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Immunology

RAG-2-deficient blastocyst complementation: An assay of gene function in lymphocyte development

(recombination-activating gene/embryonic stem cells/targeted mutation/somatic chimeras/lymphocyte differentiation)

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ABSTRACT **We describe a system to evaluate the function of lymphocyte-specific and generally expressed genes in the differentiation and/or function of lymphocytes. RAG-2 (recombination-activating gene 2)-deficient mice have no mature B and T lymphocytes due to the inability to initiate VDJ recombination. Blastocysts from RAG-2-deficient mice generate animals with no mature B and T cells following implantation into foster mothers. However, injection of normal ES cells into RAG-2-deficient blastocysts leads to the generation of somatic chimeras with mature B and T cells all of which derive from the injected ES cells (referred to as RAG-2-deficient blastocyst complementation). Complementation of RAG-2-deficient blastocysts with mutant ES cells heterozygous for a targeted mutation that deletes all immunoglobulin heavy-chain joining (J_H) gene segments ($J_H^{+/-}$) also leads to generation of chimeras with normal B and T cells. However, complementation with ES cells homozygous for the J_H mutation ($J_H^{-/-}$) generates animals with normal T cells but no B cells, due to a block in B-cell development at a very early stage. Transfection of a functionally assembled μ heavy-chain gene into the $J_H^{-/-}$ ES cells prior to blastocyst injection rescues the $J_H^{-/-}$ mutation and allows the generation of both mature T and mature B cells. The rescued B cells express IgM but not IgD and respond normally to bacterial lipopolysaccharide stimulation by proliferating and by secreting IgM.**

The immune system is particularly amenable to gene-targeted mutational analysis because it is dispensable if animals are maintained in pathogen-free conditions (1). However, the full characterization of gene function by such methods often requires not only the inactivation of the gene by debilitating mutation but also an efficient method to complement such mutations by introduction of modified genes into the null background. For such analyses, germ-line gene mutations can only be complemented by breeding with appropriately constructed transgenic lines. Further, certain genes cannot be tested for lymphocyte-specific functions by introduction of germ-line mutations because their encoded activities are required for early developmental events in nonlymphoid cells (e.g., refs. 2 and 3). In this report we describe an efficient method to assess the role of almost any gene or regulatory element in the differentiation and/or function of lymphocytes and use this approach to further define the function of the IgM heavy chain (μ) in B-cell development.

B- and T-lymphocyte differentiation occurs through an ordered series of steps which include the assembly of immunoglobulin (Ig) variable-region genes from variable (V), diversity (D), and joining (J) gene segments in precursor B cells and T-cell antigen receptor variable-region gene in precursor T cells (4, 5). Initiation of the VDJ recombination process responsible for these gene assembly events is dependent upon the activities of the products of the recombination-activating genes RAG-1 and RAG-2 (6, 7). Homozygous mutations that inactivate either of these genes in the mouse germ line result in a severe combined immunodeficiency—the animals do not develop either mature T or mature B lymphocytes (8, 9). However, such animals have no other obvious defects. We now show that injection of normal embryonic stem (ES) cells into blastocysts from RAG-2-deficient mice leads to the generation of somatic chimeras which contain normal populations of ES cell-derived lymphocytes. We refer to this procedure as RAG-2-deficient blastocyst complementation.

During B-cell differentiation, assembly and expression of Ig heavy-chain genes generally precedes that of Ig light-chain genes (10–12). Various lines of evidence have suggested that expression of the Ig heavy chain is involved in the progression of B cells through this ordered differentiation pathway (reviewed in refs. 13 and 14). Assembly of a complete Ig heavy-chain variable-region gene involves the joining of a D segment to a J_H segment followed by joining of a V_H segment to the DJ_H complex (10–12). Therefore, elimination of all J_H gene segments from a chromosome should block its ability to generate functional Ig heavy-chain genes. We have generated ES cells homozygous for a mutation that deletes all of their J_H segments. When tested in the RAG-2-deficient blastocyst complementation system, the ability of such cells to develop into B cells is blocked at an early stage but they generate normal T-cell populations. Significantly, we can rescue the ability of the homozygous mutant ES cells to generate B cells by stably transfecting them with μ-chain expression vector prior to the complementation experiment. By analogy, this method similarly will permit a rapid assay of potential roles of almost any gene in lymphocyte differentiation and/or function.

MATERIALS AND METHODS

Mice. RAG-2-deficient mice in the mixed background of 129Sv and C57BL/6 were maintained in a pathogen-free environment. Four- to 12-week-old females were used as blastocyst donors. Blastocyst injection and implantation were performed as described (15).

Targeting and Expression Vectors. The internal 1.7-kb *Apa* I–*Nae* I fragment containing all four J_H gene segments in the 5.7-kb *Eco*RI–*Ssp* I genomic fragment was replaced by a 1.2-kb pMC1-neo (neomycin phosphotransferase) gene cassette (Stratagene), and a 2.8-kb PGK–tk (phosphoglycerate kinase promoter–thymidine kinase) gene cassette (16) was added to the 5′ end of the construct for selection against random integrants (17). The VDJ–C_μ expression vector (where C_μ represents the constant region) was constructed by

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Abbreviations: ES, embryonic stem; DN, double negative; DP, double positive; SP, single positive; LPS, lipopolysaccharide.

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ligating a 4.2-kb *Eco*RI fragment containing a functionally assembled VDJ region (kindly provided by M. Reth; ref. 18) to the 10-kb *Eco*RI–*Xho* I genomic fragment containing the C_μ and μ_m (μ membrane) exons (kindly provided by C. C. Goodnow; ref. 19). The expression vector uses the normal V_H promoter and μ enhancer (Fig. 1.).

Transfection and Screening of ES Cells. CCE ES cells were maintained, transfected with the targeting construct, and selected for homologous integrants as described (8). Homozygous mutant ES cells were obtained by selecting heterozygous mutants for growth in increased concentrations of the neomycin analogue G418 (20). For further transfections, homozygous mutant ES cells were co-electroporated with linearized VDJ–C_μ and PGK-hyg (hygromycin phosphotransferase) expression vectors and selected for clones that grew in medium containing hygromycin B at 170 μg/ml.

Cell Analyses. Southern blotting and flow cytometry were done exactly as described (21). Monoclonal antibodies were obtained from PharMingen, San Diego: fluorescein-conjugated R6-60.2 (IgM), AMS9.1 (IgD^a), RA3-6B2 (CD45R/B220), S7 (CD43), and 53-6.7 (CD8α); phycoerythrin-conjugated DS-1 (IgM^a), RA3-6B2 (CD45R/B220), and Rm-4-5 (CD4); and bi-conjugated R6-60.2 (IgM) and M1/69 (heat-stable antigen). Assays for proliferation and Ig secretion of splenic B cells from normal and chimeric mice after depletion of T cells in the presence or absence of bacterial lipopolysaccharide (LPS, 20 μg/ml) were as described (22). For ELISA, the plates were coated with rabbit anti-mouse IgM and developed with alkaline phosphatase-conjugated rabbit anti-mouse μ chain (Zymed Laboratories).

RESULTS

Normal ES Cells Can Rescue the RAG-2-Deficient Phenotype. Blastocysts derived from RAG-2-deficient female mice were injected with various types of ES cells and then implanted into foster mothers. The T- and B-cell populations of the resulting pups were assayed 3–12 weeks after birth. Normal mice had substantial numbers of $CD4^+CD8^+$ (double positive, DP) and $CD4^+$ or $CD8^+$ (single positive, SP) thymocytes, as well as populations of SP T cells in their spleens, representing 15–30% of spleen cells depending on the age of the mice (Fig. 2*A*; Table 1). Approximately 50–60% of normal spleen cells were $B220^+IgM^+$ B cells, of which most also expressed surface IgD (sIgD) (Fig. 2*A*). Mice derived from RAG-2-deficient blasto-

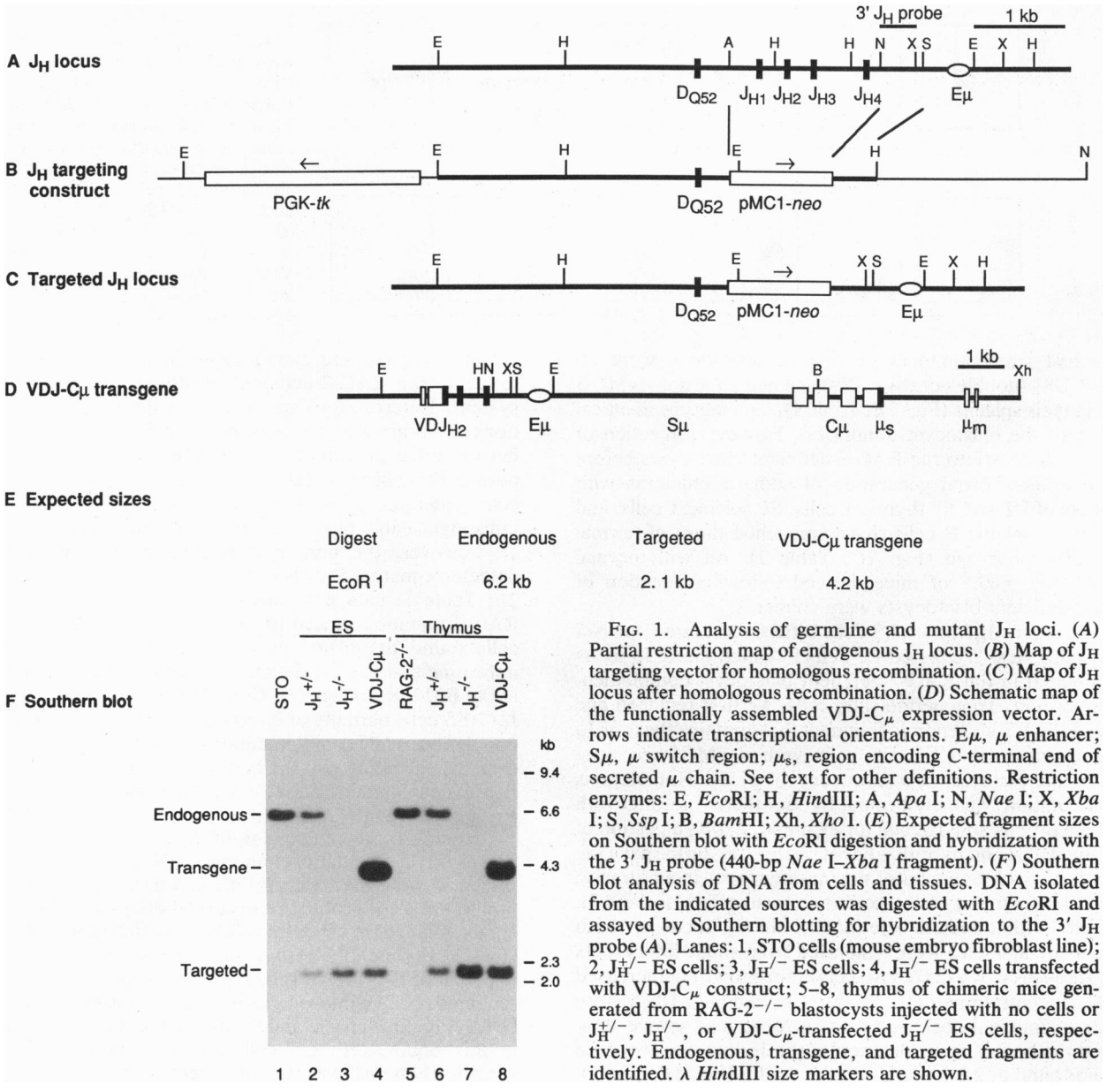

FIG. 1. Analysis of germ-line and mutant J_H loci. (*A*) Partial restriction map of endogenous J_H locus. (*B*) Map of J_H targeting vector for homologous recombination. (*C*) Map of J_H locus after homologous recombination. (*D*) Schematic map of the functionally assembled VDJ-C_μ expression vector. Arrows indicate transcriptional orientations. Eμ, μ enhancer; Sμ, μ switch region; μ_s, region encoding C-terminal end of secreted μ chain. See text for other definitions. Restriction enzymes: E, *Eco*RI; H, *Hin*dIII; A, *Apa* I; N, *Nae* I; X, *Xba* I; S, *Ssp* I; B, *Bam*HI; Xh, *Xho* I. (*E*) Expected fragment sizes on Southern blot with *Eco*RI digestion and hybridization with the 3′ J_H probe (440-bp *Nae* I–*Xba* I fragment). (*F*) Southern blot analysis of DNA from cells and tissues. DNA isolated from the indicated sources was digested with *Eco*RI and assayed by Southern blotting for hybridization to the 3′ J_H probe (*A*). Lanes: 1, STO cells (mouse embryo fibroblast line); 2, $J_H^{+/-}$ ES cells; 3, $J_H^{-/-}$ ES cells; 4, $J_H^{-/-}$ ES cells transfected with VDJ-C_μ construct; 5–8, thymus of chimeric mice generated from RAG-$2^{-/-}$ blastocysts injected with no cells or $J_H^{+/-}$, $J_H^{-/-}$, or VDJ-C_μ-transfected $J_H^{-/-}$ ES cells, respectively. Endogenous, transgene, and targeted fragments are identified. λ *Hin*dIII size markers are shown.

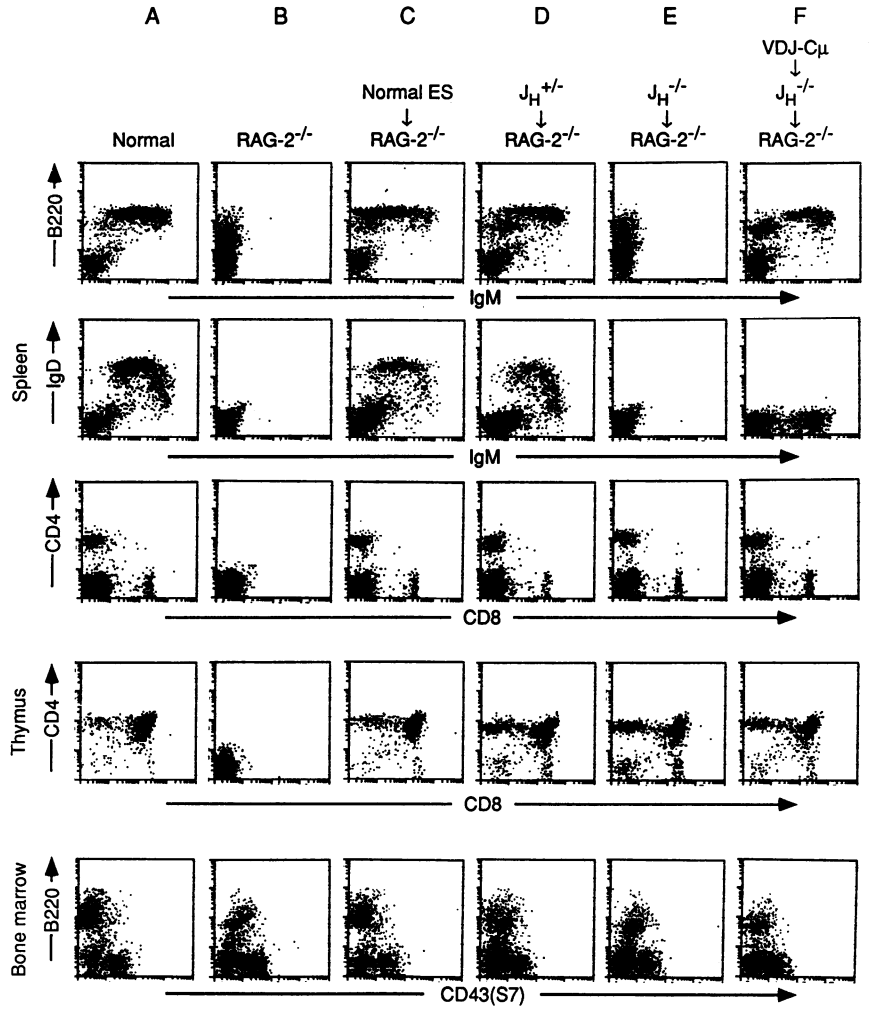

FIG. 2. Flow cytometry analysis of cells from spleen, thymus, and bone marrow. Spleen cells were stained with the following combinations of fluorescein (fl)- and phycoerythrin (PE)-labeled antibodies: fl-anti-B220 and PE-anti-IgM; fl-anti-IgD and PE-anti-IgM; or fl-anti-CD8 and PE-anti-CD4. Thymocytes were stained with fl-anti-CD8 and PE-anti-CD4. Bone marrow cells were stained with fl-anti-CD43 (S7) and PE-anti-B220. Results are presented as a two-dimensional dot plot in which each dot represents an individual cell. (*A*) Normal mice. (*B*) RAG-2-deficient mice. (*C–F*) Chimeric mice generated by injection of normal, $J_H^{+/-}$, $J_H^{-/-}$, or VDJ-C_μ-transfected $J_H^{-/-}$ ES cells into the RAG-2-deficient blastocysts, respectively.

cysts had small numbers of thymocytes which were all $CD4^-CD8^-$ (double negative, DN) and neither T nor $sIgM^+$ B cells in their spleens (Fig. 2*B*; Table 1), a phenotype identical to that of the blastocyst donors (8). However, injection of normal ES cells into the RAG-2-deficient blastocysts before implantation allowed generation of somatic chimeras with numbers of DP and SP thymic T cells, SP splenic T cells, and IgM^+IgD^+ splenic B cells that approached those of normal mice of similar age (Fig. 2*C*; Table 1). As with normal blastocysts, ≈60% of mice derived following injection of RAG-2-deficient blastocysts were chimeras.

Homozygous J_H Mutant ES Cells Differentiate into T but Not B Lymphocytes. We tested ES cells in which the Ig J_H locus was deleted in the RAG-2-deficient blastocyst complementation system. To generate such cells, we first replaced one copy of the 1.7-kb genomic fragment that contains the four J_H gene segments in CCE ES cells with a pMC1-neo gene expression cassette (at a frequency of 1/50). Homologous recombination was confirmed by Southern blotting with multiple probes and digests; for example, *Eco*RI digestion of genomic DNA and hybridization with the 3′ J_H probe gave the expected 2.1-kb fragment of the mutated locus in addition to the 6.2-kb fragment of the germ-line locus (Fig. 1*F*, lane 2). ES cells with targeted deletions of the J_H locus on both chromosomal alleles were generated from single knock-outs (at a frequency of 1/25) by selection for growth in increased G418 concentrations (20). Analysis of genomic DNA from putative double knock-out cells as described above confirmed the homozygous deletion of the J_H loci; we observed a major band at 2.1 kb and none at 6.2 kb (Fig. 1*F*, lane 3).

Heterozygous and homozygous mutant ES cells were tested in the RAG-2-deficient blastocyst complementation system. Heterozygous mutant ES cells generated populations of mature B and T cells indistinguishable from those of normal mice or chimeric mice generated by injection of normal ES cells (Fig. 2*D*; Table 1). Homozygous mutant ES cells generated populations of thymic and splenic T cells indistinguishable from those of normal mice (Fig. 2*E*; Table 1); however, the homozygous mutants did not generate detectable mature B cells in the spleen or bone marrow (Fig. 2*E*; Table 1; data not shown). Mice homozygous for the RAG-2 mutation lacked the population of $B220^+S7^-$ pre-B cells found in normal mice (compare Fig. 2 *A* and *B*), indicating that RAG-2 deficiency blocks B-cell development at the $B220^+S7^+$ stage (8). Complementation with normal or $J_H^{+/-}$ ES cells partially or completely restored this $B220^+S7^-$ population, while complementation with $J_H^{-/-}$ ES cells did not (Fig. 2), indicating that the homozygous J_H deletion blocked B-cell development at least as early as the homozygous RAG-2 mutation.

To confirm the cellular origin of the T- and B-cell populations in the somatic chimeras, DNA from thymus and spleen was digested with *Eco*RI and assayed for hybridization to the 3′ J_H probe. We observed only the 6.2-kb endogenous fragment in DNA from RAG-2-deficient mice (Fig. 1*F*, lane 5). Thymic DNA from somatic chimeras generated by injection of $J_H^{+/-}$ ES cells had germ-line and mutant bands at the same relative intensity as in heterozygous mutant ES cell DNA (Fig. 1*F*, lanes 2 and 6), indicating that nearly all cells in this organ were ES cell-derived. Thymic DNA from somatic chimeras generated by injection of $J_H^{-/-}$ cells gave a

Table 1. Comparison of lymphocyte reconstitution in chimeric mice

	Age, weeks	Spleen				Thymus	
		B cells		T cells		(SP and DP)	
		%	No. × 10^{-7}	%	No. × 10^{-7}	%	No. × 10^{-7}
Normal mice							
	4	56	4.3	15	0.9	98	11.2
	4	55	4.1	16	1.1	97	12.1
	6	57	5.0	21	1.8	98	13.1
	12	59	5.9	21	2.1	97	11.0
RAG-2$^{-/-}$ mice or mice derived from RAG-2$^{-/-}$ blastocysts							
	Any age	0	0	0	0	0	0
Chimeric mice derived from injection of RAG-2$^{-/-}$ blastocysts with various ES cells							
Normal	12	64	7.7	24	2.9	97	9.8
	12	34	2.7	24	1.9	97	4.9
$J_H^{+/-}$	4	31	1.2	15	1.6	93	13.0
	4	61	4.1	15	1.0	98	12.2
	4	61	4.9	16	1.3	96	9.2
	12	38	2.8	22	1.8	97	9.4
	12	29	2.9	26	2.6	97	4.9
$J_H^{-/-}$	4	0	0	56	0.7	97	10.7
	6	0	0	23	1.5	93	10.4
	6	0	0	24	1.2	86	8.2
$J_H^{-/-}$ transfected with VDJ-C_μ	3	6	0.3	24	1.2	96	7.4
	5	31	1.9	26	1.6	94	8.7
	6	4	0.2	23	1.2	93	10.5
	6	6	0.4	24	1.5	86	9.4

Total cell numbers in respective organs were determined by making single-cell suspensions and counting with a hemocytometer. The percentage of lymphocytes in a given organ was determined by flow cytometry with splenic B and T cells counted as IgM$^+$IgD$^+$ or IgM$^+$ cells and CD4$^+$ or CD8$^+$ (SP) cells, respectively. Thymocytes were counted as both SP and DP (CD4$^+$CD8$^+$) cells. The total number of B or T cells was calculated by multiplying the total number of cells in the organ by the percentage of that cell type. The RAG-2$^{-/-}$ thymus has 2–4 × 10^6 CD4$^-$CD8$^-$ (DN) thymocytes.

predominant targeted band and a very faint germ-line band (Fig. 1*F*, lane 7), confirming the derivation of the T-cell populations from the homozygous mutant ES cells. A targeted band also was clearly present in DNA from the spleens of chimeras derived following injection of heterozygous or homozygous J_H-deleted ES cells; however, as expected due to the substantial number of nonlymphoid cells in this organ, its intensity relative to that of the germ-line band was significantly less than observed in DNA from the corresponding thymus (data not shown).

Rescue of J_H-Deficient Phenotype by Introduction of a Functionally Assembled μ Heavy-Chain Gene. To confirm that the inability of $J_H^{-/-}$ ES cells to differentiate into mature B cells is due to the J_H deletion, a functionally assembled VDJ-C_μ expression vector was introduced into the homozygous mutant ES cells. Independent transfectants were tested for ability to generate B and T cells following injection into RAG-2-deficient blastocysts. In all cases such ES cells generated normal populations of thymocytes and splenic T cells (Fig. 2*F*; Table 1) indicating that this further manipulation did not affect ability of the cells to differentiate along the T-cell lineage. Strikingly, the transfected ES cells regained the ability to generate B220$^+$IgM$^+$ splenic B cells, although their numbers were more variable than those obtained with normal or $J_H^{+/-}$ ES cells (Table 1; see *Discussion*); further, all these B cells were IgM$^+$IgD$^-$, indicating that they were derived from the transfected mutant ES cells (Fig. 2*F*). Southern blot analyses of thymic and splenic DNA from these animals gave results consistent with the ES cell origin of their thymocytes and mature splenic lymphocytes (Fig. 1*F*, lane 8; data not shown). Finally, the VDJ-C_μ-transfected $J_H^{-/-}$ ES cells also restored significant levels of B220$^+$S7$^-$ pre-B cells in the bone marrow (Fig. 2*F*).

Functional Analysis of IgM$^+$IgD$^-$ B Cells from Somatic Chimeras. IgM$^+$IgD$^-$ B cells from somatic chimeras generated from VDJ-C_μ-transfected $J_H^{-/-}$ ES cells were further analyzed for ability to proliferate and secrete IgM after stimulation with LPS for 3 days. IgM$^+$IgD$^-$ B cells from somatic chimeras were able to proliferate and secrete IgM in response to LPS stimulation to a degree similar to that seen with normal, IgM$^+$IgD$^+$ B cells (Fig. 3).

DISCUSSION

We have defined the ability of ES cells with a homozygous J_H deletion to generate lymphocytes in the RAG-2-deficient blastocyst complementation system. These studies demonstrate the potential efficacy of this system for assaying the role of almost any gene in lymphocyte development. To be generally applied, this approach requires only the ability to

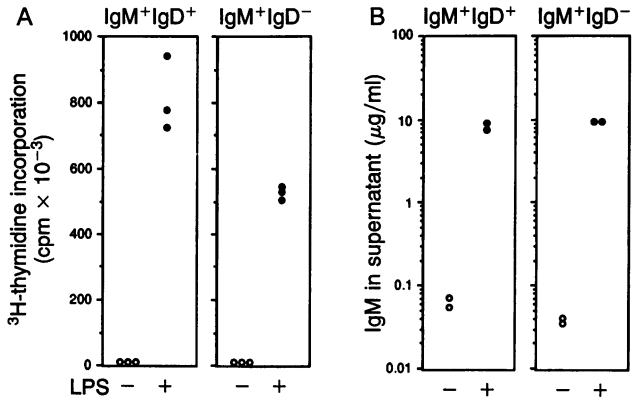

FIG. 3. Response of splenic B cells to LPS stimulation. T-cell-depleted splenic B cells from normal (IgM$^+$IgD$^+$) and chimeric (IgM$^+$IgD$^-$) mice generated by injection of VDJ-C_μ-transfected $J_H^{-/-}$ ES cells into the RAG-2-deficient blastocyst were cultured with or without LPS (20 μg/ml). After 3 days, culture supernatant was harvested for ELISA for IgM secretion (*B*) and the cells were incubated with [^{3}H]thymidine for 12 hr to assay for proliferation (*A*).

construct double mutants of the desired test gene, which is readily accomplished by the increased G418 selection method (20) or by successive targeting strategies (23).

Analysis of μ Heavy-Chain Expression in B-Cell Development. Homozygous deletion of the J_H locus in ES cells blocks their ability to generate mature B cells but not T cells. The blockage clearly is due to targeted mutation and not unknown defects that might have occurred during the various manipulations. Thus, transfection of a functionally assembled μ-chain expression vector into the $J_H^{-/-}$ ES cells rescues their ability to move from the more immature $B220^+S7^+$ stage to the later $B220^+S7^-$ stage in the bone marrow of the chimeras and allows generation of large numbers of cells that have undergone assembly and expression of endogenous Ig light-chain variable-region genes. These cells migrate into the periphery, where they can clearly be identified as ES-derived because they are IgM^+ but IgD^-, in contrast to normal B cells, which generally are IgM^+ and IgD^+. Further, the splenic B cells derived from the transfected ES cells respond normally to LPS stimulation by proliferation and IgM secretion. Introduction of additional heavy-chain gene constructs with mutations in different regions will rapidly permit tests of signaling and interaction domains in various processes. Likewise, appropriately constructed vectors could readily allow tests of other functions such as Ig heavy-chain class switching or somatic mutation in the background of a single expressed heavy-chain gene.

Chimeras generated by injection of normal or heterozygous mutant ES cells generally had normal numbers and percentage of T-lineage cells in thymus and spleen and slightly lower, although extensive, reconstitution of splenic B-cell numbers. However, chimeric mice generated by injection of VDJ-C_μ-transfected $J_H^{-/-}$ ES cells generally had lower numbers of splenic B cells but normal numbers of T cells. The variable reconstitution of the B-cell compartment may be due to the efficiency of transgene expression or the limited V_H repertoire of these cells (a single VDJ rearrangement), as a 2- to 5-fold reduction in B cell numbers has been observed in mice transgenic for Ig heavy-chain gene (24). However, the slightly lower and apparently more variable reconstitution of the B-cell versus T-cell populations by normal and $J_H^{-/-}$ ES cells also suggests that other factors, perhaps dependent on the extent of ES cell contribution, may differentially affect filling of the B- or T-cell compartments. In any case, the current level and reproducibility of reconstitution are more than sufficient for experimental analysis of mutant gene function.

General Utility of the RAG-2-Deficient Blastocyst Complementation System. The RAG-2-deficient blastocyst complementation should be amenable for assaying the role of almost any gene in the generation and/or function of B cells beyond the $B220^+S7^+$ stage and T cells beyond the DN stage. The advantage of this approach over alternatives such as complementing homozygous germ-line mutant (e.g., $J_H^{-/-}$) mice by breeding with mice transgenic for test constructs is obvious. In our system, the complementation requires only transfection of the appropriate construct into the mutant ES cells, followed by the highly reproducible construction of the chimeras. Large numbers of modified gene constructs can be easily transfected into the mutant ES cells, providing a rapid and efficient means to elucidate gene function under normal developmental conditions. In a similar approach, lymphocytes were generated following injection of heterozygous mutant ES cells into blastocysts from homozygous *scid/scid* mice (25); however, this system was not explored in detail and may be compromised by the leakiness and pleiotropic nature of *scid* mutation (26, 27). Recently, several groups have reported success in differentiating ES cells into lymphocyte precursors in culture (28, 29). When perfected, such methods could be particularly advantageous for examining early stages of lymphoid development.

The system we described is ideally suited for assaying the roles of cis-acting regulatory sequences, such as enhancer elements, in processes such as VDJ recombination, somatic mutation, or class switching. Further, the method is uniquely suited for analyzing lymphocyte-specific functions of genes that are required in many different cell types and whose ablation, therefore, leads to early embryonic lethality. For example, homozygous mutations of the N-*myc* genes leads to embryonic lethality at a stage too early to assay for effects on lymphoid lineages (2). However, homozygous N-*myc* mutant ES cells generate normal B- and T-cell populations in the RAG-2-deficient blastocyst complementation system (B. A. Malynn and F.W.A., unpublished data), providing homozygous mutant populations of mature lymphocytes for further functional analyses.

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