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Markedly impaired humoral immune response in mice deficient in complement receptors 1 and 2

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ABSTRACT **Complement receptor 1 (CR1, CD35) and complement receptor 2 (CR2, CD21) have been implicated as regulators of B-cell activation. We explored the role of these receptors in the development of humoral immunity by generating CR1- and CR2-deficient mice using gene-targeting techniques. These mice have normal basal levels of IgM and of IgG isotypes. B- and T-cell development are overtly normal. Nevertheless, B-cell responses to low and high doses of a T-cell-dependent antigen are impaired with decreased titers of antigen-specific IgM and IgG isotypes. This defect is not complete because there is still partial activation of B lymphocytes during the primary immune response, with generation of splenic germinal centers and a detectable, although reduced, secondary antibody response. These data suggest that certain T-dependent antigens manifest an absolute dependence on complement receptors for the initiation of a normally robust immune response.**

Once activated, individual complement components can interact with specific receptors on the surfaces of cells and help to regulate the humoral immune response (1). Two of these receptors, complement receptor 1 (CR1, CD35) and complement receptor 2 (CR2, CD21), are part of a family of membrane-bound and serum proteins designated the regulators of complement activation (2, 3). These proteins interact with C3 and C4 and contain a common structural motif consisting of ≈60 amino acids and designated a short consensus repeat (SCR).

In humans, the C3b/C4b receptor CR1 is a transmembrane glycoprotein whose major allelic form contains 30 SCR and has a molecular mass of 190 kDa. CR1 is present on erythrocytes, macrophages, neutrophils, B cells, follicular dendritic cells (FDC), and a subset of T cells. On erythrocytes, CR1 is responsible for the immune adherence phenomenon by which C3b coated circulating immune complexes are bound and transported to the reticuloendothelial system. On macrophages and neutrophils, CR1 facilitates the phagocytosis of complement opsonized particles. CR1 has also been implicated as a regulator of B-cell proliferation and differentiation (4). In addition, this receptor serves as a cofactor for factor I-mediated cleavage and inactivation of C3 and C4. CR1 can also regulate the C3 and C5 convertases by a process known as decay-acceleration.

The C3d,g receptor CR2 is a 15-SCR-containing 150-kDa membrane protein. CR2 is present on B cells, a subset of T cells, and on FDC, and is thought to participate in the regulation of B-cell activation and differentiation (5). Human CR2 also interacts with CD23, the low-affinity receptor for IgE. Binding of CR2 by CD23 in the presence of interleukin-4 and CD40-ligand results in enhanced B-cell IgE production (6).

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Mouse CR2 is 67% homologous in nucleotide sequence to its human counterpart (7). Mouse CR2 is present on the surface of FDC and B cells, and binds human and mouse C3d with similar affinities (8). Mouse CR1 is also present on B cells, binds mouse C3b with high affinity, and has cofactor activity for C3b cleavage by murine factor I (9). In spite of these striking functional similarities, certain structural differences are evident between the two species. In humans, CR1 and CR2 are proteins encoded by distinct, but related, genes. In the mouse they are the alternatively spliced products of a common gene designated *Cr2* (10, 11). In addition, mouse CR1 is not present on the membranes of erythrocytes or platelets and, thus, is not the mouse immune adherence receptor (12).

In both species, CR1 and CR2 immunoregulatory activities have been studied (13). For example, polymeric C3d,g or anti-CR2 antibodies (Abs) enhance proliferation of human B cells when primed with T-cell-dependent factors, phorbol esters, or anti-IgM. An increase in intracellular Ca^{2+} is seen when human B cells are treated with anti-CR2 Ab in the presence of suboptimal amounts of anti-IgM. Cross-linking of CR1 enhances the production of immunoglobulins when B cells are stimulated with low doses of mitogen. Finally, an impairment in the specific immunoglobulin response against low doses of injected antigens has been observed when *in vivo* functional interference of these proteins is achieved through the use of specific blocking rat anti-mouse CR1 and CR2 monoclonal Abs (mAbs) or by competitive inhibition using a soluble form of human CR2 (14–18). Experiments using the T-cell-dependent antigens from sheep red blood cells (SRBC) and keyhole limpet hemocyanin have suggested that the primary immune response deficit in mice treated with anti-mouse CR1 and CR2 mAb can be overcome by using high doses of the immunogen (14).

Here we have further explored the role of these receptors in the development of humoral immunity by generating CR1- and CR2-deficient mice using gene targeting. Our data show that CR1 and CR2 play important roles in the B-cell response and are necessary for appropriate B-cell activation and Ab production at both low and high doses of antigen. In addition, we demonstrate that germinal center formation is retained in the absence of these proteins. These mice should provide an excellent model to study in detail the specific roles of CR1 and CR2 in the immune response.

MATERIALS AND METHODS

Gene Targeting. A portion of the murine *Cr2* gene was isolated from an NIH 3T3 cell genomic library by using the

Abbreviations: CR1, complement receptor 1; CR2, complement receptor 2; SCR, short consensus repeat(s); FDC, follicular dendritic cell; FITC, fluorescein isothiocyanate; PE, phycoerythrin; AP, alkaline phosphatase; SRBC, sheep red blood cells; RU, relative units; Ab, antibody; mAb, monoclonal antibody; HSV TK, herpes simplex virus thymidine kinase.

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mouse CR2 cDNA pBSMCR2-5 as a probe (19). A 4.5-kb genomic *Pst* I DNA fragment encoding SCR 12 to SCR 17 of the *Cr2* gene was subcloned into pBluescript KS (Stratagene). The coding sequence was interrupted by replacing a 0.5-kb *Stu* I fragment that includes exons encoding SCR 14 with a 1.6-kb neomycin resistance cassette pGKneobpA (20) (Fig. 1). In this targeting vector, the pGKneobpA is flanked by a 1.1-kb genomic *Cr2* fragment on the 5′ side and a 2.5-kb genomic *Cr2* fragment on the 3′ side. The herpes simplex virus thymidine kinase (HSV-TK) gene was added 3′ of the 2.5-kb *Cr2* sequence to select against random integration events (21). Transfection of embryonal stem cells and blastocyst injection were as described (22).

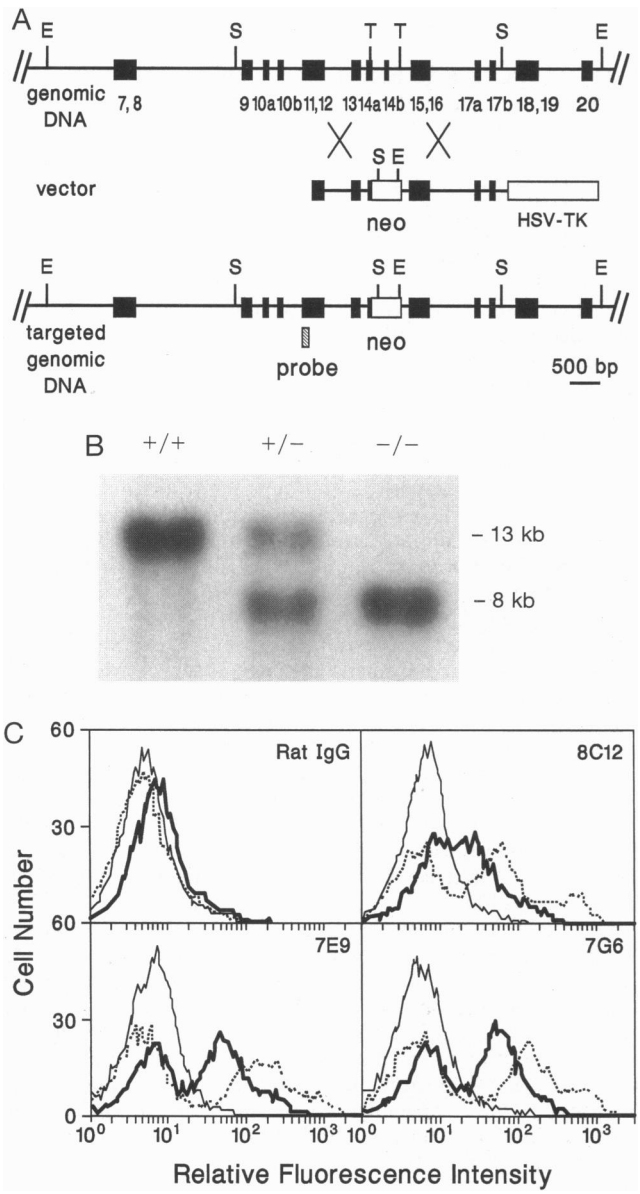

FIG. 1. Targeted disruption of the *Cr2* gene. (*A*) The targeting vector and the configuration of the targeted genomic DNA are shown. Large black boxes represent exons, and the numbers below represent the SCRs encoded. The pGKneobpA (neo) and HSV-TK genes are shown as open boxes. Restriction endonuclease cleavage sites are indicated. E, *Eco*RI; S, *Sph* I; and T, *Stu* I. The hybridization probe is indicated by the hatched box. (*B*) Southern blot analysis of *Eco*RI-digested genomic DNA from offspring of CR1/CR2 +/− intercrosses. (*C*) Flow cytometry of +/+ (dashed line), +/− (thick solid line), and −/− (thin solid line) splenocytes using anti-mouse CR1/CR2 mAb 8C12, 7G6, or 7E9; or control Rat IgG polyclonal Ab. The *x* axis is relative fluorescence intensity; the *y* axis is cell number.

Southern Blotting. DNA from individual double-resistant stem cell clones or from tails of 4-week-old mice were prepared according to the method of Laird *et al.* (23). Southern blotting was done according to standard protocol (24). Filters were hybridized overnight with a 0.2-kb probe derived from *Cr2* SCR 11.

Flow Cytometry. 7E9 and 7G6 are rat anti-mouse CR1 and CR2 mAb, and 8C12 is a rat anti-mouse CR1 mAb (12). Phycoerythrin (PE)-conjugated anti-mouse B220, PE-conjugated anti-mouse CD5, and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgM were from PharMingen. PE-conjugated anti-mouse CD4 and FITC-conjugated anti-mouse CD8 were from Becton Dickinson.

Single-cell suspensions were prepared from spleen, thymus, bone marrow, and peritoneum. CR1/CR2 staining was done as previously described (10). Binding was detected by the addition of FITC-conjugated polyclonal goat anti-rat Ab (Southern Biotechnology Associates). Flow cytometry was performed on a FACScan (Becton Dickinson). For the two-color immunofluorescence, Ab were added according to manufacturer's instructions.

Immunohistochemistry. Spleens were removed and frozen quickly in OCT. compound (Miles, Elkhart, IN). Ten-micrometer-thick sections were cut and fixed in acetone. Endogenous peroxidase was quenched with 0.2% H_2O_2/methanol. Sections were first incubated with peanut agglutinin conjugated to biotin (Vector, Burlingame, CA) and rat anti-IgD (Southern Biotechnology Associates) polyclonal antiserum. After being washed, these sections were further incubated with alkaline phosphatase (AP) conjugated to streptavidin (Zymed) and rabbit anti-rat IgG conjugated with horseradish peroxidase (Southern Biotechnology Associates). Bound AP and horseradish peroxidase were detected with AP reaction (Vector) and diaminobenzidine. Sections were counterstained with 1% methylgreen and covered with crystal-mount (Biomedia, Foster City, CA).

Immunization of Mice. Mice (10–12 weeks old) were immunized i.v. with 100 μl of phosphate-buffered saline containing 5×10^5 or 1×10^8 SRBC at day 0 and were then boosted at day 20. Serum was obtained before and at the indicated intervals after the first immunization.

ELISA. Serum immunoglobulin levels were measured by using Immulon 4 plates (Dynatech) and the clonotyping system/horseradish peroxidase (Southern Biotechnology Associates). The relative concentration of immunoglobulin in individual samples was calculated by comparing the mean OD obtained from triplicate wells to a standard curve using linear regression analysis.

Anti-SRBC Ab were measured by the method of Heyman *et al.* (25). The detecting antibody was 100 μl of a 0.2 μg/ml AP-conjugated goat anti-mouse isotype-specific antibody (Southern Biotechnology Associates), added for 1 hr, followed by AP substrate *p*-nitrophenyl phosphate (Sigma) at 1 mg/ml. The mean OD at 405 nm from triplicate wells was compared to a standard curve of titrated serum to calculate the relative units (RU) (17, 26).

RESULTS

Generation of CR1/CR2-Deficient Mice. CR1/CR2 −/− mice were generated by mutation of the *Cr2* gene in embryonic stem cells using homologous recombination. A portion of the first and all of the second exon encoding SCR 14 were replaced with a neomycin resistance marker in the antisense orientation with respect to the *Cr2* gene. Three transfected stem cell clones out of 576 screened had the predicted mutation as detected by Southern blot analysis. One clone generated chimeric mice which transmitted the mutation to their progeny. The wild-type genotype generates a 13-kb *Eco*RI fragment when hybridized with a probe derived from SCR 11, Fig. 1*A*). The

Table 1. Frequency of B lymphocytes from CR1/CR2-deficient mice

	Bone marrow			Spleen	Peritoneum	
Mouse	IgM^-B220^+	IgM^+B220^{lo}	IgM^+B220^{hi}	IgM^+B220^+	IgM^+B220^+	IgM^+CD5^+
+/+	38 ± 4	10 ± 6	9 ± 4	27 ± 8	64 ± 8	32 ± 10
+/−	27 ± 1	6 ± 2	6 ± 1	24 ± 9	52 ± 2	26 ± 16
−/−	31 ± 11	15 ± 5	9 ± 4	22 ± 11	58 ± 13	33 ± 14

Data are from five 9- to 12-week-old mice. Numbers represent the percentage of lymphocytes (± SD) expressing the indicated cell-surface marker as determined using two-color immunofluorescence staining. For all values, $P > 0.05$ as determined using the paired Student's t test.

targeted DNA shows an 8-kb hybridizing fragment (Fig. 1*B*). Targeting was confirmed by Southern blotting of *Sph* I-digested genomic DNA and by PCR analysis (data not shown).

Mice heterozygous for the targeted allele were mated, and CR1/CR2 +/− and CR1/CR2 −/− mice were obtained at the expected Mendelian frequency. CR1/CR2 −/− mice were deficient in the expression of the receptors as determined by flow cytometry of spleen cells using three different rat anti-mouse CR1/CR2 mAbs (Fig. 1*C*), and by immunostaining of FDC in spleen tissue sections (data not shown). Mice heterozygous for the mutation had intermediate expression of the receptors. By Northern blotting, there was no detectable CR1/CR2 mRNA expression in the −/− mice (data not shown). CR1/CR2 −/− mice thrived and reproduced as well as their wild-type littermates and showed no obvious phenotypic abnormalities.

B-Cell Development and Serum Immunoglobulin Levels in CR1/CR2–Deficient Mice. We determined the percentage of B-cell precursors in the bone marrow by using two-color flow cytometry and Abs against various B-cell surface proteins (Table 1). There was no significant difference between CR1/CR2 +/+ and CR1/CR2 −/− mice in the percentage of IgM^-B220^+ pro-B and pre-B cells, IgM^+B220^{lo} immature B cells, and IgM^+B220^{hi} mature B cells. There was also no significant difference in the percentage of IgM^+B220^+ splenic and peritoneal B cells or in the percentage of IgM^+CD5^+ peritoneal cells. The spleen size and the total number of splenocytes were similar in CR1/CR2 +/+ and CR1/CR2 −/− mice. The thymic and splenic T-cell populations were also indistinguishable (data not shown). Furthermore, there was no significant difference in the levels of total serum immunoglobulins (Table 2).

Ab Responses. To determine whether CR1/CR2 contributes to the regulation of the humoral immune response, we immunized mice with the particulate T-cell-dependent antigen SRBC. CR1/CR2 −/− mice showed a moderate decrease in the IgM response as compared to their wild-type littermates, with ≈28% lower response after immunization with a low dose (5×10^5) of SRBC and 37% lower response after immunization with a high dose (1×10^8) of SRBC (Fig. 2). In contrast, there was dramatic impairment in the antigen-specific IgG response seen with either low or high doses of antigen. There was a 92% and 89% reduction in IgG1 titers, 61% and 75% in IgG2a titers, 68% and 84% in IgG2b titers, and 85% and 94% in IgG3 titers after immunization with low and high doses of antigen, respectively.

After secondary immunization, CR1/CR2 −/− mice showed an IgG response several-fold higher than during the primary response, but still substantially decreased as compared to controls (Fig. 3). There was a 58% and 70% reduction in IgG1 titers, 26% and 68% in IgG2b, and 84% and 51% in IgG3 using low and high doses of antigen, respectively. There was no detectable difference in the IgG2a secondary response using high doses of antigen, although there was a 47% reduction in the IgG2a response with the low dose. Surprisingly, despite the substantial differences in IgG responses, germinal center morphology in the CR1/CR2 −/− mice immunized with 5×10^5 SRBC was indistinguishable from their identically immunized CR1/CR2 +/− and +/+ littermates (data not shown).

DISCUSSION

We have generated mice deficient in CR1 and CR2 using gene targeting and have used these mice to investigate the role of CR1 and CR2 in B-cell development and in the humoral immune response. CR1/CR2 −/− mice have normal serum levels of total IgM and of the different IgG isotypes and show no evidence of altered B- or T-cell development. Nevertheless, when challenged with a T-cell-dependent antigen, their humoral immune responses are markedly impaired with a decrease in the titers of antigen-specific immunoglobulin isotypes. CR1/CR2 −/− mice immunized with either low or high doses of antigen show significantly lower levels of anti-SRBC antibodies, especially in IgG1 and IgG3 (Figs. 2 and 3). This result is consistent with previous reports concerning the Ab responses of mice treated with anti-mouse CR1/CR2 Ab during primary immunization (14–18) and suggests that CR1/CR2 acts importantly to enhance the efficiency of the B-cell response. Previous reports have suggested, though, that the deficit in the primary immune response could be overcome with a high immunogen dose, specifically the same 1×10^8 SRBC dose we have used. We interpret the difference between our data and previously published studies as due to incomplete removal of CR1/CR2 from the surface of B cells and/or FDC when using the anti-mouse CR1 and CR2 Ab, thus obscuring the defect we found at high antigen doses.

In this experimental model, we find only a moderate decrease in IgM responses compared to the marked decrease in IgG responses. Studies by Wiersma *et al.* (15), using anti-mouse CR1/CR2 mAb, and Hebell *et al.* (17), using soluble human CR2 to block the interaction of endogenous murine CR2 with its ligand, demonstrate similar patterns of antibody responses. However, other studies by the same groups, as well as by other investigators using similar experimental conditions, have reported the nearly complete inhibition of the antigen-specific IgM response (14–18). The reason for these apparently conflicting results are not known but may reflect the specific antigen used, the antigen dose, and/or the particular mouse strain used.

The severe impairment of IgG responses in the context of only a moderately affected IgM response in CR1/CR2 −/− mice suggests that, in addition to the role of these receptors in B-cell activation, CR1 and/or CR2 are also important for

Table 2. Serum immunoglobulin levels, μg/ml

	Wild-type	CR1/CR2 deficient
IgM	1134 ± 214	1131 ± 202
IgG1	414 ± 214	488 ± 144
IgG2a	341 ± 277	117 ± 129
IgG2b	452 ± 143	366 ± 160
IgG3	197 ± 95	112 ± 154
IgA	149 ± 30	164 ± 73

Mean (± SD) immunoglobulin levels of six 10-week-old mice per group were determined by ELISA. For all values, $P > 0.05$ as determined using the paired Student's t test.

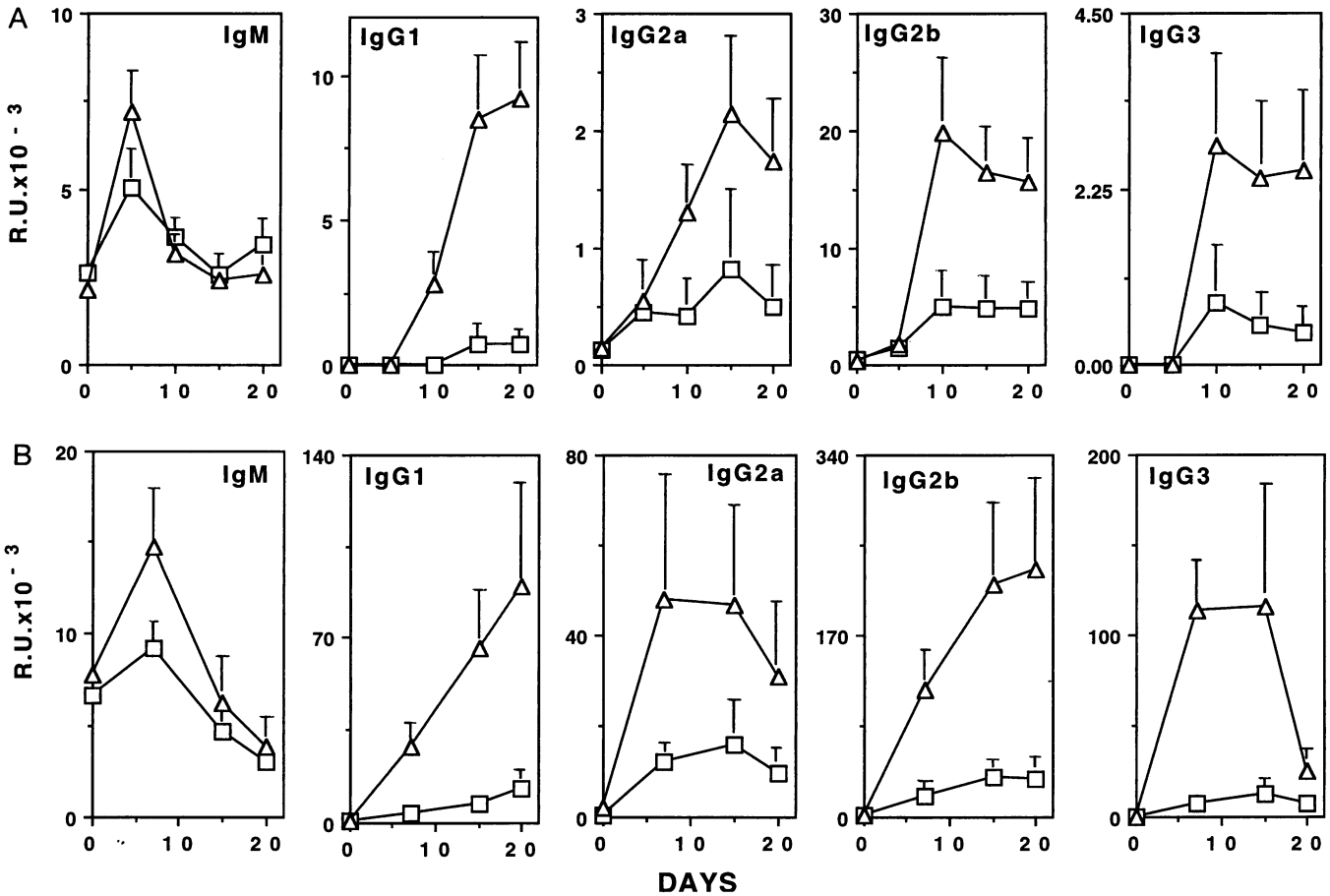

FIG. 2. Humoral immune response in CR1/CR2 +/+ (triangles) or −/− (squares) mice. Groups of four to five 10-week-old mice were immunized at day 0 and bled at the indicated times. Results represent means ± SEM. (*A*) Mice injected with 5×10^5 SRBC. IgG isotype levels are significantly decreased in CR1/CR2 −/− mice compared to wild-type littermates, $P < 0.05$ for day 10, 15, and 20; IgM $P = 0.11$ for day 5; as determined using the paired Student's *t* test. (*B*) Mice injected with 1×10^8 SRBC. For IgG1 and IgG2b, $P < 0.05$ for day 7 and 15; for IgG3, $P < 0.05$ for day 7; for IgM, $P = 0.054$ for day 7.

normal B-cell maturation into IgG-secreting cells. Interestingly, the action of CR1/CR2 in isotype switching varies for the different IgG isotypes. IgG1 and IgG3 are the most dramatically dependent on CR1/CR2, especially during the primary response. Further analysis will be required to define at which stage of the B-cell maturation pathway CR1/CR2 acts.

Our results show that the complement receptors are not absolutely required for B-cell activation. In the absence of CR1/CR2, several features of the B-cell response remain intact. (*i*) Germinal center formation is retained. Thus, CR1/CR2 does not appear directly to regulate the formation of these organized lymphoid structures. (*ii*) Antigen-specific titers of IgG are increased after booster immunization. Thus, secondary responses are less impaired, suggesting that alternate B-cell activation pathways can be recruited to partially compensate for the CR1/CR2 deficiency. Finally, CR1/CR2

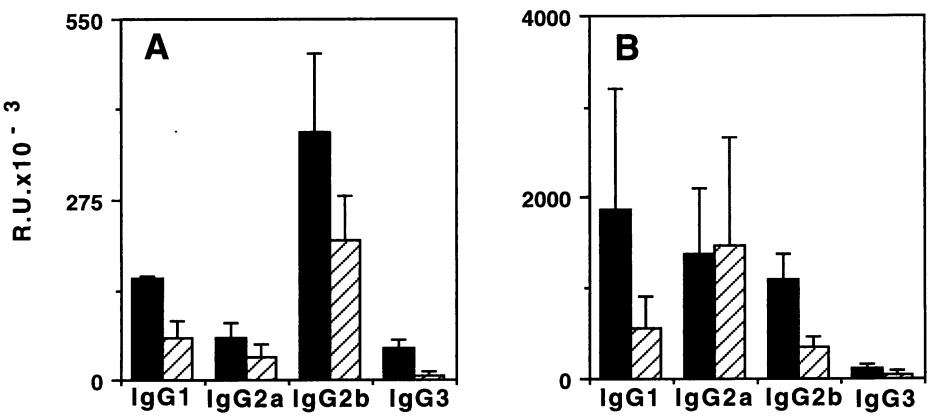

FIG. 3. Secondary immune response. CR1/CR2 +/+ (solid bar) or −/− (hatched bar) mice. Groups of four to five 10-week-old mice were boosted at day 20 after the primary immunization and anti-SRBC immunoglobulin titers determined at day 27. Results represent means ± SEMs. (*A*) Mice injected with 5×10^5 SRBC. IgG1 and IgG3 responses of CR1/CR2 −/− mice were significantly decreased as compared with wild-type littermates, $P < 0.01$; IgG2a, $P = 0.12$; IgG2b, $P = 0.24$; as determined using the paired Student's *t* test. (*B*) Mice injected with 1×10^8 SRBC. For all values $P > 0.05$.

−/− mice show a dose-dependent increase in antigen-specific IgG following i.v. immunization with SRBC.

CR2 noncovalently associates with other molecules to form a multi-molecular signal transduction complex on the B-cell surface (27, 28). One of these molecules is CD19, a 95-kDa immunoglobulin superfamily glycoprotein involved in the activation and proliferation of B cells. CD19 −/− mice share some characteristics with the CR1/CR2 −/− mice (29, 30). CD19 −/− mice mount modest primary IgM and IgG1 responses after immunization with T-cell-dependent antigens. Like CR1/CR2 −/− mice, the secondary response of CD19 −/− mice is also reduced compared to wild-type controls. These results underscore the importance of the physical interactions between these two molecules. CR2 has been proposed to serve as the receptor interacting with C3d-coated, surface IgM-bound antigens. CD19 is then thought to act as the signalling component initiating a series of intracellular events that amplifies and facilitates B-cell activation through surface IgM. Our results establish that CD19 alone cannot compensate for a loss of CR1/CR2.

Although CD19 −/− mice develop antibody responses similar to CR1/CR2 −/− mice, important differences are seen between the two experimental models. CD19 −/− mice fail to generate germinal centers after antigenic stimulation. In addition, they have reduced numbers of B cells in peripheral lymphoid tissues, especially of the B1 subpopulation of peritoneal lymphocytes. Furthermore, serum immunoglobulin levels in nonimmunized mice are significantly reduced. These disturbances are not present in the CR1/CR2-deficient mice. These contrasting results suggest that there are additional independent roles of CD19 and CR1/CR2 in B-cell physiology. For example, CD19 is expressed very early in B-cell ontogeny, whereas CR1/CR2 is limited to mature B cells. Moreover, CD19 and CR1/CR2 can be present in the B-cell surface independently and can each associate with other molecular complexes (31). In humans, CR2 can also be independently associated with CR1. Although CR2 has an intracytoplasmic tail believed to be too short for signal transduction, this tail has been suggested to interact with the p53 antioncoprotein and the p68 calcium-binding protein (32). The conservation of the cytoplasmic domains of human and murine CR2 also implies constraints on the structure of this portion of the molecule consistent with a direct role of the receptor in intracellular events (27).

B-cell responses can be subdivided into different phases (33). Priming of B cells is a consequence of antigen engagement. Recruitment of additional stimulatory signals leads to proliferation of the antigen-binding B cells followed by their differentiation into immunoglobulin-secreting cells or memory cells. In this model, CR1/CR2 can directly modify the initial cognitive phase of this activation pathway by amplifying the subsequent costimulatory signals delivered through CD19. In addition, not only the quantity but the quality of the initial signal could be affected, as CR2 co-crosslinking with surface IgM has been shown to rescue resting B cells from anti-IgM-induced apoptosis (34). Direct signalling through CR2 or a CR1/CR2 molecular complex on B cells, or via C3d-coated antigen bound to CR1 and CR2 on FDC, could further shape the nature of the ongoing immune response. Different effects of CR1/CR2 deficiency on each isotype suggest that T-cell function may also be altered, as isotype switching is largely controlled by the types of T-cell cytokines produced. The CR1/CR2 −/− mice described here establish an excellent experimental model in which to study in detail the function of these receptors during each phase of the humoral immune response.

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