Antibody Response to a T-dependent Antigen Requires B Cell Expression of Complement Receptors

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Summary

Several lines of evidence indicate that antibody responses to T-dependent antigens require complement receptors expressed on either B lymphocytes or follicular dendritic cells. We have used RAG-2–deficient blastocyst complementation to create mice specifically lacking B cell complement receptors. Despite normal expression of complement receptor 1 (CR1 [CD35]) and CR2 (CD21) on follicular dendritic cells, these mice have a profound defect in their capacity to mount a T-dependent antibody response. This is the first direct demonstration in vivo that B cell expression of complement receptors is required for a humoral immune response. This suggests that CD21 and/or CD35 on B lymphocytes may be required for cellular activation, adsorptive endocytosis of antigen, recruitment to germinal centers, and/or protection from apoptosis during the humoral response to T-dependent antigens.

A critical role for the complement system in generating antibody responses to T cell–dependent (TD)[1] antigens was first suggested more than 20 years ago by the demonstration that in vivo depletion of complement protein C3 with cobra venom factor suppressed the antibody response of mice to SRBCs (1–3). The capacity of C5- and C6-deficient animals to mount normal antibody responses and the impaired responses (including absent secondary responses and isotype switching) of humans, guinea pigs, and dogs genetically deficient in C2, C4, or C3 suggested that the effect of cobra venom factor was the direct result of C3 deficiency (for review see reference 4). More recent studies have determined that the in vivo antibody response to TD antigens can be inhibited by administration of either the anti–complement receptor mAb 7G6 (5–7), or a soluble form of CR2 (8) before immunization. Together, these observations have identified the interaction of complement receptors with ligands derived from C3 as a critical point at which the immune and complement systems intersect.

In mice, expression of complement receptor 2 (CR2 [CD21]) (Fig. 1) is limited to B cells and follicular dendritic cells (FDCs). This observation has generated two principal hypotheses to explain the critical role of the C3–CR2 interaction during TD antibody responses. First, immune complexes bearing C3 ligands may act directly on B lymphocytes. The capacity of C3-derived ligands to stimulate B cell growth and activation is thought to occur through coligation of CR2, which participates with CD19, TAPA-1, and Leu-13 in a B cell–specific complex (9), and the antigen receptor, membrane IgM. Cross-linking CD19 to IgM on B cells lowers the number of antigen receptors required for inducing DNA synthesis by two orders of magnitude (10), and signals transduced through this complex have been shown to trigger recruitment of phosphatidylinositol kinase (11), augment activation of phospholipase C (12), and enhance cellular adhesion (13). In vivo, this coligation is thought to result from antigen and/or antigen–antibody complexes bearing iC3b and/or C3dg as a result of activating the classical and/or alternative pathways of complement.

The second potential site at which a C3–CR2 immunomodulatory event might occur is at the surface of FDCs, unique APCs that play a major role in trapping and preserving native antigens within peripheral lymphoid tissue (for review see reference 14). After primary immunization, immune complexes activate complement, and these antigen–Ab–C3 complexes traffic to FDCs, where they are captured by C3 receptors. It has been demonstrated that C3 is essen-

[1] *Abbreviations used in this paper:* ES, embryonic stem; FDC, follicular dendritic cell; MCR, murine complement receptor; NP, 4-hydroxy-3-nitrophenyl acetyl; SCR, short consensus repeat; TD, T cell–dependent.

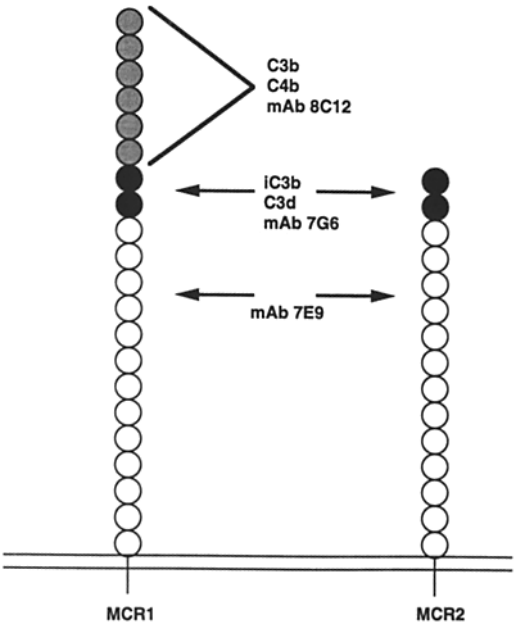

Figure 1. Structure and function of murine CRs (29–33). Murine CR1 (MCR1, CD35) and murine CR2 (MCR2, CD21), alternative transcripts of the *Cr2* gene, are cell surface glycoproteins that specifically recognize proteolytic fragments of complement components C3 and C4. MCR1, M_r 190,000, is expressed by B cells, FDCs, peritoneal macrophages, and activated granulocytes and serves to bind C3b, iC3b, C3d, and C4b. MCR2, M_r 150,000, is expressed by B cells and FDCs and serves to bind iC3b and C3d. MCR2 has an extracellular domain composed entirely of 15 short consensus repeats (SCRs), a 27-amino acid transmembrane region, and a 35-residue cytoplasmic tail. The structure of MCR1 is identical to that of MCR2, except for the presence of an additional six SCRs at the amino terminus that contain a binding domain for C3b, C4b, and mAb 8C12. SCR-1 and SCR-2 of MCR2, which are common to both receptors, mediate binding of iC3b, C3d, and mAb 7G6, which blocks binding of both ligands to the receptors.

tial for targeting of TD antigens to murine lymphoid follicles and for the subsequent development of memory B cells (15, 16). The undegraded antigen that is captured and retained by FDCs is thought to provide the stimulus for the formation of germinal centers. This antigen depot may also provide a necessary stimulus for the generation and perpetuation of memory B cells through continued stimulation without additional administration of antigen (17). Thus, the immunomodulatory effect of a C3–CR2 interaction might be via localization and retention of C3-bearing immune complexes on the reticulum of CR1- and CR2-bearing cytoplasmic processes of FDCs. These studies were undertaken to characterize the antibody response to a TD antigen in mice specifically deficient in B cell CR1 and CR2 and thereby to dissect the relative contributions to humoral immunity of complement receptors expressed on B cells as compared with those on FDCs.

Materials and Methods

Creation of Mice with CD21- and CD35-deficient B Cells. The targeting vector used in these studies was assembled from a 6.5-kb fragment of the *Cr2* gene that was isolated from a genomic library prepared from the 129 mouse strain (Fig. 2). The genomic DNA was subcloned into the plasmid pNT, which bears a *neo* selection marker under control of the PGK promoter. The murine *Cr2* gene was disrupted through insertion of the *neo* gene into a BamHI site within the coding region for SCR-10 of murine complement receptor (MCR)2, which introduces a frameshift and a stop codon, resulting in disruption of MCR1 and MCR2 expression. 2×10^7 embryonic stem (ES) cells (cell line AB1, originated by Dr. A. Bradley, Baylor College of Medicine, Houston, TX) were transfected by electroporation with 20 μg of linearized pNT.CR2, were plated onto five 10-cm dishes coated with Sto feeder cells (treated with mitomycin C to prevent cell division), were allowed to recover for 6 h, and were cultured for 6 d in double selection medium (0.5 mg/ml G418 and 2.0 μM gancyclovir in DMEM supplemented with recombinant leukemia inhibitory factor and 15% FCS). 250 colonies were grown to confluence in 96-well plates that were coated with feeder cells. Genomic DNA was prepared from them, and targeted clones were screened for identification of positives as described (18). DNA samples were digested with BamHI and analyzed by Southern blot hybridization. A cDNA probe was used that recognizes an 8-kb fragment within the wild-type *Cr2* gene, which is expected to increase to 10 kb after incorporation of the targeting vector. After primary screening, eight positives were identified, each of which was expanded and further analyzed in secondary screening. All eight were heterozygous for the disrupted *Cr2* allele, and six of these had a normal number of 40 chromosomes (data not shown). One of these clones was cultured for 14 d in 3 mg/ml G418 selection medium to obtain a clone homozygous for the *Cr2* null allele. 10 colonies were picked and screened by Southern blot analysis to identify clones homozygous for the *Cr2*-disrupted allele. One clone harboring homozygous disruption of the *Cr2* locus was identified, expanded, and used for microinjection of RAG-2–deficient embryos as described (19, 20).

Flow Cytometry. Splenic mononuclear cells were obtained by layering through Ficoll–Paque (Pharmacia Biotech, Inc., Piscataway, NJ) and were washed with PBS, 2% BCS, 10 mM Hepes, 0.01% sodium azide. For each sample, 5×10^5 to 1×10^6 cells were used for flow cytometric analysis. Cells were sequentially incubated with biotinylated anti–mouse CR1/CR2 mAb 7E9 followed by avidin–PE and FITC-conjugated anti–mouse CD19 mAb 1D3 and were analyzed on a FACScan® flow cytometer. Data analysis was performed with the Consort 30 program. Shown are the results of at least 50,000 events analyzed for each sample.

Immunohistochemistry. Spleens were snap frozen in OCT (Biomedia, Foster City, CA) cooled in liquid nitrogen. Frozen sections (5–6 μm) were thaw-mounted and stored at −20°C. Sections were fixed either in 4% formaldehyde for 6 min at room temperature (CD19, CR1/2) or in acetone for 10 min at 4°C (CD3, 4-hydroxy-3-nitrophenyl acetyl [NP]), rehydrated in PBS, incubated in normal goat (CD19, CR1/2) or rabbit (CD3, NP) serum for 20 min at room temperature, and were incubated for 1 h at room temperature with rat anti–mouse CD19 mAb 1D3, rat anti–mouse CR1/CR2 mAb 7G6, hamster anti–mouse CD3 mAb $500A_2$, or NIP_5–BSA. Sections incubated with rat or hamster mAbs were incubated with biotinylated rabbit Ab specific for rat Ig or biotinylated goat Ab specific for hamster immunoglobulin, respectively. All sections were subsequently incubated with avidin–biotin complex for 30 min at room temperature, incubated with diaminobenzidine substrate (brown) (Vector Laboratories, Burlingame, CA) for 5 min at room temperature, counterstained with hematoxylin, dehydrated, and mounted with Permount (Fischer Scientific, Fair Lawn, NJ).

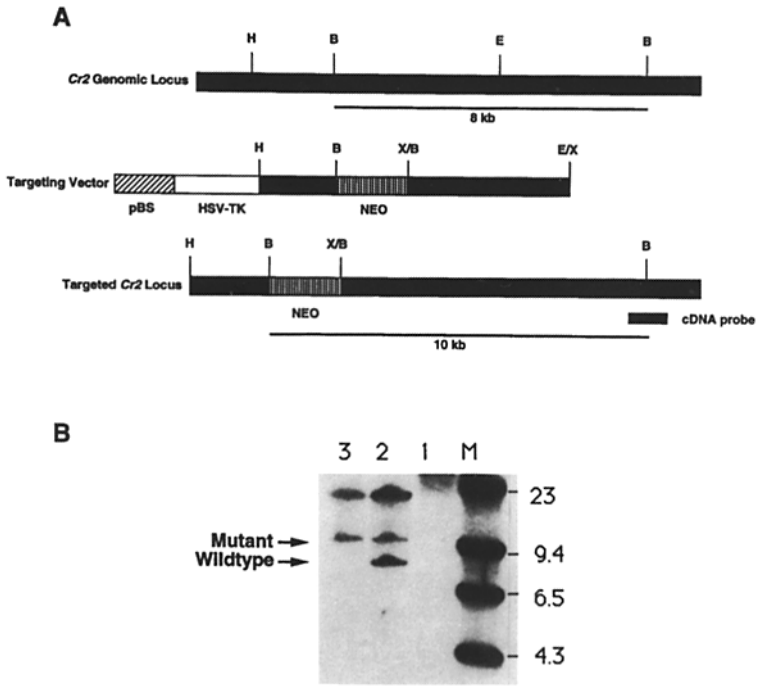

Figure 2. Targeted disruption of murine *Cr2*. (*A*) The murine *Cr2* locus encodes both MCR1 and MCR2, which are generated by alternative splicing (22, 23). Shown from top to bottom are partial restriction maps of the mouse *Cr2* genomic locus, the targeting vector in which the *neo* gene has been inserted at a BamHI site within the exon encoding SCR-10 of mouse CR2, and the anticipated result of successful targeting of the locus. H, HinDIII; B, BamHI; X, Xho I. (*B*) DNA samples were digested with BamHI and analyzed by Southern blot hybridization. A cDNA probe was used that recognizes an 8-kb fragment within the wild-type *Cr2* gene which is expected to increase to 10 kb after incorporation of the targeting vector. Shown are the results of Southern blot hybridization of an ES cell line that is heterozygous (lane *2*) and one that is homozygous (lane *3*) for *Cr2* disruption.

ELISA. ELISAs were performed by coating microtiter wells with 100 μl NP-KLH (50 μg/ml) in 0.1 M $NaHCO_3$ pH 9.8, at 4°C, followed by sequential incubations with sera diluted in bovine lactotransfer technique optimizer (BLOTTO)–Tween, goat anti–mouse IgG–AP conjugate, and substrate. Plates were read at OD_{405}.

Results and Discussion

We have taken advantage of the RAG-2 blastocyst complementation experimental system developed by Alt and colleagues (21) to determine that expression of complement receptors by murine B cells is specifically required for humoral immunity. RAG-2–deficient mice are homozygous for the null allele at the RAG-2 locus, which results in failure to form mature T and B lymphocytes, but are otherwise normal (22). This defect can be corrected by microinjection of embryos with ES cells that contain a normal RAG-2 allele. However, if the ES cells that are used to complement the deficient embryos are homozygous for some other null allele, it becomes possible to study the functional effects of this specific mutation in T and B lymphocytes without affecting other tissues directly. Thus, all mature lymphocytes must be homozygous for the specific mutation being studied, because they can only arise from the injected ES cells, whereas all other tissues are potentially chimeric.

We have created ES cells homozygous for targeted disruption of the *Cr2* gene (which encodes both murine complement receptors CR1 [CD35] and CR2 [CD21]) and injected these *Cr2*-deficient ES cells into RAG-2–deficient blastocysts to generate mice with a B cell–specific deficiency of complement receptors (Fig. 2). If RAG-2–deficient mice are reconstituted with normal ES cells, the offspring should express complement receptors on both B lymphocytes and FDCs. In contrast, RAG-2–deficient mice reconstituted with ES cells homozygous for disruption of the *Cr2* locus should express complement receptors on FDCs, but all mature B cells should be complement receptor negative.

We first determined whether mature B cells in the targeted mice were deficient in surface expression of CR1 and CR2. Splenic mononuclear cells were prepared and assayed for reactivity with the B cell marker CD19 and for expression of complement receptors as determined by reactivity with mAb 7E9, which binds to an epitope common to both murine CR1 and CR2 (see Fig. 1). As shown in Fig. 3, most CD19-positive cells derived from a BALB/c mouse also express complement receptors, whereas mononuclear cells purified from the spleen of a RAG-2–deficient mouse demonstrate total deficiency of mature CD19-positive B cells. If a RAG-2–deficient mouse is reconstituted with normal ES cells, mature splenic B cells reappear in normal numbers with a CD19-positive, complement receptor–positive phenotype. However, when *Cr2*-deficient ES cells are used for blastocyst complementation, mature CD19-positive B cells populate the spleen, but they are complement receptor negative, as expected.

As shown in Table 1, the numbers of CD19-positive splenic B cells and the numbers of CD3-positive splenic B cells observed in the CR2-deficient mice ranged from 29 to 55% and 23 to 40%, respectively, slightly below the numbers observed in the normal controls. Complement receptors were detected on 37–55% of the spleen cells in the normal mice, which was nearly identical to the percentages of cells that expressed CD19. In contrast, splenic mononu-

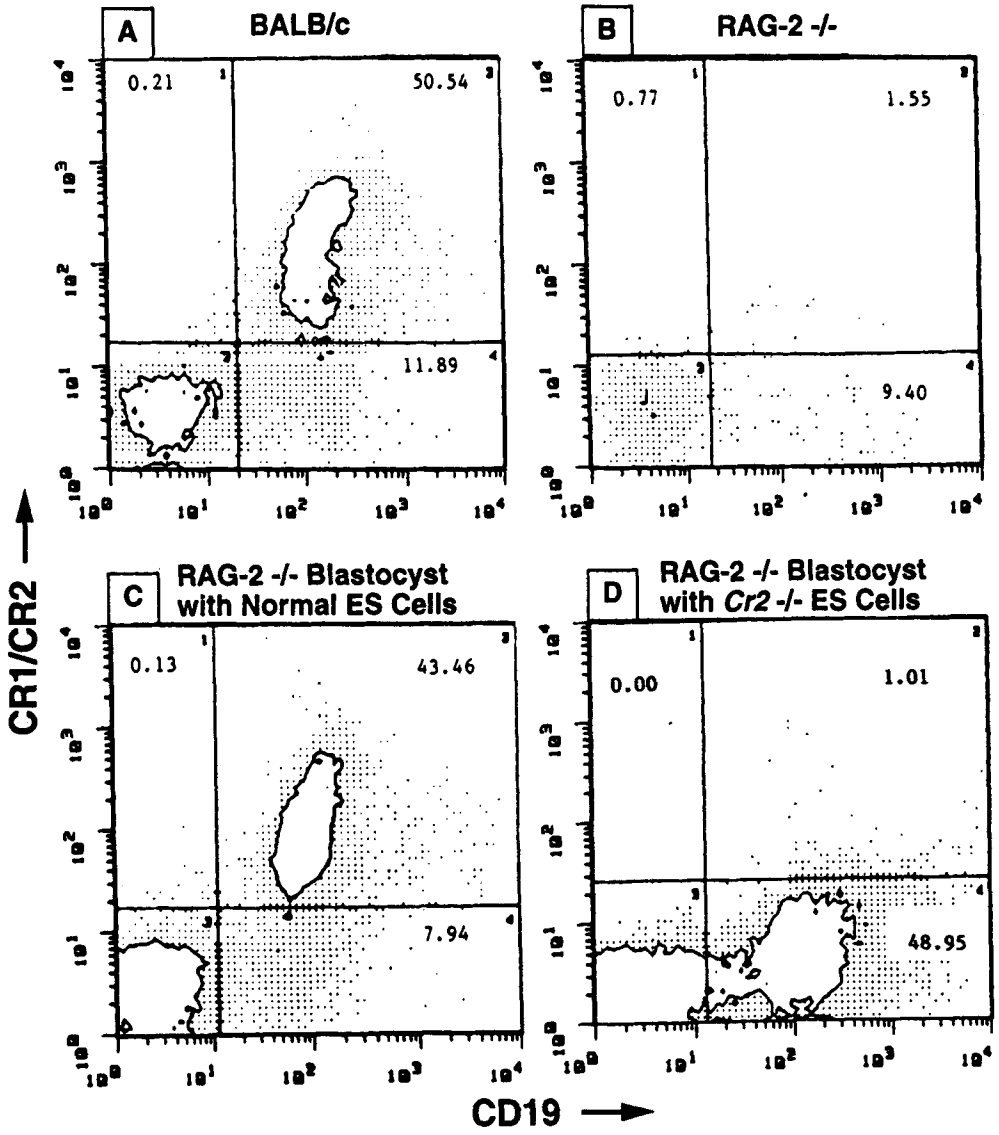

Figure 3. Flow cytometric analysis of splenic mononuclear cells. Single splenic mononuclear cell suspensions were prepared and assayed for expression of CD19 and CR1/CR2 by dual color flow cytometric analyses. (*A*), wild-type BALB/c, (*B*), RAG-2–deficient, (*C*), RAG-2–deficient reconstituted with normal ES cells, and (*D*) RAG-2–deficient reconstituted with *Cr2*-deficient ES cells. Shown are the results of at least 50,000 events, analyzed for each sample.

clear cells from the targeted mice were consistently complement receptor negative. Significant quantities of all Ig isotypes studied were detected in each of the experimental mice; however, the levels of several subclasses, such as IgG2a and IgG2b, were markedly lower in the complement receptor–deficient animals.

Mice reconstituted with *Cr2*-deficient cells and control mice reconstituted with normal ES cells were immunized intraperitoneally with 10 μg of NP–KLH in alum at days 0 and 21. All mice were killed on day 28, at which time antibody responses to the immunogen were determined. RAG-2–deficient mice that had been reconstituted with normal ES cells generated anti-NP titers (Table 1) similar to those seen with normal mice (data not shown). In contrast, RAG-2–deficient mice that had been reconstituted with *Cr2*-deficient ES cells generated no detectable antibody response to the hapten NP as measured by ELISA using NP–BSA. When the antibody response to NP–KLH was measured by ELISA using NP–KLH, the titers observed in control mice were again similar to those of wild-type mice, whereas mice lacking B cell expression of complement receptors were unable to generate significant antibody responses (Fig. 4).

Immunohistochemical studies were performed on the spleens of all mice in an attempt to further understand the cellular basis for this failure of humoral immunity. As shown in Fig. 5, CD19 expression in $CR^{+/+}$ mice was limited almost exclusively to the peripheral or B cell region of the follicles, sparing the central T cell zone, a pattern nearly identical to that observed for expression of complement receptors. As observed with the control mice, CD19 expression was limited to the peripheral B cell region of $CR^{-/-}$ mice. In striking contrast with the pattern observed with normal mice, expression of complement receptors in the spleens of the targeted mice did not parallel CD19 expression. Instead, we observed an intense reticular pattern

Table 1. *Characterization of the Immune Response to NP–KLH by $CR^{+/+}$ and $CR^{-/-}$ Mice*

Mouse	Anti-NP titer	Percentage of cells positive for expression of				Serum levels of Ig isotypes						Splenic mononuclear cells
		CD3	CD19	CR1/CR2	CD19 + CR1/CR2	IgM	IgG1	IgG2a	IgG2b	IgG3	IgA	
						μg/ml						× 10^6
$CR^{+/+}$ 1	70,000	31	48	47	43	900	2,400	1,400	500	450	1,640	13.6
$CR^{+/+}$ 2	3,000	34	56	54	49	510	1,350	5,200	600	530	1,940	13.6
$CR^{+/+}$ 3	70,000	33	61	55	52	700	1,500	1,800	500	640	1,860	12.1
$CR^{+/+}$ 4	3,000	44	42	37	38	700	620	1,220	125	340	1,340	2.7
$CR^{-/-}$ 1	0	38	55	1	1	400	800	920	100	230	1,640	3.4
$CR^{-/-}$ 2	0	29	39	0	0	950	400	480	40	230	790	7.9
$CR^{-/-}$ 3	0	40	46	0	0	600	250	580	100	215	1,080	8.8
$CR^{-/-}$ 4	0	23	29	0	0	225	3,500	950	60	300	226	9.5

Each of four $CR1/CR2^{+/+}$ mice and each of four $CR1/CR2^{-/-}$ mice was immunized with 10 μg of NP–KLH in alum on days 0 and 21. On day 28, all mice were killed and assayed for serum levels of Ig isotypes, serum titers of IgG anti-KLH, total numbers of splenic mononuclear cells, and percentages of splenic mononuclear cells positive for surface expression of either CD3, CD19, or CR1/CR2, or positive for coexpression of CR1/CR2 and CD19.

of staining of cells with anti-CR1/CR2 mAb 7E9 in a limited peripheral area of the follicle. This pattern was observed in splenic follicles of all *Cr2*-deficient mice, consistent with expression of complement receptors limited to FDCs. Further identification of these complement receptor–bearing cells as FDCs was achieved by demonstrating their reactivity in serial sections with the FDC-specific mAb FDC-M1, which produced the same staining pattern (data not shown).

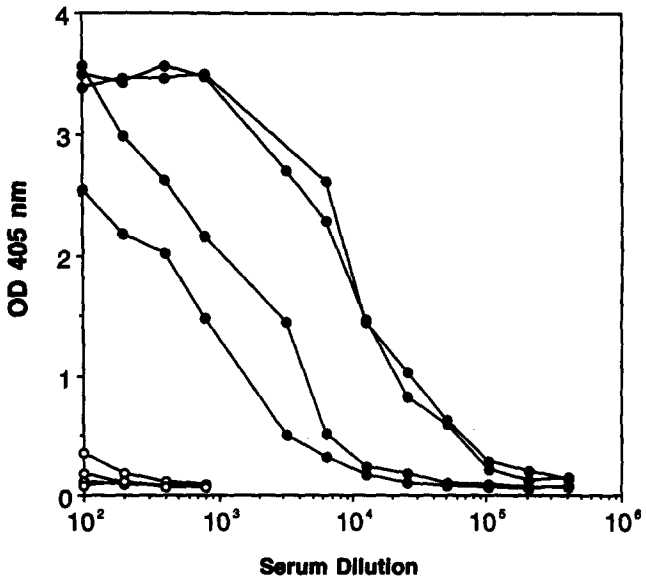

Figure 4. Immune response to NP–KLH. RAG-2–deficient mice reconstituted with normal ES cells (●) and RAG-2–deficient mice reconstituted with *Cr2*-deficient ES cells (○) were immunized intraperitoneally with 10 μg NP–KLH in alum on day 0 and on day 21. Mice were killed on day 28, at which time serum titers to NP–KLH were determined by ELISA. Mice complemented with normal ES cells generate titers comparable to those observed after immunization of wild-type mice (data not shown), whereas no significant anti–NP–KLH response was observed in mice lacking B cell expression of complement receptors.

Although the flow cytometric studies indicated that the experimental mice had somewhat decreased numbers of CD3-positive splenic mononuclear cells, no apparent differences in number or distribution were detected by immunohistochemistry (Fig. 5).

Finally, the spleens of all mice were assayed for reactivity with NP–BSA to identify antibody-forming B cells. Spleens of all control mice were filled with numerous intensely positive NP-reactive B cells, located primarily in the red pulp. In contrast, although NP-positive cells were detected in the spleens of all immunized complement receptor–deficient mice, they were consistently far fewer, and their reactivity with NP–BSA was much less intense than was observed in the spleens of control mice.

Together, these findings demonstrate that mice expressing complement receptors on FDCs but lacking B cell CR1 and CR2 generate insignificant antibody responses after immunization with NP–KLH. However, these mice are capable of generating NP-specific B cells, suggesting that their immune response is profoundly impaired, rather than absolutely deficient. There appear to be at least four explanations for the essential role played by B cell complement receptors during generation of antibodies to TD antigens. First, as described above, immune complexes that activate complement may stimulate B cell activation and proliferation by triggering signals transduced through the CR2–CD19 membrane complex (9). Recent observations that CD19-deficient mice exhibit a similarly profound defi-

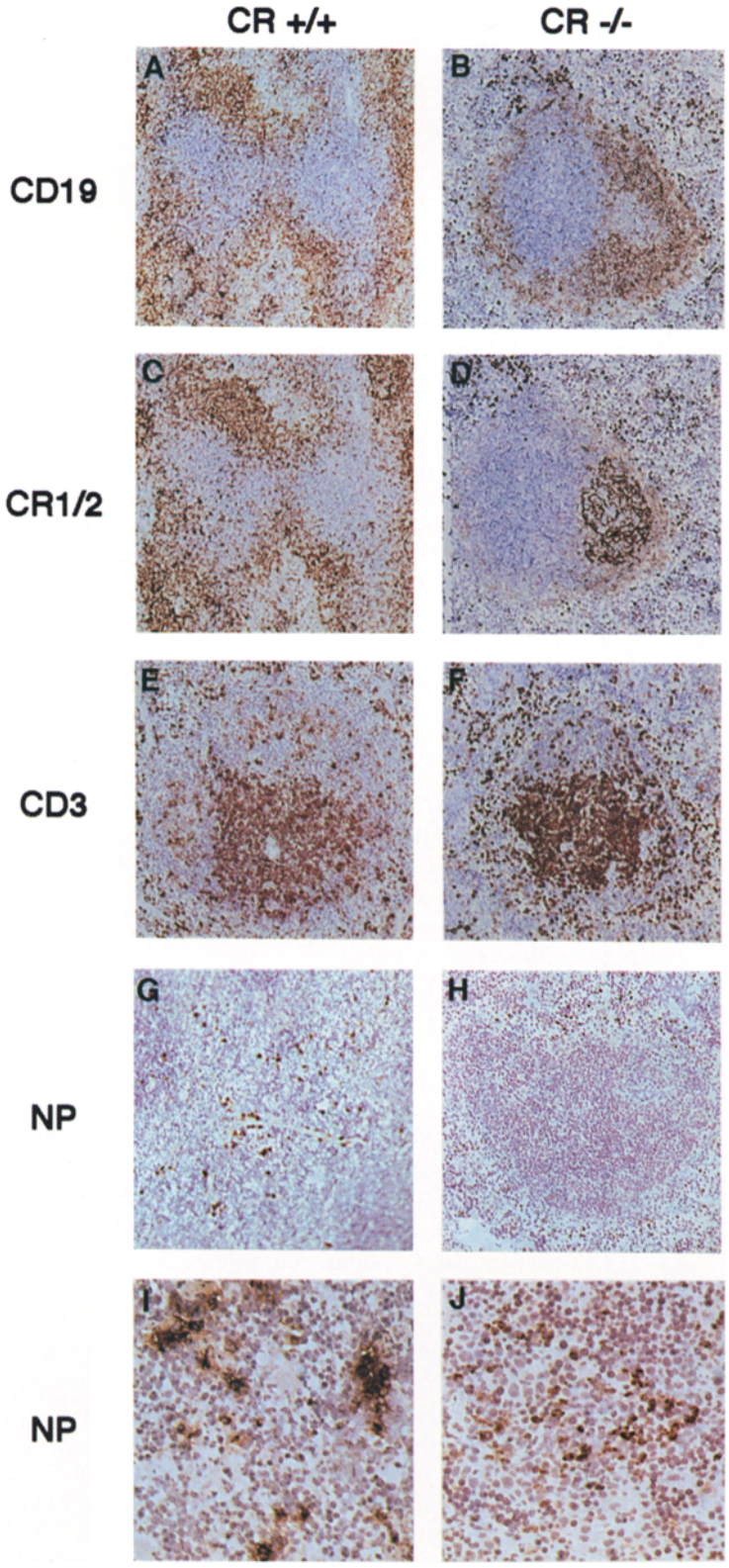

Figure 5. Immunohistochemical analysis of mouse spleens. Spleens from RAG-2–deficient mice reconstituted with normal ES cells ($CR^{+/+}$) (*A*, *C*, *E*, *G*, and *I*) and RAG-2–deficient mice reconstituted with CR1/CR2-deficient ES cells ($CR^{-/-}$) (*B*, *D*, *F*, *H*, and *J*) were assayed for expression of CD19 (*A* and *B*), CR1/2 (*C* and *D*), CD3 (*E* and *F*) and for the presence of NP-specific B cells (*G–J*). Representative serial sections demonstrate similar distributions of CD19-positive (*A*) and complement receptor–positive (*C*) cells in the spleen of a $CR^{+/+}$ mouse. In striking contrast, serial sections from the spleen of a $CR^{-/-}$ mouse demonstrate normal expression of CD19 in the B cell regions (*B*), with complement receptor expression limited to FDCs within a limited peripheral area of the follicle (*D*). As shown at lower (*G* and *H*) and higher (*I* and *J*) magnifications, numerous NP-reactive cells, presumably NP-specific B cells, were observed in the spleens of $CR^{+/+}$ mice, primarily in the red pulp (*G* and *H*). In contrast, although NP-reactive cells were present in the spleens of $CR^{-/-}$ mice, they were more difficult to find (*H* and *J*). No reactivity with NP–BSA was observed in the spleens of normal mice that were not immunized (data not shown). The numbers and distribution of CD3-positive cells were comparable in $CR^{+/+}$ (*E*) and $CR^{-/-}$ (*F*) mice. (*A* and *C*) ×80; (*B*, *D*, *E*, *F*, *G*, and *H*) ×128; (*I* and *J*) ×320.

ciency in generating antibodies to TD antigens further supports that at least one role of B cell complement receptors is mediated through this pathway (23, 24). Second, complement receptors may provide a mechanism for B cell adsorptive endocytosis of antigen, which is then processed and presented to T cells during development of germinal centers. Third, B cells that have been activated extrafollicularly may depend upon complement receptors for their recruitment to peripheral lymphoid follicles, where they interact with antigen that has been trapped by FDCs. Fourth, positive selection of antigen-specific B cells in germinal centers is dependent upon their ability to bind antigen and survive apoptosis. C3-derived ligands may provide a bridge between complement receptors expressed on FDCs and those expressed on B cells, facilitating this interaction. Alternatively, it may be that B cell complement receptors must interact with CD23, a ligand for CR2 that is expressed at high levels on a subset of FDCs (25–28); however, this alone does not explain the known requirement for C3 in the process.

This experimental system can now be expanded to distinguish the relative contributions of murine CR1 and CR2 to the humoral immune response, by reconstituting null mice differentially with each receptor, respectively. It should also be possible to rescue the immune response of these deficient mice with B cell–specific expression of mutant receptors, such as those lacking the domains that have been identified as critical for interaction with C3, CD19, and CD23. This will enable further definition and dissection of the roles of complement and complement receptors during generation of a humoral immune response.

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