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Immunology

Immunoglobulin D-deficient mice can mount normal immune responses to thymus-independent and -dependent antigens

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ABSTRACT **To examine the *in vivo* function of IgD we generated mice deficient for IgD by gene targeting. The IgD$^-$ mice show a reduced B-cell compartment with 30–50% less B cells in the spleen and lymph nodes but show a normal pre-B-cell compartment. The surface-IgD$^-$ B cells express two to three times more surface IgM than B cells of control animals. Serum concentrations of the immunoglobulin isotypes of IgD$^-$ mice are almost normal, indicating that surface-IgD expression is not necessary for class switching of B cells. Immunization experiments showed that IgD$^-$ mice could respond well to thymus-dependent and -independent antigens. After immunization normal germinal centers developed in the IgD$^-$ mice. These data suggest that IgD is not necessary for the induction of immune responses but may be important in homeostasis of cells in the B-cell compartment.**

In adult mice normal B cells are produced in the bone marrow. They first express only IgM as antigen receptor on their surface. When leaving the bone marrow as mature B cells, they coexpress a second antigen receptor, IgD, of the same specificity as IgM (1–3). Naive B cells and B cells located in primary follicles express both IgD and IgM, whereas splenic marginal-zone B cells and a large proportion of memory B cells only express IgM (5, 6). Upon activation, B cells further differentiate to plasma cells that are surface-immunoglobulin negative. This strict regulation of IgD expression suggests a functional role distinct from IgM. Experiments *in vitro* have shown that the signals mediated by engagement of surface IgD (sIgD) differ from those mediated by surface IgM: only the latter caused apoptotic death of the cells (7, 8). *In vivo* the functions attributed to the engagement of sIgD vary from the acquisition of resistance to tolerance induction (10–12) and the initiation of the B-cell response (13) to a role in B-cell memory (6, 14, 15). Some of these functions were studied by using chronic anti-IgD treatment from birth (16, 17), by *in vitro* sorting of sIgD$^+$ and sIgD$^-$ B cells (10, 18, 19), by enzymatic removal of sIgD *in vitro* (11), or by transgenic mouse models (20). Discrepancies of results were found in these studies that could be generated by the nature of the indirect experimental systems used. Roes and Rajewsky (21) presented preliminary evidence that chimeric mice in which one of the δ heavy-chain constant region (C_H) alleles was deleted could respond to TD antigens. We show here that mice germ line deficient for IgD can respond to TD and TI antigens, have normal germinal-center formation, but have a smaller B-cell compartment.

MATERIALS AND METHODS

Targeting Construct. The targeting vector for IgD contained one exon of the constant region (Cδ3) and exons for the secretory and membrane-spanning part of the protein (δS, δX, δM1, and δM2) (Fig. 1*A*). Into the *Sst* I site within exon Cδ3 a translational stop codon as well as a neomycin-resistance gene cassette of the vector pMC1neo-polyA (22) (Stratagene) was introduced. The herpes simplex virus–thymidine kinase gene of pIC19R/MC1-tk (23) was added 5′ of Cδ3.

Cell Culture and Transfections. Embryonic stem (ES) cells of clone D3 (derived from the 129/Sv agouti mouse strain) (24) were maintained on feeder layers from primary embryonic fibroblasts in medium containing leukemia inhibitory factor (25). The ES cells were transfected with the linearized targeting construct by electroporation and selected with medium/G418/gancyclovir (23).

Embryo Manipulations. Blastocysts (3.5 days postcoitus) were collected from C57BL/6 mice and injected with 15–30 trypsinized ES cells. Injected blastocysts were reimplanted to the uteri of 2.5-day pseudopregnant females.

PCR and Southern Blots. ES cell clones were picked and prepared for a nested primer PCR analysis, as described (35). Genomic DNA for Southern blotting was prepared from ES cells or from the tail ends of mice.

Flow Cytometric Analysis (FCM). FCM of single-cell suspensions of lymphatic organs was done on a FACScan flow cytometer (Becton Dickinson). The antibodies used for staining were either (*i*) biotinylated and counterstained with streptavidin–phycoerythrin 11-26C (anti-IgD, from J. Kearney, University of Alabama, Birmingham) (Fig. 2 *a* and *b*), M41 (anti-IgM) (27) (Fig. 2 *c* and *d*), B7/6 (anti-IgM) (27) (Fig. 2 *g* and *h*) or (*ii*) conjugated with fluorescein isothiocyanate M41 (anti-IgM) (Fig. 2 *a* and *b*), M1/69 [anti-heat-stable antigen (HSA)] (28), AMS 15.1.5 (anti-IgD^a) (29), AFS 122.2.51 (anti-IgD^b) (29), and 11-26C (Fig. 2 *e* and *f*) or (*iii*) directly phycoerythrin-labeled: RA3-6B2 (anti-B220) (30).

ELISA Analysis and Immunizations. (*i*) Immunoglobulin-isotype levels. Serum levels of 10-week-old untreated mice were determined by ELISA. Class-specific unlabeled and alkaline phosphatase (AP)-labeled antibodies were obtained from Southern Biotechnology Associates (Birmingham, AL). Anti-IgE monoclonal antibodies (mAb) 84-1C (unlabeled) and EM95.3 (AP-labeled) were from Z. Eshhar (Wiezmann Institute, Rehovot, Israel). (*ii*) Dextran immunizations. The +/− F_1 mice (Ig^a, IgD$^-$/Ig^b) and +/+ F_1 controls (Ig^a/Ig^b) were immunized i.p. or i.v. with 100 μg of 1,3 α-dextran (from P. Slodki, Northern Regional Research Lab., Peoria, IL). Plates were coated with dextran and λ-bearing antibody was detected with AP-coupled GaM λ antibody by ELISA. Serum concentrations were calculated by using mAb MOPC 104E as standard. (*iii*) 2,4-Dinitrophenol–ovalbumin (DNP-ova) immunizations. Eight-week-old F_1 mice [+/− (Ig^a, IgD$^-$/Ig^b) and +/+ (Ig^a/Ig^b)] were immunized i.p. with 100 μg of

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Abbreviations: sIgD, surface immunoglobulin D; C_H, constant region of heavy chain; ES cells, embryonic stem cells; FCM, flow cytometric analysis; GaM, goat anti-mouse antibody; mAb, monoclonal antibody; TD, thymus-dependent; TI, thymus-independent; AP, alkaline phosphatase; HSA, heat-stable antigen; DNP-ova, 2,4-dinitrophenal-ovalbumin.

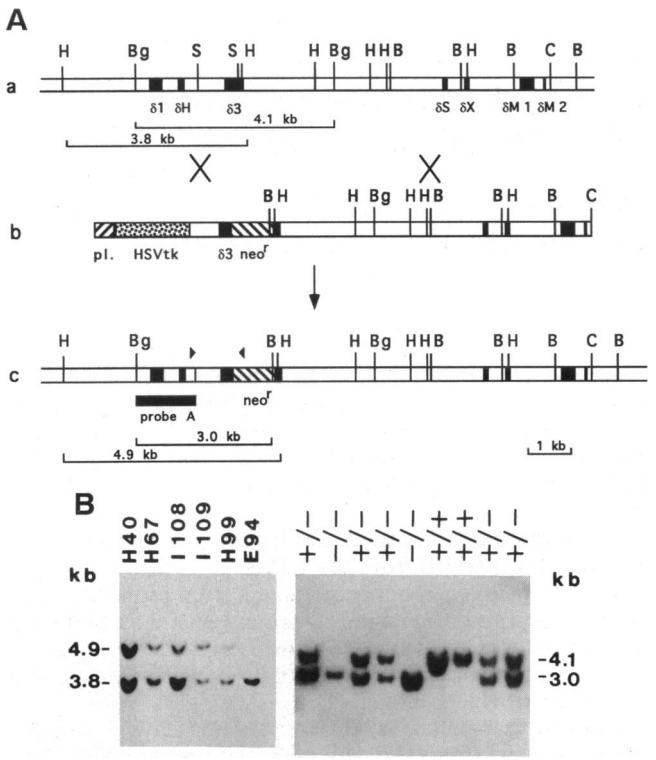

FIG. 1. (*A*) (*a*) Genomic structure of constant region of Cδ gene; exons are represented by black boxes. The lengths of two diagnostic restriction fragments are shown. (*b*) Targeting vector. HSVtk, herpes simplex virus thymidine kinase; neo^r, neomycin resistance. (*c*) Predicted structure of targeted locus. PCR primers are shown by triangles. The length of diagnostic restriction enzyme fragments and the hybridization probe A are indicated. B, *Bam*HI; Bg, *Bgl* II; C, *Cla* I; H, *Hin*dIII; S, *Sst* I. (*B*) (*Left*) Southern blot analysis of five PCR-positive ES cell clones (H40, H67, I108, I109, H99) and a negative control (E94). Ten micrograms of genomic DNA was digested with *Hin*dIII; the predicted size of an additional 4.9-kb fragment is visible. (*Right*) Analysis of a litter of nine mice of the F_2 generation, derived from clone H67. Tail DNA was digested with *Bgl* II/*Bam*HI. Predicted sizes of 4.1 kb (wild-type allele) and 3.0 kb (targeted allele) are visible.

alum-precipitated DNP-ova with *Bordatella pertussis* as an adjuvant (31) at day 0 and boosted at days 7 and 14. The 10- to 12-week-old F_2 −/− (Ig^a,IgD$^-$/Ig^a,IgD$^-$) or F_2 +/+ (Ig^b/Ig^b) mice were primed s.c. with 20 μg of alum-precipitated DNP-ova with *Bordatella pertussis* and boosted i.v. and above each hind footpad at day 14. The primary (day 7 or day 14) and secondary responses (day 21) were measured by using an ELISA system with DNP-ova-coated plates. GaM IgM, GaM IgG1, GaM IgG2b (all Southern Biotechnology Associates), mAb RS3-1 (anti-IgM^a) (30), mAb 20.9.10.1 (anti-IgG1^a) (32), mAb 20.8.3 (anti-IgG2a^a) (32), all AP-labeled, were used for detection.

Immunohistochemistry. Cryostat sections were prepared from spleens and lymph nodes. The sections were double-stained for either (*i*) IgM$^+$ cells (sheep anti-mouse IgM conjugated to Texas red, Southern Biotechnology Associates) and T cells (rat anti-mouse Thy-1, T24) (33) or (*ii*) IgM$^+$ cells and major histocompatibility class II$^+$ cells (rat anti-mouse Ia, M5/114) (4). Rat antibodies were detected by fluorescein isothiocyanate-conjugated mouse anti-rat IgG F(ab')$_2$ fragments (Jackson ImmunoResearch). The sections were evaluated and photographed by using a Zeiss Axioscop microscope.

RESULTS

Gene Targeting. After transfecting ES cells, six positive ES cell clones out of 420 G418- and gancyclovir-resistant clones were detected by PCR. Targeting to the correct locus was confirmed by genomic Southern blotting with three different restriction enzyme digests and probes, one of which is shown (Fig. 1*B*, *Left*). Chimeras derived from three injected ES cell clones transmitted the mutation with 80–100% agouti offspring. Mice were screened by Southern blotting (Fig. 1*B*, *Right*). The phenotype described in experiments below was identical in the offspring of the three chimeras.

FCM Analysis of Peripheral Lymphoid Organs of IgD$^-$ Mice. Mice carrying the mutation (IgD$^-$) were born with expected frequencies. Spleen cells of homozygous (IgD$^-$/IgD$^-$) and heterozygous mice (IgD^{a-}/IgD^{b+}) showed no remaining sIgD when stained with an isotype-specific (Fig. 2 *b* and *d*) or allotype-specific (Fig. 2*c*) mAb. Northern blot analysis of spleen cells of IgD$^{-/-}$ mice confirmed the successful targeting. mRNA specific for δ C_H was not detectable (data not shown).

Heterozygous animals that are IgM^a, IgD$^-$/IgM^b, IgD^b (allotype a derived from 129/sv mice, allotype b derived from

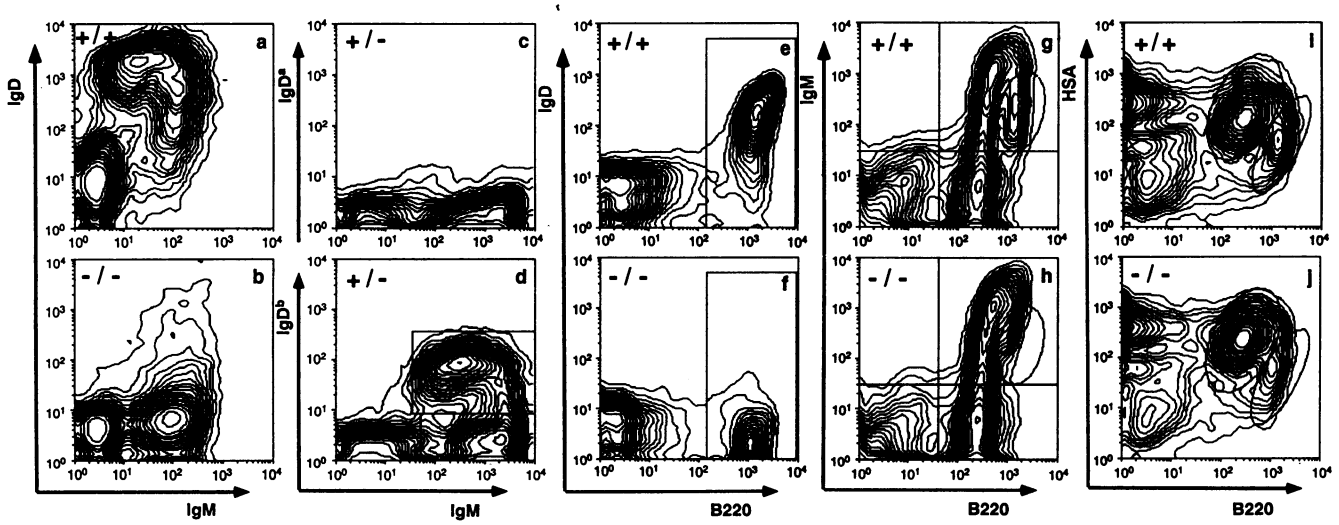

FIG. 2. FCM of spleen cells (*a–d*), of lymph node cells (*e* and *f*) and of bone-marrow cells (*g–j*) of 10-week-old (*a* and *b*) or 6-week-old (*e–j*) +/+ and −/− F_2 mice, or of 10-week-old +/− F_1 mice (Ig^a, IgD$^-$/Ig^b) (*c* and *d*). (*d*) Mean fluorescence intensity (mfi) of IgM: upper box = 494, lower box = 997. (*e*) mfi of B220 = 1506. (*f*) mfi of B220 = 1103. (*g*) Cell numbers: upper right, 22%; lower right, 34%; oval, 5%. (*h*) Cell numbers: upper right, 29%; lower right, 43%; oval, 3%. (*i*) Cell numbers: left oval, 46%; right oval, 6%. (*j*) Cell numbers: left oval, 63%; right oval, 4%.

Table 1. FCM of cell numbers

	F_1 mice				F_2 mice			
					Experiment 1		Experiment 2	
	C5.3 (+/+)	C5.10 (+/+)	C5.11 (+/−)	C5.11 (+/−)	CC32.12 (+/+)	CC32.11 (−/−)	DD4.2 (+/+)	DD4.4 (−/−)
Spleen cell, no.*	6.2	5.6	4.3	4.2	11	7.0	9.4	4.6
$B220^+$, IgM^{a+}	50%	50%	30%	31%				
$B220^+$, IgM^{b+}	46%	46%	65%	66%				
$B220^+$, IgM^+					60%	62%	64%	52%
$CD8^+$					7%	7%	8%	11%
$CD4^+$					16%	15%	17%	26%
LN								
$B220^+$, IgM^{a+}	49%	47%	35%	36%				
$B220^+$, IgM^{b+}	46%	44%	59%	58%				
$B220^+$, IgM^+					33%	19%	29%	27%
$CD8^+$					19%	23%	19%	18%
$CD4^+$					41%	55%	40%	50%
BM cell, no.*					0.86	0.82	1.1	1.5
$B220^+$, IgM^+					21%	25%	27%	24%
$B220^+$					56%	70%	61%	52%

Cells of F_1 mice (12-week-old littermates) were analyzed by 3-color FCM. Lymphocytes were gated for $B220^+$ cells, and IgM^{a+} and IgM^{b+} cell numbers were compared. F_2 mice [6-week (experiment 1) or 8-week (experiment 2) old littermates] were analyzed by 2-color FCM for the markers shown. Mice designated C5 or CC32 are derived from ES clone H67 (Fig. 1*B*); mice designated DD4 are derived from independent ES clone E97. The values indicated (%) are percentages of positive cells of a lymphocyte gate. LN, lymph node; BM, bone marrow.
*Cell number $\times 10^7$.

C57BL/6 mice) had ≈50% fewer B cells of the Ig^a allotype than of the Ig^b allotype in spleen and lymph nodes. F_1 controls (IgM^a, IgD^a/IgM^b, IgD^b), however, showed an allotype ratio of B cells of ≈1:1 (Table 1). Homozygous mice (IgD^-/IgD^-) showed a reduction of splenocytes and lymph node cells to 50–70% of the cell numbers of control animals. The B-cell, as well as the T-cell compartments, were affected by this reduction. Distribution of T cells in $CD4^+$ and $CD8^+$ cells was unaffected (Table 1).

The $sIgD^-$ B cells carried two to three times more sIgM (Fig. 2*d*). This result was seen on mature, recirculating B cells of the bone marrow and on spleen and lymph node B cells. B220, a pan-B lineage marker, was slightly (20%) reduced on mature IgD^- B cells. This reduction, shown on lymph node cells (Fig. 2 *e* and *f*), is from the missing population of the mature IgD^+, IgM^+ phenotype. The expression of the low-affinity receptor for IgE (CD23), HSA (CD24), Lyb-2 (CD72), and major histocompatibility complex class II, all markers for specific stages of B-cell maturation, were not affected by sIgD loss (data not shown).

FCM of Pre-B Cell Compartment. The cell number of B-lineage cells in the bone marrow was the same in −/− F_2 and +/+ F_2 mice (Table 1 and Fig. 2 *g* and *h*; compare numbers of IgM^+ cells). The population of B cells with higher expression of B220 (IgD^+ cells) was missing (Fig. 2 *g* and *h*, indicated by ovals). Staining of bone-marrow cells for antigens HSA and B220 showed a small reduction of the more mature, recirculating $B220^{hi}$ HSA^{lo} B cells in the $IgD^{-/-}$ mice (Fig. 2 *i* and *j*). From these experiments we conclude that the disruption of the δ C_H locus does not affect the development of pre-B cells. In summary, IgD^- B cells can differentiate to mature B cells and express most typical B-cell markers. The total number of B cells in the periphery is reduced, and sIgM is elevated.

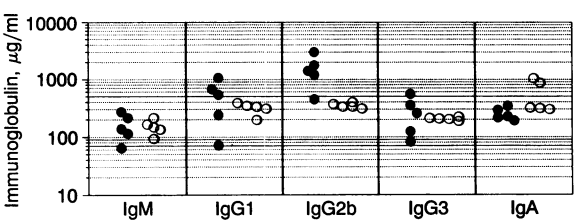

FIG. 3. Serum concentration of immunoglobulin isotypes in −/− F_2 (Ig^a, IgD^-/Ig^a, IgD^-) (○) and +/+ F_2 (Ig^b/Ig^b) mice (●). Immunoglobulin concentrations of 10-week-old mice were determined by ELISA, by using mAbs of different isotypes as standards.

Determination of Immunoglobulin-Isotype Levels. Serum levels of immunoglobulin isotypes of homozygous mice (Ig^a, IgD^-/Ig^a, IgD^-) were compared with F_2 controls (Ig^b/Ig^b) (Fig. 3). Isotype levels of IgD^- mice were comparable to control mice, except for IgG2b, which was four and one-half times lower and for IgA which was two times higher. The titer of IgE of both groups of mice was close to the limit of detection (not included in Fig. 3).

Immunization with TI and TD Antigens. To study the response to TI antigens, IgD^- mice were immunized with 1,3 α-dextran. Only mice of the Ig^a allotype respond to 1,3 α-dextran, and the response is always composed of λ antibodies (9). The +/− F_1 mice (Ig^a, IgD^{a-}/Ig^b) and F_1 controls (Ig^a/Ig^b), which are identical in genetic background, were injected i.p. and i.v. The dextran-specific λ or IgM^a response was analyzed. IgD^- B cells responded to dextran after i.p. injection (Fig. 4 *Left*); the medium titer of dextran-specific λ antibodies was slightly reduced compared with control littermates. After i.v. immunization no significant difference in anti-dextran titer was seen (Fig. 4 *Right*).

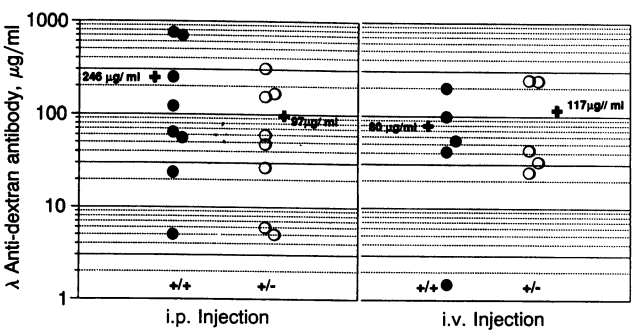

FIG. 4. Serum levels of anti-dextran λ antibodies on day 5 determined by ELISA. Values are arithmetic means. (*Left*) +/− F_1 mice (Ig^a, IgD^-/Ig^b) and +/+ F_1 controls (Ig^a/Ig^b) were immunized i.p. with 100 μg of α1,3-dextran; the mean titer at day 0 (before injection) was <1 μg per ml. (*Right*) F_1 mice were immunized i.v. with 100 μg of dextran; the mean titer at day 0 was 5.5 μg per ml.

Table 2. Immune response to DNP-ova

	IgM*	IgG2†	IgG1‡
		F_2 mice	
d 0 +/+	100 ± 41	100 ± 123	1
d 0 −/−	230 ± 231	42.7 ± 73	1
d 14 +/+	170 ± 250	890 ± 647	1999 ± 1374
d 14 −/−	504 ± 293	344 ± 437	1050 ± 1138
d 21 +/+	706 ± 443	5540 ± 6038	105,527 ± 69,590
d 21 −/−	693 ± 185	1838 ± 393	52,165 ± 33,710
		F_1 mice	
d 0 +/+	100 ± 23	100 ± 76	1
d 0 +/−	46.1 ± 43	96.3 ± 89	1
d 7 +/+	210 ± 51	ND	ND
d 7 +/−	87.1 ± 61	ND	
d 21 +/+	ND	2481 ± 2240	220 ± 274
d 21 +/−		2088 ± 1798	190.6 ± 254

−/− F_2 (Ig^a, IgD^-/Ig^a, IgD^-) mice (n = 4) and +/+ F_2 (Ig^b/Ig^b) mice (n = 4) were immunized with DNP-ova and boosted at day 14. +/− F_1 (Ig^a, IgD^-/Ig^b) mice (n = 5) and +/+ F_1 (Ig^a/Ig^b) mice (n = 4) were immunized with DNP-ova at day 0 and boosted at days 7 and 14. The primary response (day 7 or day 14) and secondary response (day 21) were measured by ELISA. Values are arithmetic means ± SD, given in arbitrary units per ml compared with the arithmetic mean of F_1 or F_2 control mice (day 0: set = 1 for IgG1 and $IgG1^a$; set = 100 for other isotypes). ND, not done.
*For F_1 mice, IgM^a.
†For F_2 mice, IgG2b; for F_1 mice, $IgG2a^a$.
‡For F_2 mice, $IgG1^a$.

To examine the response to TD antigens homozygous F_2 and heterozygous F_1 mice were immunized with DNP-ova, and total or allotype-a-specific antibody titers, respectively, were measured. Table 2 shows that IgD^- B cells could mount good primary and secondary responses. No significant difference was seen in the DNP-ova-specific antibody titers. The immune responses of −/− F_2 mice were, however, slightly lower than the responses of +/+ F_2 controls (Table 2). The anti-DNP-ova antibodies of the classes IgG3, IgE, and IgA were also measured and showed a low, but comparable, increase in both −/− and +/+ F_2 mice (data not shown). Immunization of heterozygous F_1 (+/− and +/+) mice with identical genetic background confirmed the results obtained with F_2 mice (Table 2).

Histology of Lymphoid Organs of Immune Mice. Evaluation by light microscopy showed a normal development of lymphoid-cell compartments in spleens and lymph nodes of −/− F_2 and +/+ F_2 mice after immunization with DPN-ova (Fig. 5). By using IgM to assess B-cell distribution, the marginal zones appeared similar in both groups. The number of germinal centers that developed seemed quantitatively equal, as were the T-cell zones. The number of IgM^+ plasma cells was also comparable. Histological evaluation provided no obvious differences in phenotype or distribution of B and T cells.

DISCUSSION

IgD is present in low quantities in human serum, virtually absent in mouse serum, but the most prominent surface immunoglobulin on mature mouse B cells. The δ C_H locus is not preceded by a classical switch region. Expression of IgD seems to be regulated by transcriptional and posttranscrip-

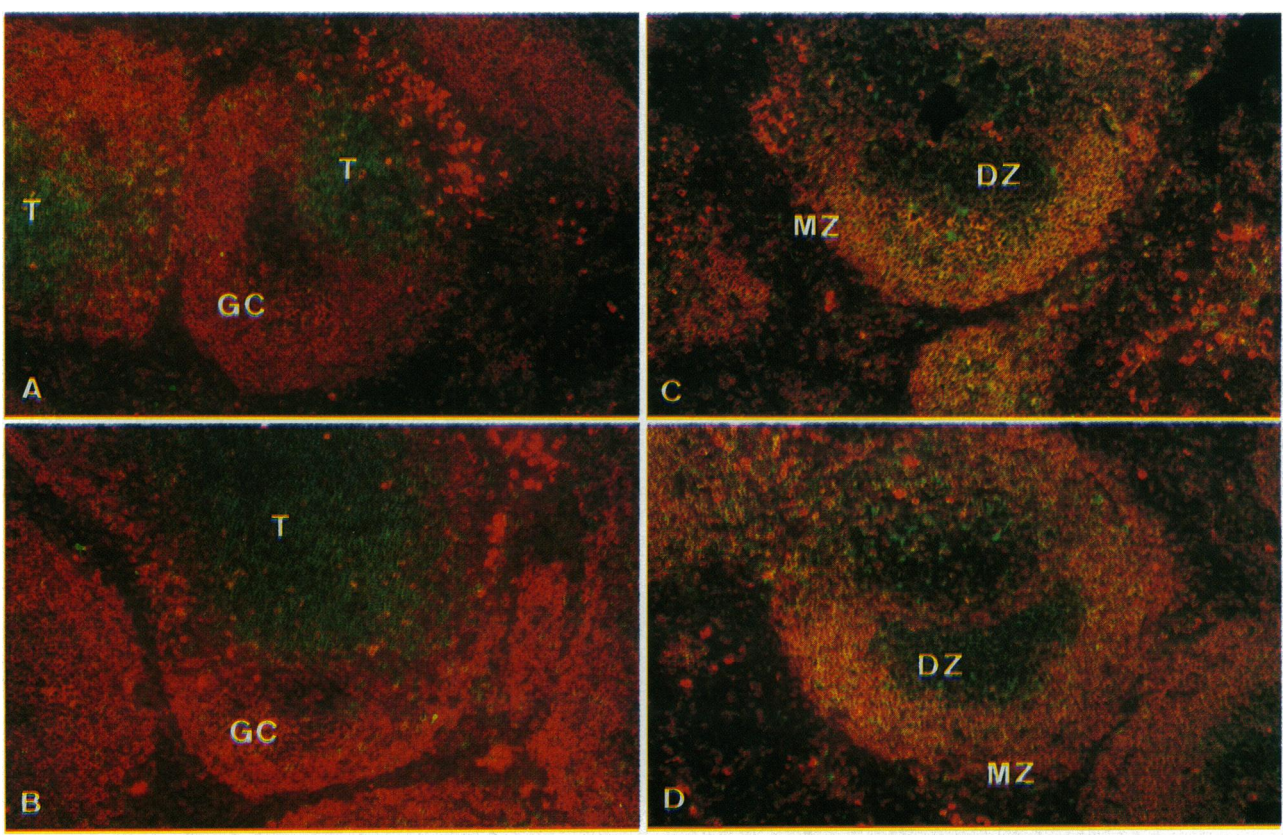

FIG. 5. Light micrographs showing the distribution of T cells or major histocompatibility complex class II^+ cells and IgM^+ cells in spleens of mice primed and boosted with DNP-ova (from group in Table 2). (*A* and *C*) −/− F_2 mice. (*B* and *D*) +/+ control F_2 mice. In *A* and *B*, IgM^+ B-cell areas (red) and T-cell zones (T, green) are clearly developed in both groups. Also, germinal centers (GC) with their characteristic dark and light zones are visible in both groups. In *C* and *D*, expression of class II allows delineation between the Ia^{lo},IgM^+ population of B cells in the marginal zones (MZ) and the double-staining Ia^{hi}, IgM^+ B cells of the dark zone of the germinal center. Above this area is the Ia^{hi},IgM^- dark zone (DZ) of the germinal center. Dendritic cells, highly positive for class II (green), can be seen in the T-cell zone above the germinal center.

tional modification (3). This regulation has led to the hypothesis that IgD is not primarily involved in the humoral defense but rather in the regulation of B-cell responses.

A notable effect of disrupting the δ C_H locus was the reduction of the peripheral B-cell population. This result was most clearly seen in the F_1 animal. Cells expressing the immunoglobulin locus of the targeted allele were reduced to one-third of all B cells. This relative reduction of affected B cells was less clearly seen in $IgD^{-/-}$ F_2 mice. In these mice, however, a reduction in total lymphoid cells was found.

Our results indicate that sIgD plays a role in the homeostasis of the B-cell, and perhaps the T-cell, compartment. Pre-B cells were present in normal numbers, and their cell-surface markers showed a normal distribution. Also unaffected was the population of IgM^+, IgD^- cells. Reduced in number, however, were the more mature recirculating, normally IgD^+, $B220^{++}$ cells (Fig. 2*h*). We conclude that disruption of the δ C_H locus does not affect B cells in their early stages of development. These data are not compatible with a lower production as cause for the decreased number of peripheral B cells. A more likely explanation is shorter survival or limited expansion.

IgD^- B cells are, however, able to differentiate to plasma cells. Serum immunoglobulin isotypes of IgD^- mice were found in normal amounts, showing that IgD is not a prerequisite for class switching. Immune responses to both TI and TD antigens could be generated. The ability of IgD^- B cells to respond to TI antigens contradicts earlier *in vitro* results (10, 19), in which $sIgD^+$ cells were shown necessary for the response to TI antigens. These discrepancies can be explained by differences in treatments and protocols. Isolation of IgD^- cells or suppression by anti-IgD antibodies most likely selects for cells of an immature phenotype that are unable to participate efficiently in an immune response.

IgD^- cells from both heterozygous and homozygous mice responded to TD antigens with adjuvant. The normal germinal-center formation agrees with these results. Previous experiments with chronic anti-IgD treatment from birth both confirm (16, 17) or conflict (17, 26) with our results. Suppression of one allotype in an F_1 mouse by allotype-specific anti-IgD treatment almost abrogated the immune response derived from this allotype. Suppression of both allotypes, however, resulted in a normal immune response (17). These results suggested an advantage of the nonsuppressed allotype and the emergence of compensatory mechanisms in the double-suppressed animals. Analysis of our $IgD^{-/+}$ F_1 animals did not show a clear dominance of the immune response from the wild-type allotype. Even if compensatory mechanisms exist for the lack of IgD *in vivo*, our results show that IgD is not indispensable for immune responses.

sIgD has been shown to interfere with tolerance induction (10–12). We have shown that the HSA^{hi}, $B220^{lo}$, IgM^+ population in the bone marrow is not sensitive to tolerance induction, but that the later stage, the HSA^{lo}, $B220^{hi}$, IgM^+ population, is sensitive to this induction (34). This latter population is reduced in the IgD^- mice to some extent (Fig. 2*j*). We have, however, not yet specifically addressed tolerance induction in these mice. B-cell memory and affinity maturation, functions also attributed to IgD, do not seem to be grossly disturbed, but this analysis awaits further studies.

In conclusion, we find no serious impairment of the immune response when the mice are challenged with TI antigens or TD antigens. However, a reduced B-cell compartment in the F_1 mice and a reduction in total lymphoid cells in the F_2 animals point to a role of IgD in B-cell homeostasis.

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