Immunoglobulin D (IgD)-deficient Mice Reveal an Auxiliary Receptor Function for IgD in Antigen-mediated Recruitment of B Cells

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Summary

To assess the role of immunoglobulin D (IgD) in vivo we generated IgD-deficient mice by gene targeting and studied B cell development and function in the absence of IgD expression. In the mutant animals, conventional and CD5-positive (B1) B cells are present in normal numbers, and the expression of the surface markers CD22 and CD23 in the compartment of conventional B cells indicates acquisition of a mature phenotype. As in wild-type animals, most of the peripheral B cells are resting cells. The IgD-deficient mice respond well to T cell-independent and -dependent antigens. However, in heterozygous mutant animals, B cells expressing the wild type IgH locus are overrepresented in the peripheral B cell pool, and T cell–dependent IgG1 responses are further dominated by B cells expressing the wild-type allele. Similarly, in homozygous mutant (IgD-deficient) animals, affinity maturation is delayed in the early primary response compared to control animals, although the mutants are capable of generating high affinity B cell memory. Thus, rather than being involved in major regulatory processes as had been suggested, IgD seems to function as an antigen receptor optimized for efficient recruitment of B cells into antigen-driven responses. The IgD-mediated acceleration of affinity maturation in the early phase of the T cell–dependent primary response may confer to the animal a critical advantage in the defense against pathogens.

IgD is the major antigen receptor isotype coexpressed with IgM on the surface of most peripheral B cells in mouse, human, and a variety of other species (1–7). The genes encoding the constant region of the μ (Cμ) and the δ (Cδ) chain reside in a single transcription unit and can be expressed simultaneously, by alternative splicing of the V region exon to the first exon of the Cμ or the Cδ gene (8). The regulation of IgM and IgD expression appears to follow a strict, developmentally controlled program.

Newly arising "immature" B cells in the bone marrow or fetal liver express IgM but not IgD on their surface, whereas the vast majority of "mature" peripheral B cells of an adult mouse is characterized by surface expression of IgM and IgD (9, 10). The amount of IgD expressed exceeds that of IgM by about 10-fold (11). During ontogeny, the first μ*δ* B cells appear about 4 d after birth, and the frequency of these cells gradually increases to adult levels by 4–6 wk of age (3, 4, 12, 13). IgM as well as IgD can mediate B cell activation (14), and it seems that both receptors can transduce signals by the same mechanism (15).

Upon activation of B cells by antigen, IgD is downregulated (16, 17) and the cells enter the pathways of plasma cell or memory cell generation (18). The downregulation of IgD is accompanied by an increase in IgM expression, and subsequent secretion of this isotype in the early primary response.

Since its discovery (19), the biological role of IgD has remained enigmatic despite continuing experimental effort aimed at defining its role in B cell development. Data obtained from experiments applying anti-IgD antibodies or proteolytic enzymes in vivo or in vitro were interpreted as evidence for an important role of IgD in the transition from a stage of susceptibility to tolerance induction to one of responsiveness (20–24) or in triggering B cells by particular types of antigen. However, contradictory results have been reported (13, 25–30). A distinct function of IgM and IgD was suggested by the observation that treatment with anti-IgM but not anti-IgD in vitro induced growth arrest of cell lines or normal mature B cells expressing IgM and IgD on the surface (31–33). Furthermore, a requirement for surface expression of IgD for the propagation of immunological memory has been suggested (34), but contradictory results were reported (35, 36). If, however, IgM and IgD would deliver the same signal (15) or would not signal at all but just serve as an antigen-focusing device allowing efficient antigen processing and presentation (37), the coexistence of two receptor isotypes might serve simply to improve antigen binding (38, 39). A specific role for IgD in the interaction between B and T cells, mediated by IgD binding receptors expressed on T cells, was proposed based on the observation of rosette formation of IgD-coated erythrocytes with a subpopulation of T cells (40). The deter-
minants recognized by this IgD receptor were reported to be sugar residues on the C61 and C63 domain (41).

An interesting and unexpected phenomenon, with respect to expression of IgM and IgD, was observed in mice transgenic for rearranged H and L chain genes encoding a self-reactive antibody. Autospecific B cells, found to be anergic, had downregulated surface expression of IgM, whereas the level of IgD expression remained high. These data led to the suggestion that IgD may play a role in maintaining B cell tolerance (42). Along these lines, coincidence of low IgD surface expression and autoreactivity of B cells has been observed in some mouse strains prone to the development of autoimmune disease and in the CD5 B cell population of normal mice (43).

To more directly assess the role of IgD in B cell development, we generated IgD-deficient mice utilizing the gene-targeting technique. We have previously shown, in chimeric mice, that B cells expressing the IgH allele bearing a non-functional Cδ gene appear as μ−δ− B cells in the periphery at the same frequency as their counterparts expressing the wild-type allele (μ−δ+) (44). These μ−δ− B cells seemed to mature normally and respond to a TD antigen, ruling out an essential role for IgD in the transition from a state of susceptibility to tolerance induction to one of responsiveness. Although the analysis of chimeric mice established that B cells can develop and respond to antigen without ever having expressed IgD, it did not allow for a detailed functional comparison of IgD-expressing and IgD-deficient B cells, nor did it exclude a possible biological role of IgD apart from serving as a receptor on the surface of the cell producing it (45). Therefore, we generated a mouse line deficient for IgD through germline transmission of the mutated Cδ allele. Results obtained using this system are reported in the present paper.

Materials and Methods

Gene Targeting. Gene targeting of the Cδ gene was performed as described (44) except that the 129/ola-derived embryonic stem (ES) cell line E14-1 (46) was used. A total of 10⁶ E14-1 ES cells were transfected with the targeting vector designed to replace a large part of the C61 exon and to insert framenshift mutations in C63 by filling in restriction sites present in this exon (44). The introduction of the mutations into the germline would result in functional inactivation of both δ chain Ig domain exons. This was considered important to exclude the possibility of expression of a truncated δ H chain that could compete with μ for L chains and be secreted. The presence of the framenshift in the mouse germline confirmed the structure of the targeted locus. Homologous recombinants were obtained at a frequency of 1/17 double-resistant or 1/103 G418-resistant clones. Targeted ES cell clones were injected into blastocysts isolated from C57Bl/6 mice and transferred to (C57Bl/6 × BALB/c) fosters. Male chimeric offspring were mated with C57Bl/6 females for germline transmission of the δmut mutation. Offspring derived from ES cells were identified by coat color.

Mice. Animals were bred in our own conventional mouse facility.

Southern Blot Analysis. Southern blot was performed as described (48).

Northern Blot Analysis. Total RNA was isolated from spleens using guanidiniumisothiocyanate-containing lysis buffer with subsequent CsCl gradient purification (48). Poly(A)+ RNA was enriched using oligo-dT spin columns (Pharmacia Fine Chemicals, Freiburg, FRG) size fractionated by formaldehyde agarose gel electrophoresis as described (48) and transferred to a nylon filter. An RNA ladder (Gibco BRL, Egggenstein, FRG) was included to determine the sizes of the various RNA species. The probe used for hybridization was a 1-kb Cu cDNA fragment obtained by PCR amplification using Jδ4- and Cδm-specific primers. This fragment was digested with BglII, cutting in Cδ1, to remove the 5'-part containing Jδ4. The Cδm-specific probe was a 700-bp-long HindIII/BglIII genomic fragment including the δml exon, intervening sequence, δm2, and a part of the 3'-untranslated region (39). The neomycin’ (neo) gene was detected with a 1.1-kb XhoI/SalI fragment isolated from pMClneo (49), and the Cδ3-specific probe was a genomic 1.2-kb NcoI/BglI fragment spanning from the 5' end of the Cδ3 exon through a region of the 3' intron (47). To remove probes for sequential hybridization, filters were stripped to background levels of radioactivity by boiling in 0.1× SSC/0.1% SDS.

Flow Cytometry. Analyses were performed on a FACScan® cytometer (Becton Dickinson & Co., Mountain View, CA). The reagents and staining procedures have been described (44, 50, 51). CD22 was revealed by staining cells with rat mAb NIM-R6 (52) supernatant (gift of Dr. R. M. E. Parkhouse, Institute for Animal Health, Pirbright, UK) and detected with FITC-conjugated mAb Mar18.5 (anti-rat x; 53). IgD was revealed using FITC-conjugated polyclonal rabbit anti-mouse IgD antiserum (Nordic Immunological Laboratories, Capristano Beach, CA), and normal rabbit serum was used to block nonspecific binding. Phosphatidylcholine (PtC) binding cells were detected by staining cell suspensions with FITC-conjugated PtC liposomes (54; gift of Dr. A. Kantor, Stanford University Medical Center, Stanford, CA).

Analysis of B Cell Life Span. 5'-bromo-2'-deoxyuridine (BrdU; Sigma Chemical Co., St. Louis, MO) labeling experiments were performed as described (55) except that the concentration in the drinking water was 1 mg/ml.

Immunization of Mice. 10, 20, or 100 μg (4-hydroxy-3-nitrophenyl) acetyl (NP) coupled to chicken gamma-globulin (CG) (NP-CG) precipitated in alum/PBS, 50 μg α(1-3)-dextran (gift of Dr. J. Kearney, University of Alabama, Birmingham, AL) in PBS or 5 μg NP-Ficoll in PBS were injected intraperitoneally. 50 μg NP-CG in alum were injected intraperitoneally 6 wk after priming to elicit a secondary response (see also reference 51).

Titration of Serum Ig. Serum Ig isotypes IgM, IgG3, IgG2b, IgE, and IgA were determined by a direct plate binding assay as described (46). IgG1 and IgG2a were determined by direct binding to plates coated with mAb Ig-4a(10.9) (44; anti-IgG1) or rat anti-mouse IgG2a antiserum (Nordic Immunological Laboratories). Bound Ig was revealed by biotinylated goat anti-mouse IgG2a (Southern Biotechnology Associates, Birmingham, AL). mAbs of the various isotypes served as standards. Antigen-specific titters were determined on plates coated with NP-C-BSA or CG at 10 μg/ml or α(1-3)-dextran-BSA at 50 μg/ml. Anti-NP and anti-CG titters were determined by ELISA.

Abbreviations used in this paper: BrdU, bromodeoxyuridine; CG, chicken globulin; ES, embryonic stem; NP, (4-hydroxy-3-nitrophenyl)acetyl; PtC, phosphatidylcholine.
in preimmune sera were <1 μg/ml for IgM and χ and <0.2 μg/ml for IgG isotypes. Concentrations of NP-, CG-, or α(1-3)-
dextran–specific serum antibodies were quantified relative to NP-
binding standard antibodies of the various isotypes.

Affinity Measurements. These were done as described (36, 56)
using microtiter plates coated with NP-BSA and NP, BSA. Anti-
NP mAbs of known affinities, from primary and secondary re-
 sponses, served as standards (56). Bound antibody was revealed by 
a biotinylated anti-IgGl (Ig4a-10.9; 44) or anti-M (LS136; 46)
 mAb. As reported for anti-DNP antibodies (36), we have previ-
ously shown that the relative binding to coats of high and low 
epitope density correlated with the log of the affinity binding con-
stants of IgG anti-NP antibodies (56; T. Tókuihisa, unpublished results).

Results

Generation of IgD-deficient Mice. The strategy of Cδ gene 
 inactivation was the same as previously described (44). Re-
striction maps of wild-type and mutated Cδ loci as well as 
restriction analysis of HindIII-digested genomic DNA from 
five individual candidate recombinants (Fig. 1 a, lanes 2–6) 
and control cells (Fig. 1 a, lane 1) are shown. A homologous 
recombination event would result in a 4.4- or 6.0-kb band

Figure 1. Southern blot analysis of (a) PCR-positive E14-1 embryonic 
stem cell clones transfected with the targeting vector; and (b) tail DNA 
of mice obtained through germline transmission of the mutated Cδ allele. 
Genomic DNA samples were digested with HindIII (a) or BamHI (b), 
respectively. (Bottom) Restriction maps and the genomic organisation of 
(c) the Cμδ locus and (d) the mutated allele. The probe indicated in (c) 
is a XhoI/EcoRV fragment derived from the Cμ-δ intron. (b) BamHI; 
(D) DraIII (not unique); (E) EcoR1; (H) HindIII; (X) XhoI; (X') 5'-end 
of the targeting vector; and (H") HindIII site deleted in the targeting vector 
generating a frame shift mutation (44).

in addition to the 3.8-kb germline band, depending on the presence or absence of the frame shift mutation in Cδ3 that 
results in loss of the HindIII site (Fig. 1, c and d). 9 of 10 
homologous recombinant clones showed the 6.0-kb fragment, 
indicating that the breakpoint of recombination was located 
3' of the Cδ3 exon. Thus, the Cδ1 and Cδ3 exons were ren-
dered nonfunctional in these clones. One clone retained the 
HindIII restriction site in the Cδ3 exon, resulting in a diag-
nostic restriction fragment of 4.4 kb (Fig. 1 a, lane 6). The 
structure of the targeted locus was confirmed using a variety 
of other probes and restriction enzymes (data not shown). 
Four clones harboring mutated Cδ1 and Cδ3 exons were in-
jected into blastocysts and the resulting male chimeras were 
mated with C57Bl/6 females for germline transmission of 
the mutated allele. Offspring derived from ES cells were 
identified by coat color and analyzed for the presence of the 
mutation which we call δT, by Southern blotting (Fig. 1 b) 
or phenotypically, by flow cytometry. Homozygous mutant 
mice (δT/δT) were obtained by the interbreeding of hetero-
zygous offspring (Fig. 1 b, lanes 3 and 6).

mRNA Potentially Able to Encode an IgD-like Molecule Is 
Undetectable in Homozygous Mutant Mice by Northern Blot Anal-
ysis. The δT mutation results in functional inactivation of 
both exons encoding Ig domains of the H chain. The trans-
membrane and the hinge region exons, however, remain in-
tact and potentially functional. To exclude that aberrant 
splicing of precursor RNA encompassing both the Cμ and 
the Cδ genes results in the generation of a significant amount 
of chimeric Ig transcripts encoding the extracellular domains 
of the Cμ gene and the transmembrane and cytoplasmic por-
tion of Cδ, we analyzed poly(A)+ RNA isolated from 
spleens of homozygous mutant (δT/δT) and wild-type mice 
by Northern blotting (Fig. 2). mRNA containing Cμ exons 
spliced to the Cδ transmembrane exon would be larger than 
the normal Cμ transcripts of 2.4 (μ) or 2.7 kb (μm) (57), 
because the 3'-untranslated region of the δ message is 600-bp 
longer than that of the μ message (39). Hybridization of 
splicen poly(A)+ RNA of homozygous mutant mice with 
a Cδ transmembrane-specific probe reveals bands of 4.8-, 4.0-, 
3.8-, and 3.0-kb. However, none of these bands hybridized 
with the Cμ-specific probe (Fig. 2, A and B). The detection 
limit of the two probes was similar within a factor of two 
(1.2 × 10^6 copies for the Cμ and 0.6 × 10^6 for the δm 
probe) as judged by the signals obtained from hybridization to 
standard plasmid DNA (data not shown). The Cμ probe 
is a cDNA fragment of 1 kb with complete match to the 
mRNA and the standard plasmid, whereas the δm probe hy-
bridizes to the mRNA over a stretch of only 480 bp, but 
700 bp on the plasmid standard because it contains the 0.6m/m2 
intronic. Consequently, a mRNA representing Cμ exons spliced 
to the δm exons, if detected with the δm probe, should also 
be revealed with the Cμ probe. Because the 3.0-, 3.8-, 4.0-, 
and 4.8-kb bands are clearly above the δm probe detection 
limit, they should, if containing Cμ sequences, also be de-
tected with the Cμ probe. This, however, is not the case (Fig. 
2 B, lanes a and b; right).

Furthermore, sequential hybridization of the same blot with 
a neo' gene and a probe specific for the Cδ3 exon
Figure 2. Northern blot of poly(A)^+ RNA from spleens of wild-type (+/+ ) and homozygous mutant (δT/δT) mice. (A) Northern blot of splenic poly(A)^+ RNA from homozygous mutant (δT/δT) and control mice (+/-) hybridized with a Cμ cDNA and a δm-specific probe. (B) poly(A)^+ from spleens of (δT/δT) mice was sequentially hybridized with a Cμ cDNA, a δm, a neo', and a Cδ3-specific probe.

Figure 3. B cell maturation in IgD-deficient mice. Flow cytometric analysis of (a) lymph node and (b-d) spleen cells of wild-type mice (left, IgH^+, +/IgH^+, +), and mice heterozygous (center, IgH^+, δT/δH^+, +) or homozygous (right, IgH^+, δT/δH^+, δT) for the δT mutation. Dots and numbers (%) in the fluorescence windows refer to cells in the lymphocyte gate as defined by light scatter.

demonstrates that the 3.8-, 4.0-, and 4.8-kb bands also contain sequences derived from the neo' gene (Fig. 2 B, lane c), indicating that they represent aberrant splice products. The 3.0-kb band hybridizes with the probe specific for the Cδ3 exon (Fig. 2 B, lane d) which is nonfunctional in the targeted allele because of frame shift mutations. In addition, with the neo' probe, mRNA of 2.4 kb, which does not hybridize with the δm probe, is also detected (Fig. 2 B, lanes b and e). Low abundant mRNAs of 1.6 and 2.0 kb hybridizing with a Cδm-specific probe are detectable in 10 μg poly(A)^+ RNA from both normal and mutant mice (Fig. 2 A). These mRNAs, however, are smaller than the normal Cμ message and therefore, unlikely to encode a functional Ig molecule.

Taken together, mRNA species representing potentially functional chimeric δ/δ molecules are undetectable in Northern blots using as much as 10 μg of splenic poly(A)^+ RNA of δT/δT mice. Because mRNA encoding the δ H chain can be detected with the Cδm-specific probe in as little as 300 ng of poly(A)^+ RNA of spleens of normal mice (Fig. 48 Immunoglobulin D-deficient Mice
Table 1. Frequency of Cells Recovered from Organs of Wild-type (+/+) and Homozygous Mutant Mice (δT/δT)

<table>
<thead>
<tr>
<th>Cell type</th>
<th>+/+</th>
<th>δT/δT</th>
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<tbody>
<tr>
<td>Bone marrow (x 10⁷)*</td>
<td>2.4 ± 0.3 (n = 3)</td>
<td>2.2 ± 0.1 (n = 3)</td>
</tr>
<tr>
<td>pre-B* (%)</td>
<td>15.5 ± 3.0</td>
<td>21.7 ± 11.0</td>
</tr>
<tr>
<td>immature B* (%)</td>
<td>12.2 ± 0.3</td>
<td>12.8 ± 3.2</td>
</tr>
<tr>
<td>mature B* (%)</td>
<td>16.8 ± 6.3</td>
<td>16.9 ± 5.5</td>
</tr>
<tr>
<td>Peritoneum (x 10⁶)</td>
<td>3.0 ± 1.0 (n = 4)</td>
<td>3.8 ± 2.1 (n = 4)</td>
</tr>
<tr>
<td>CD5 B* (%)</td>
<td>39.7/46.2</td>
<td>35.7/39.8</td>
</tr>
<tr>
<td>PtC binding* (%)</td>
<td>21.4/20.7</td>
<td>10.7/21.7</td>
</tr>
<tr>
<td>Spleen (x 10⁷)</td>
<td>9.1 ± 1.7 (n = 5)</td>
<td>12.4 ± 3.0 (n = 5)</td>
</tr>
<tr>
<td>B* (%)</td>
<td>48.9 ± 5.8 (n = 9)</td>
<td>46.9 ± 5.1 (n = 9)</td>
</tr>
<tr>
<td>T* (%)</td>
<td>21.9 ± 2.2 (n = 3)</td>
<td>32.2 ± 4.0 (n = 4)</td>
</tr>
<tr>
<td>CD4/CD8 (ratio)</td>
<td>2.8 ± 0.3 (n = 4)</td>
<td>2.1 ± 0.7 (n = 7)</td>
</tr>
<tr>
<td>CD5 B (%)</td>
<td>&lt;1/&lt;1</td>
<td>&lt;1/&lt;1</td>
</tr>
<tr>
<td>PtC binding (%)</td>
<td>&lt;1/&lt;1</td>
<td>&lt;1/&lt;1</td>
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</table>

When more than two animals were analyzed, mean and SD are given.
* Total nucleated cells recovered.
† CD45R/B220+/IgM⁻.
§ CD45R/B220+/IgM⁺.
‖ CD45R/B220+/IgM⁻.
§§ CD45R⁻/CD5⁻.
* Stained with fluorescein-containing PtC-liposomes (54).
† IgM⁺ and/or IgD⁺.
§§ Thy-1⁺.

on μ⁺δ⁻ and μ⁺δ⁺ B cells as determined by staining with an anti-χ L chain antibody (data not shown).

CD22 and CD23 are expressed on virtually all mature μ⁺δ⁺ B cells, whereas immature B cells in the bone marrow (μ⁺δ⁻) lack these surface markers (52, 58, 59). Surface expression of CD22 coincides with that of IgD, although CD22 seems to be expressed intracellularly early in B cell development (60), whereas CD23 is acquired later than IgD in ontogeny (58). Spleen cells of wild-type, heterozygous, and homozygous mutant mice were stained for CD23 and CD22 in combination with an anti-IgM antibody recognizing the a allotype of the targeted allele (Fig. 3, c and d). In heterozygous mutant mice that are IgH b on the wild-type allele, the level of surface expression of CD22 and CD23 can be directly compared between μ⁺δ⁺ B cells expressing the wild-type allele (IgM⁺) and μ⁺δ⁻ B cells expressing the targeted allele (IgM⁺⁺). The results show that in both heterozygous and homozygous mice, the latter express the same levels of the CD23 and CD22 antigens as μ⁺δ⁺ B cells. Thus, expression of IgD is not a prerequisite for CD22 and CD23 expression, and the B cell population acquires a mature phenotype in IgD-deficient mice, with respect to expression of these markers.

A naturally occurring B cell population that expresses low levels of surface IgD are the CD5 B cells residing in the peritoneum of normal mice (61). A large fraction of these cells express IgM antibodies binding to isologous RBCs pretreated with the proteolytic enzyme bromelain (62). One of the determinants recognized by these antibodies is PtC, a membrane component exposed on senescent- or bromelain-treated red blood cells (63). Using fluorescein-containing PtC liposomes, PtC-specific cells can be directly analyzed by flow cytometry (54). The data in Table 1 show a normal frequency of CD5 B cells and of PtC-binding cells in the peritoneum of δT/δT mice. In the spleen of δT/δT mice, such cells are undetectable as they are in the controls. Thus, the δT mutation does not affect the recruitment of B cells into the compartment of CD5 or conventional B cells.

The frequency of peripheral T cells and the ratio of CD4/CD8-positive cells in IgD-deficient mice (Table 1) was also found to be normal. The slight differences between control animals and IgD-deficient mice are within the range observed between the parental strains C57Bl/6 (33% ± 6.7% LN T cells; CD4/CD8 ratio = 2.0 ± 0.2%) and 129 (see Table 1, +/+ ) and, therefore, most likely due to the variable genetic background of the mutant mice.

A Long-lived Peripheral B Cell Pool Is Established in IgD-deficient Mice. The majority of mature resting μ⁺δ⁻ B cells of an adult mouse is "long-lived" with a half-life of several weeks or months (55). To examine the life span of peripheral B cells in homozygous δT/δT mice, we measured BrdU incorporation into spleen cells of mice fed for 6 d with BrdU in the drinking water. The result (Fig. 4) shows the same degree of labeling of splenic B cells in homozygous mutant (δT/δT)
Figure 4. B cell life span in IgD-deficient mice. Spleen cells of wild-type (+/+) and homozygous mutant mice (ST/ST) fed for 6 d with 1 mg BrdU per ml drinking water (left and center) were analyzed by flow cytometry for BrdU incorporation. (Right) Unlabeled control. The numbers in the upper right quadrant are percent of B cells (CD45R/B220+) staining with the anti-BrdU antibody. Each number refers to one individual mouse.

and wild type (+/+) animals, indicating that expression of IgD is not required for establishing the long-lived peripheral B cell pool of an adult mouse.

μδδ- B Cells Expressing the Targeted Allele Are Underrepresented in Heterozygous Mutant Mice. Comparison of the frequency of B cells expressing the targeted or the wild type allele in mice heterozygous for the ST mutation reveals an underrepresentation of B cells expressing the targeted allele (Fig. 3; Table 2). This underrepresentation, observed in PBLs as well as splenic B cells, indicates that B cells expressing the wild type allele have an advantage in populating the peripheral B cell pool over the μδδ- population. This becomes apparent only in the case of competition between μδδ- and μδδ+ B cells in heterozygous mutant mice. In homozygous mutant mice, in the absence of competing μδδ+ B cells, μδδ- B cells are present in normal numbers (Table 1; Fig. 3).

Serum Ig in IgD-deficient Mice. Serum Ig isotype levels of 10-wk- and 6-mo-old homozygous mutant and wild-type mice are shown in Fig. 5, a and b. Igs of the various isotypes except IgE are present in homozygous mutant mice at levels equal or close to those of controls. The lower level of IgG2a in 10-wk-old mutant mice compared with 13-wk-old controls (Fig. 5 a) could be due to the age difference of the animals. IgG2a levels were the same in 6-mo-old homozygous mutant and control mice (Fig. 5 b). It is interesting, however, that the IgE levels were lower in IgD-deficient than control mice at both time points. This may indicate that triggering of B cells by antigens that elicit an IgE response in IgD-deficient mice is less effective in IgD-deficient mice.

IgD-deficient Mice Respond Efficiently to TI-II Antigens. We investigated the immune responsiveness of IgD-deficient mice to the TI-II antigens α(1-3)-dextran and NP-Ficoll. The response to α(1-3)-dextran of IgH* allotype mice is dominated by κ L chain-bearing antibodies and appears largely derived from CD5 B cells, whereas the anti-NP response originates from conventional B cells (51). The results are shown in Fig. 6. It is evident that IgD-deficient mice are capable of mounting efficient immune responses against TI-II antigens.

IgD-deficient Mice Respond to T Cell-dependent Antigen. Fig. 7 shows that the levels of antigen-specific Ig in primary and

Table 2. Frequency (percent) of B Cells Expressing the IgH* or IgH* Allele in Wild-type F1 (IgH*, +/IgH*, +) and Heterozygous Mutant F1 (IgH*, ST/IgH*, +) Mice at 3 or 6 Mo of Age

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<tr>
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<th>PBL</th>
<th>Spleen</th>
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<td></td>
<td>3 mo</td>
<td>6 mo</td>
</tr>
<tr>
<td>IgH*, +/IgH*, +</td>
<td>16.1%/13.7 jerk</td>
<td>11.9 ± 0.8/17.0 jerk ± 1.9 (n = 5)</td>
</tr>
<tr>
<td>IgH*, ST/IgH*, +</td>
<td>0.7 ± 0.06</td>
<td>0.59 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>0.99</td>
<td>0.75</td>
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When more than two animals were analyzed, mean and SD are given.

* Percent B cells expressing the IgH* allele (μδ+ and/or δδ+).
† Percent B cells expressing the IgH* allele (μδ+ and/or δδ+).
§ Percent IgH* divided by percent IgH*.

Figure 5. Serum Ig isotypes in IgD-deficient mice. (a) Concentration (μg/ml) of Ig isotypes in sera of 10-wk-old homozygous mutant (closed circles) and 13-wk-old control animals (open circles) and (b) sera obtained from 6-mo-old animals.
Figure 7. Serum concentrations of hapten (NP)- and carrier (CG)-specific antibodies of ST/ST (closed circles) and control mice (open circles) immunized with NP-CG. (a) Primary response day 6; (b) primary response day 14; and (c) secondary response day 6. NP-specific, as well as CG-specific titers are given relative to anti-NP mAbs of the various isotypes used as standards. 1 unit corresponds to ~1 μg.

Secondary responses of IgD-deficient mice to the TD antigen NP-CG reach those of control mice; only at day 6 are lower CG-specific titers observed in IgD-deficient mice (Fig. 7 a). This may reflect a delayed onset of the carrier-specific response in IgD-deficient compared with control mice.

Affinity Maturation of Serum Antibodies Is Delayed in IgD-deficient Mice. Relative affinities of serum antibodies were determined using a plate binding assay that is based on the correlation of the affinity constant with the relative binding of antibodies to coats of low and high epitope densities (36). This assay offers a sensitive test to compare relative affinities of serum antibodies, and standardization using mAbs of known affinity allows an indirect measurement of the average affinity of serum antibodies in a first approximation. Fig. 8 shows titers of NP-specific IgG1 (top) and the corresponding ratios of NP-binding at low and high hapten density as a relative measurement of antibody affinity (bottom) in immune sera obtained at different time points after primary (Fig. 8, a–c) and secondary immunization (Fig. 8 d). In the control groups as well as in IgD-deficient mice, binding ratios of NP-specific IgG1 increase, indicating affinity maturation in the course of the primary response. Furthermore, virtually the same level of affinity is reached in secondary response sera of wild-type and IgD-deficient mice. It is interesting, however, that when comparing the responses of both groups at a given time point—although the level and kinetics of IgG1 production by IgD-deficient mice are the same as that of the controls—a significantly lower binding ratio of primary response IgG1 in IgD-deficient mice is consistently observed in three independent experiments using different doses of antigen and including mice of different genetic background (Fig. 8, a–c). Although the differences in affinity are not dramatic, it would...
appear that the early primary IgG1 response (of IgD-deficient mice) is less effective than that of IgD-competent animals, because affinity maturation, i.e., production of high affinity antibodies, is delayed by 3–4 d (Fig. 8, a–c), imposing a possibly critical disadvantage on IgD-deficient mice in the defense against pathogens.

\[\mu^-\delta^- B\] Cells Are Preferentially Recruited into the T cell-dependent Immune Response in Heterozygous Mutant Mice. The analysis of mice heterozygous for the \(\delta T\) mutation allows a direct comparison of the relative contribution to the anti NP-CG response of B cells expressing the targeted allele and B cells expressing the wild type allele. Fig. 9 shows NP-specific IgG1 derived from either allele in control F1 (\(\mu^+\delta^+\), \(+/\delta^+\), +) and heterozygous mutant F1 mice (\(\mu^+\delta^-\), \(\delta^T/\delta^H^b, +\)), measured at various time points after immunization. The amount of NP-specific IgG1 derived from the \(a\) allele and the \(b\) allele is similar in control F1 mice, resulting in a ratio in the geometric mean values of 1.18 for IgG1/IgG1 (416/352 \(\mu G/ml\)) at day 10 of the response (Fig. 9 c). In contrast, a ratio of 0.23 (148/643 \(\mu G/ml\)) is observed in heterozygous mutant mice bearing the \(\delta T\) mutation on the IgH \(a\) allele. This results from a reduction of NP-specific IgG1\(a\)-derived from the targeted allele, accompanied by a compensatory increase in NP-specific IgG1\(b\) produced by B cells expressing the wild type allele.

Discussion

Gene Targeting Technology Provides a Tool to Directly Assess the Role of IgD In Vivo. Experiments conducted in the past to assess the biological role of IgD in B cell development were largely based on the selective removal of IgD from the B cell surface by treatment with proteolytic enzymes or anti-IgD antibodies in vivo or in vitro. The results, however, were contradictory and a consistent picture did not emerge (13, 22, 23, 25–30). This may be partly due to the fact that these experimental approaches involved proteolytic digestion of surface proteins, likely to affect surface proteins other than IgD on the one hand, and to be incomplete on the other, or that engagement of surface Ig which — apart from removing IgD — might alter the activation state of the B cell. The disruption of the Ca gene in the mouse germline allows a more direct investigation of the biological role of IgD. Extending our previous observations (44), the present results exclude an essential role for IgD in B cell development and maturation, and suggest that the major function of IgD resides in its capacity to improve the efficiency of the early antibody response.

Models Assigning to IgD a Major and Unique Regulatory Function Are Not Supported by the Phenotype of IgD-deficient Mice. The fact that IgD-deficient mice respond to TI-II and TD antigens rules out a critical role for IgD in the acquisition of immune responsiveness. Thus, models proposing IgD as an essential triggering receptor in general (22–24) or for the activation of B cells by TI-II (25) or TD (26) antigens do not gain support from the analysis of IgD-deficient mice. Furthermore, an important role for IgD in maintaining B cell tolerance (42) is called into question, because IgD-deficient mice do not develop any obvious symptoms of autoimmunity.

IgD As an Optimized Surface Receptor Improving B Cell Recruitment by Antigen. The analysis of heterozygous mutant mice allows a direct comparison within the same animal of the effect of the \(\delta T\) mutation, because in these animals \(\mu^-\delta^-\) and \(\mu^-\delta^-\) B cells compete with each other. We have previously shown that the peripheral pool of long-lived \(\mu^-\delta^-\) cells in normal mice is strongly selected for the dominant expression of certain antibody specificities and have interpreted this as antigen-driven B cell selection (64). In the heterozygous mutant mice, the IgD-deficient B cells are losers in this competition in that they are underrepresented in comparison with their IgD-positive counterparts (Table 2). Similarly, lower levels of IgG1 are produced in these animals from the targeted allele in the primary response to NP-CG, accompanied by a compensatory increase of IgG1 produced by B cells expressing the wild-type allele (Fig. 9). This likely reflects more efficient recruitment of the latter cells into the response rather than simply their more abundant occurrence in the peripheral pool, because the difference in response is fivefold at day 10 compared with a 1.5-fold difference in pool size (Table 2; Fig. 9). It thus appears that IgD facilitates B cell recruitment into certain differentiation pathways. This is also and perhaps most strikingly observed at the level of affinity maturation in the early TD primary response as discussed below.

It is tempting to speculate that IgD may represent a particularly efficient antigen-binding receptor because of its unique structural flexibility. This is conferred on the molecule by an extended hinge region. Additionally, the H chains of the dimeric receptor are covalently linked by only one disulfide bridge close to the carboxy terminus. These structural characteristics allow for a higher degree of freedom of the antigen binding sites than observed for other Ig isotypes (8). The role of hinge regions in generating flexibility of Ig molecules, facilitating antigen binding has been established (65), and the high degree of susceptibility to proteolysis of IgD may be merely a consequence of its flexibility (65), rather than being of functional importance per se (24, 45). Because of its flexibility, IgD would allow more efficient binding of multimeric antigen than the more rigid IgM, in that it would more easily bind target epitopes with both V domains simultaneously. Receptor occupancy could be further increased if both antigen binding sites were able to move independently of each other as "half molecules" (H1/L1). Significantly, the existence of IgD half molecules (H1/L1) in equilibrium with monomers on the B cell surface has been demonstrated (66, 67). In light of these data, IgD could be regarded as an optimized surface antigen receptor allowing high-avidity binding of B cells to antigen presented in multimeric form, e.g., any antigen displayed on the surface of APCs.

A Role for IgD in Affinity Maturation of the Antibody Response. Affinity maturation and the establishment of high affinity B cell memory is apparently taking place in IgD-deficient mice, but affinity maturation is delayed compared to wild-type animals (Fig. 8). Although reservations may have
to be placed on the precision of a plate binding assay in determining absolute affinities, the data clearly show a reduced capability to trap antigen and a delayed maturation of serum antibodies in the early primary response of IgD-deficient mice compared to controls. Additional support to the significance of the data is provided by the fact that no differences in binding ratios of anti-NP IgG antibodies elicited by the TI hapten of the data is provided by the fact that no differences in binding conjugate NP-Ficoll were observed between wild-type and IgD-deficient mice (data not shown).

Because affinity maturation through hypermutation occurs in germinal centers (68, 69) where IgD is downregulated in the proliferating B cells (2, 70), it is likely that IgD affects the efficiency of affinity maturation in a similar way as discussed above, namely at the stage of recruitment of the cells into this particular differentiation pathway. It is not obvious why this effect should be more apparent at the level of affinity maturation than at the level of antibody production and kinetics of the primary anti-NP response which appears to be similar in IgD-deficient mice and controls (Figs. 8 and 9; and data not shown). This seems in contrast to the advantage of B cells expressing the wild-type IgH locus in the primary response to NP-CG in heterozygous mutant mice (Fig. 9 and previous section). The latter phenomenon, however, could well be due to a more efficient affinity maturation in the wild type cells, which would further support a particular role for IgD in the recruitment of B cells into the germinal center response. For technical reasons we have not been able to determine allotype-specific affinities in heterozygous mice.

Perhaps B cells are recruited into the germinal center at a time when antigen becomes limiting and/or through the recognition of antigen-antibody complexes on the surface of APCs. In both cases, the flexibility of surface IgD may be particularly important for the B cell to efficiently interact with antigen and perhaps with the cell presenting it. The alternative possibility, namely that a signal delivered through IgD is required to recruit B cells into germinal centers is not only because of the results of Vonderheide and Hunt (71), but also because we have observed substantial numbers of histologically normal germinal centers in the spleens of IgD-deficient mice (unpublished data).

Finally, it should be pointed out that despite the occurrence of germinal centers in IgD-deficient mice and the ability of these animals to produce high affinity secondary response antibodies that are indistinguishable from those of control mice (Fig. 8d), we cannot formally rule out at this point that in the absence of surface IgD the hypermutation mechanism operating on rearranged V region genes in germinal centers (68, 69) is not turned on. Although this possibility seems remote in light of the evidence that affinity maturation in I chain-bearing anti-NP antibodies is due largely, if not exclusively, to somatic hypermutation rather than the outgrowth of preexisting, rare, high affinity clones, as was shown for antibodies of the b allotype (72) sequence analysis of rearranged antibody V region genes from the mutant animals, is required to definitively settle this point. Such an analysis is presently underway.

Our data indicate that the unique function of IgD is that of an auxiliary surface-bound antigen receptor. This is in line with the earlier work of Puré and Vitetta who reported that surface expression of IgD facilitated the response to antigens of low epitope density in vitro (38). IgD would mediate efficient interaction of B cells with their target antigens, and perhaps also with Th cells through IgD-binding receptor molecules (40, 41). The principle of dual isotype expression may have allowed an evolutionary optimization of the IgD molecule for serving this function, whereas IgM would be free to be optimized to efficiently mediate effector functions in the early humoral response.

In the initiation of the germinal center response, the contribution of IgD to B cell recruitment appears to be particularly important. Once driven into this differentiation pathway, the cells downregulate surface IgD with its presumed high avidity for antigen complexed on the membrane of follicular dendritic cells, allowing stringent selection of B cells expressing high affinity antibodies.

Although the difference in antibody affinities between control animals and IgD-deficient mice in the course of the early primary TD response are only moderate, it would seem from the data in Fig. 8 that in the mutant mice, affinity maturation, i.e., the production of antibodies of high affinity, is delayed by 3–4 d as compared with the controls. Thus, expression of IgD could confer a critical advantage in the defense against pathogens undergoing rapid expansion and mutational drift upon entry into the host.

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References


