments in the WT mice and/or N/P addition in all four strains. This was not surprising, because the anti-DEX rearrangements used a similar V<sub>H</sub> sequence and the same J<sub>H</sub> gene. Although positions 100 and 101 varied, position 99 contained an aspartic acid (D99) in all of the WT CDR-H3s. In some cases, D99 was entirely encoded by D<sub>H</sub> sequence, N addition by others, and by both for the rest of the sequences.

None of the DFL16.1, D<sub>Q52</sub>FS, or iD gene segments encode aspartic acid in their deletional reading frames. To test the extent to which N region addition could contribute to the D99 to the DEX Abs produced by the D-limited mice, we examined the deduced amino acid in this position and also looked at amino acids 100 and 101 to ascertain the extended level of junctional diversity created in the D-limited mice.

Despite the inability of D-limited mice to contribute a germline-encoded D99 to the anti-DEX Abs, all but one of the sequences obtained from these mice included this particular amino acid. The single exception was a AAC-encoded asparagine (N99) that was identified in only 1 of 35 unique sequences cloned from the ∆D-iD strain. All of the sequences obtained from the ∆D-DFL and ∆D-D<sub>Q52</sub>FS mice contained D99.

At positions 100 and 101, amino acid usage proved more variable, with each mouse strain displaying its own unique signature (Fig. 3). ΔD-DFL CDR-H3 sequences showed increased usage of serine and tyrosine at amino acid positions 100 and 101, which closely followed the amino acid signature of DFL16.1 reading frame 1. ΔD-D<sub>Q52</sub>FS CDR-H3 sequences were unique in their acquisition of an N nucleotide-generated lysine residue at position 100, as well as an increased dependence on J<sub>H</sub>4 to introduce a germline-derived tyrosine at amino acid position 101. Anti-DEX sequences from ∆D-iD mice were marked by the use of iD germline-encoded arginine at position 100 (R100), as well as by an N nucleotide-generated valine at amino acid position 100 (V100) (Figs. 3, 4).
In summary, as a population, the DEX-specific CDR-H3 sequences from the D-limited mouse strains conserved D99 usage, and each exhibited signature sequence characteristics that reflected the individual nature of the DH gene segment from which the sequences were derived. Thus, somatic selection for anti-DEX–binding capabilities at positions 100 and 101 was predicated by the sequence of the DH.

Additional likely contributes to D99 generation in D-limited mice

In contrast to positions 100 and 101, the near-universal presence of aspartic acid at position 99 in all three strains of D-limited mice, as well as WT mice, suggested that, at this particular position, somatic selection played a dominant and necessary role. Unlike WT mice, in which one fifth of the D99s could be attributed to either the DSP2.2 or DST4 gene segments, all of the D-limited mice seemed to rely on N addition to generate D99 (Fig. 4A). N addition also played a role in the introduction of arginine, lysine, valine, tyrosine, and serine at position 100 (B), tyrosine and serine at position 101 (C), as well as the nucleotides that are germline DH or JH. All are shown as the percentage of total sequences. The percent usage of each amino acid for each mouse strain is shown on top of each bar.

**FIGURE 4.** Contribution of N addition in generating crucial amino acids in CDR-H3 sequences from ∆D-DmFS, ∆D-DiD, ∆D-DFL, and WT mice. The numbers indicate the number of nucleotides that are nongermline (N nucleotides) that encode for aspartic acid at position 99 (A), arginine, lysine, valine, tyrosine, and serine at position 100 (B), tyrosine and serine at position 101 (C), as well as the nucleotides that are germline DH or JH. All are shown as the percentage of total sequences. The percent usage of each amino acid for each mouse strain is shown on top of each bar.

In contrast to positions 100 and 101, the near-universal presence of aspartic acid at position 99 in all three strains of D-limited mice, as well as WT mice, suggested that, at this particular position, somatic selection played a dominant and necessary role. Unlike WT mice, in which one fifth of the D99s could be attributed to either the DSP2.2 or DST4 gene segments, all of the D-limited mice seemed to rely on N addition to generate D99 (Fig. 4A). N addition also played a role in the introduction of arginine, lysine, valine, tyrosine, and serine at position 100 in all four mouse strains, including WT (Fig. 4B). In contrast, where present, the tyrosines at position 100 and 101 were universally encoded by either germline DH or JH sequence in the D-limited mice (Fig. 4). Among the WT mice, at least two of the three nucleotides encoding tyrosine were similarly encoded by germline DH or JH sequence.
D-limited × TdT−/− mice fail to respond to DEX

To test the extent of reliance on TdT activity to provide D99 and, by extension, to create the anti-DEX response, we bred the TdT knockout allele onto each of the D-limited mouse strains and then performed similar E. cloacae immunizations. A response to DEX 7 d after immunization could not be detected in any of the D-limited mice homozygous for the knocked-in ΔH gene (Fig. 5A). Moreover, the response elicited by the heterozygous D-limited mice was even lower than that of TdT-deficient animals (18). These results led us to conclude that the ability to include an aspartic acid in position 99 is essential for the production of E. cloacae-induced anti-DEX Abs in BALB/c mice and, that in the presence or absence of ΔH-encoded aspartic acid, TdT expression either facilitates or is absolutely required, respectively, to place this D99 in CDR-H3. By extension, these findings support the view that the ontogenetic differences in the anti-DEX response observed in BALB/c mice are directly related to physiologic control of TdT activity.

Discussion

Abs to a number of polysaccharide Ags have several common attributes: CDR-H3 is implicated in binding to Ag (28, 29), its length is strictly maintained (20, 30), and its sequence is almost invariably characterized by the presence of hydrophilic tyrosine residues (21, 31, 32). Xu and Davis (9) showed that, in VH-restricted mice, CDR-H3 diversity was sufficient for the development of specific Ab responses to a variety of hapten and protein Ags but not for two bacterial polysaccharide Ags. The latter finding was attributed to the failure of the single VH gene in mouse model to accommodate the polysaccharide-specific response, which suggested VH dependency. Work by Nakouzi and Casadevall (33) showed that, in addition to CDR-H3, CDR-H2 encoded amino acids critical for the generation of Abs specific for the polysaccharide galactoxylomannan. These studies highlighted the significant roles that both the VH, as a whole, and the CDR component of the VH, as a part, can play in the generation of polysaccharide-specific Abs.

In this study, we addressed the relative roles of ΔH gene segment content and N addition, both key components of CDR-H3 diversity, on the nature of the Ab response to the bacterial polysaccharide DEX following bacterial challenge. We previously showed that restriction of TdT alone led to a lower total anti-DEX response and drastically diminished expression of the normally dominant J558 idiotype (18). In the present study, we found that limiting the repertoire to the use of a ΔH segment that preferentially encodes for hydrophilic (ΔΔ-DFL), charged (ΔΔ-DID), or hydrophobic (ΔΔ-DμFS) amino acids does not dramatically impair the magnitude of the DEX-specific Ab response per se. We further found that, in the absence of a critical ΔH germline-encoded amino acid (D99), D-limited mice wholly depend on the activity of TdT to generate this residue, which our data suggest is absolutely required for anti-DEX Ab responses. Thus, in the presence of genetic constraints on ΔH usage, the compensatory diversifying mechanisms of N addition and exonuclease nilling permitted and influenced the magnitude of the anti-DEX response with the short CDR-H3 sequences that are typical of this response in WT BALB/c mice.

In response to E. cloacae, our D-limited mice, especially ΔΔ-DμFS, produced an anti-DEX Ab response that was similar, although not identical, to that of WT mice. The drive to generate DEX-specific Abs that contain a highly focused CDR-H3 amino acid content suggests that the physicochemical properties of the Ag play a role in selecting clones within a specific range of hydrophobicity. Polymers of glucose, such as α 1→3 and α 1→6 dextran (DEX B512), are generally neutral (34). Therefore, we propose that the interaction between DEX-specific B cells and their cognate Ag may require a preponderance of neutral to hydrophobic moieties at their binding site.

ΔΔ-DμFS mice were capable of generating higher levels of J558-expressing Abs compared with the other mouse groups. This is likely due to the preponderance of tyrosine residues in amino acid position 101 (Y101) in CDR-H3 sequences isolated from plasma blasts of DEX-immunized ΔΔ-DμFS mice. Previous reports showed that the capacity of EB3-7 Ab (anti-J558 Id Ab) to bind J558 was diminished if this tyrosine residue was chemically modified (35). This specific affinity of EB3-7 to bind Y101 may explain the higher levels of J558 Id expression in the serum of ΔΔ-DμFS DEX-immunized mice. In WT mice, Y101 was variably the product of JH1, N addition, or DQ52. In all of the sequences from the D-limited mice, Y101 was only the product of germline ΔH or JH1. Additionally, of the D-limited mice, the ΔΔ-DμFS strain used this residue most frequently. This preference for JH1 Y101 in ΔΔ-DμFS mice may reflect differences in the way that the DμFS gene segment is handled by the recombination and selection system in the bone marrow. Evidence for this was reported earlier by examining sequences of VH 7183DJCμ transcripts from different stages of B cell development, where ΔΔ-DμFS rearrangements showed increased nucleolytic loss at the 3′ end of the ΔH gene segment compared with controls (23). This was true for the minor population of B cells in ΔΔ-DμFS mice that were selected to use ΔH reading frame 1 instead of the genetically enforced ΔH reading frame 2. A tendency to compensate for a shorter ΔH reading frame 1 sequence with a JH terminal residue could provide a partial explanation for the enhanced response.
A large proportion of the DEX-specific CDR-H3 sequences that were PCR amplified from immunized D-limited mice contained no identifiable D9H gene segments. The mechanisms that can reduce or limit D9H content in CDR-H3 were thoroughly discussed by Rohatgi et al. (36). Briefly, CDR-H3s without identifiable D9H content can be generated by recombination signal sequence replacement, hence permitting direct V9H to J9H joining (37); violation of the 12/23 bp rule, as demonstrated in the D9H gene-deficient mouse (38); or excessive nucleolytic activity at the D9H gene segment. The DEX-specific CDR-H3 sequences that we obtained from plasmablasts contained many of these types of extensive nucleotide modifications, including nucleotide deletions or additions. In D-limited mice, with the exception of ΔD-DFL mice, these junctional alterations drastically modified the genetically mouse (38); or excessive nucleolytic activity at the D9H gene segment and generated a CDR-H3 region that was hydrophilic in nature and of the appropriate length to allow the host B cell to respond robustly to DEX. It is important to note that V(D)J joins that had assignable D9H gene segments were mostly in reading frame 1. Taken together, several mechanisms may be in play to ensure that the H chain Ab repertoire conforms to a certain architecture, despite genetic manipulations.

Alternative V(D)J recombination events, other than generation of CDR-H3 regions with “no D9H”, were available for D-limited mice to generate a WT DEX-specific repertoire. Examination of the WT anti-DEX response shows a propensity for use of the D9P gene family. ΔD-DiDi9H mice had the capacity to invert the iDSP2.2 gene to increase the likelihood of generating a DEX-specific repertoire that displays increased usage of the DSP2.2 gene with its component aspartic acid. Despite this fact, V(D)J recombination by inversion was not detected in DEX-specific sequences isolated from these mice. This confirmed our earlier observation that V(D)J recombination by deletion is favored over inversion, despite the more WT-like Ab repertoire that can develop if inversion is the predominant recombination event (24).

The most striking finding is the apparent absolute requirement for an aspartic acid at position 99 of the CDR-H3 sequence (D99) to generate an anti-DEX IgM response. We previously showed that, under WT conditions, D99 can be contributed by DSP2.2 or D9T4 or by TdT activity. Both DSP2.2 and D9T4 are deleted in the D-limited mice, and we showed that, in these mice, D99 was absolutely dependent on TdT activity. This absolute dependence on D99, in general, and on the need for N addition in D-limited mice, in particular, was underscored by the observation that, in the absence of TdT, the response to DEX was completely abrogated. The singular importance of particular amino acids in specific locations in T-independent responses is not unique to the DEX response. An aspartic acid at the V9H-D9H junction was shown to be essential for the binding of anti-phosphorylcholine Abs, such as the M603 clone (39).

DEX-specific sequences from WT mice showed a superior capacity to generate R100 and Y101 in the CDR-H3, which are the amino acid residues that characterize the J558 idiotype. If the generation of these two residues was absolutely dependent on TdT activity, all D-limited mice should generate an equivalent number of sequences as the WT mice. However, our analysis of the WT sequences revealed that there are two ways to generate R100 and Y101 in WT mice (Fig. 2), one of which involves the DQ52 D9H gene segment (which is only available in the WT strain). Thus, it is possible that DQ52 usage has provided WT mice with an advantage in generating the preferred D99, R100, and Y101 CDR-H3 anti-DEX sequence.

Past studies showed that D-limited mice elicit a lower Ab response to purified DEX (22–24). In this study, we analyzed the DEX response elicited by the Gram-negative commensal E. cloacae; it is very likely that pathogen-associated molecular patterns, such as LPS, engaged TLRs and other receptors involved in the innate arm of the immune response, possibly boosting the Ab response. Conversely, it is possible that the Ab response to the bacteria as a whole yields a more diverse Ab response to other bacterial Ags and epitopes that affects the relative level of response to DEX differently, depending on the composition of the D9H locus and, by extension, the Ab repertoire as a whole.

This report showed that mechanisms controlling the nature of the Ab response to DEX operate to ensure that Abs generated exhibit certain characteristics that are independent of the sequence composition of the D9H. These restrictive mechanisms are not well understood and have received little attention in immunological research (33). Possible mechanisms for such restriction include strict structural constraints for the Ag–Ab interaction, such that only some V regions, or other CDR-H component can be selected, idiotypic regulation, deletion of self-reactive clones resulting in the emergence of a dominant clone or selection of a protective clone by self- or commensal Ags (40–42)

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Disclosures
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Figure S1

Figure S1: Contribution of VH, DH, JH, N- and P-nucleotides to CDR-H3 sequences. Percent of total V(D)J rearrangements where DH gene segment could not be assigned to the single DH gene segment (in case of D-limited mice) or to germline DH genes (in the case of WT) is denoted as “No DH”. Contribution of each CDR-H3 component is shown as percent of CDR-H3 length for each mouse strain. RF1: reading frame 1 usage by DH genes that were identified.