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Targeted Inhibition of the Complement Alternative Pathway with Complement Receptor 2 and Factor H Attenuates Collagen Antibody-Induced Arthritis in Mice

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Abstract

The alternative pathway (AP) of complement is required for the induction of collagen Ab-induced arthritis (CAIA) in mice. The objective of this study was to examine the effect of a recombinant AP inhibitor containing complement receptor 2 and factor H (CR2-fH) on CAIA in mice. CR2 binds to tissue-fixed activation fragments of C3, and the linked fH is a potent local inhibitor of the AP. CAIA was induced in C57BL/6 mice by i.p. injections of 4 mAb to type II collagen (CII) on day 0 and LPS on day 3. PBS or CR2-fH (250 or 500 μg) were injected i.p. 15 min after the mAb to CII on day 0 and 15 min after LPS on day 3; the mice were sacrificed on day 10. The disease activity score (DAS) was decreased significantly ($p < 0.001$) in both groups receiving CR2-fH compared with the PBS. Histology scores for inflammation, pannus, bone damage, and cartilage damage decreased in parallel with the DAS. C3 deposition in the synovium and cartilage was significantly reduced ($p < 0.0001$) in the mice treated with CR2-fH. In vitro studies with immune complexes containing type II collagen and mAb to CII showed that CR2-fH specifically inhibited the AP with minimal effect on the classical pathway (CP) and no effect on the lectin pathway (LP). The relative potency of CR2-fH in vitro was superior to mAbs to factor B and C5. Thus, CR2-fH specifically targets and inhibits the AP of complement in vitro and is effective in CAIA in vivo.

The pathogenesis of rheumatoid arthritis (RA)[3] can be divided into three phases: initiation, perpetuation, and chronic inflammation. Innate immune mechanisms may be involved in all three stages (1–3). Both the cartilage and synovium are major targets of immune dysregulation

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[3]Abbreviations used in this paper: RA, rheumatoid arthritis; AP, alternative pathway; CAIA, collagen Ab-induced arthritis; CIA, collagen-induced arthritis; CP, classical pathway; CII, type II collagen; DAS, disease activity score; IC, immune complex; LP, lectin pathway; SCR, short consensus region; WT, wild type.

and inflammatory injury. It is now well appreciated that autoantibodies such as Abs to citrullinated protein Ags and rheumatoid factors are associated with severe disease and may be pathogenic in the human RA disease process. The major cells and molecules of the innate immune system, including complement activation fragments and its proinflammatory receptors, are all present in the rheumatoid synovium at substantially elevated levels, suggesting an important role for the complement system in tissue damage in RA (4).

The complement system can be activated by each of three distinct pathways; the classical (CP), alternative (AP), and lectin (LP) pathways. While the LP and the CP are initiated by the recognition of target molecules and engagement of the cascade, the AP is unique in that activation can occur by the process termed “tick-over” (5). Activation of the AP can also be promoted when properdin, a positively acting molecule in the pathway, recognizes targets and promotes initial C3b deposition (6,7). In addition, a fourth mechanism, the AP amplification loop, is engaged when C3b that is generated by any of the three activation pathway C3 convertases binds to target surfaces. The AP amplification loop proteins are identical with the AP itself and function by the binding of factor B to C3b, with subsequent enzymatic cleavage by factor D to form the C3bBb complex, which serves as a C3 convertase to generate more C3b. The AP amplification loop can be promoted at sites of local injury when inflammatory cells are recruited, either through a mechanism that involves the additional generation of necrotic cells that fix complement, or more likely because these infiltrating cells contain C3 and properdin that increase activation specifically at that site (8,9). Factor H is a regulatory protein specific for the AP C3 and C5 convertases.

The collagen-induced arthritis (CIA) model of human RA is induced by immunization of mice with type II collagen (CII), usually bovine, in incomplete Freund's adjuvant supplemented with mycobacteria (10). In DBA/1j mice, following immunization at day 0 and day 21, on about day 25 detectable inflammation is observed that is quantitated in a blinded fashion using standard clinical scoring scales (11). Humoral autoimmunity is measured by the development of IgG Abs to CII that react with both bovine and murine collagen, with the major pathogenic Ab isotype being IgG2a (12). T cell autoimmunity to CII develops in CIA, and the major immunodominant peptides have been identified previously (13). The effector phase can be mimicked by the passive transfer of anti-CII mAbs in collagen Ab-induced arthritis (CAIA) in the C57BL/6 mouse strain. These mice have been primarily utilized in CAIA studies from this laboratory (14–16), as well as others (17–19), in large part because gene-targeted mice have been bred onto that strain, and C57BL/6 mice are fully susceptible to joint injury in the CAIA model.

The relative roles of C3 and C5 in CIA have been explored using therapeutic interventions that interfere with one or both of these complement components. For example, inhibition of complement activation with soluble complement receptor 1 (sCR1), a potent inactivator of both the CP and AP C3 convertase, was found to block the development and progression of CIA in mice; both anti-CII Ab levels and T cell responses to collagen were reduced by this treatment (20). Mice deficient in either C3 or factor B were inhibited in the development of CIA and demonstrated decreased levels of anti-CII Abs (17,21). Additional studies have explored the specific role of C5 and the membrane attack complex in CIA. Treatment of DBA/1j mice with a mAb to murine C5 both prevented the onset of and ameliorated established CIA, and DBA/1j mice congenic for C5 deficiency also exhibited resistance to CIA despite demonstrating normal humoral and cellular immune responses to collagen (22,23). Finally, a key role for C5a and the C5a receptor has been suggested by the finding that C5aR gene-targeted mice are protected from CAIA (24).

Using gene targeted mice, we have previously shown that the AP is unique in its requirement for development of arthritis, whereas the CP and LP are dispensable. Notably, mice that have

only a functional AP, due to disabling of the other two pathways by gene targeting, are also fully capable of developing CAIA (16,25,26). Thus, using this experimental approach, the AP appears to be both necessary and sufficient for mediation of pathogenic complement activation in vivo in the joint.

To further evaluate the role of the complement system in experimental forms of disease, a novel complement therapeutic agent was used that has the capacity to be "targeted" to sites of complement activation (27). This agent contains an amino-terminal domain encoding the iC3b/C3d binding site from complement receptor type 2 (CR2), and a carboxyl-terminal domain encoding the complement regulatory protein domain of the murine protein CR2-related gene/protein y (Crry), which inhibits both the CP and AP C3 convertase (28). The use (27,29) of this novel targetable inhibitor, CR2-Crry, has been reported in several in vivo disease models. The results of these studies have shown accumulation of the inhibitor at sites of initial tissue iC3b/C3d deposition, and a prolonged tissue half-life, both consistent with the effect of the CR2 domain in mediating binding to these tissue-bound ligands. Previous studies using CR2-Crry in the active CII immunization model of CIA demonstrated that clinical disease activity and histologic injury were significantly lower in CR2-Crry treated groups (30).

The same CR2 domain has been linked to the amino terminus five short census repeats (SCRs) of the specific inhibitor of the AP, mouse factor H. These factor H SCRs encode the AP C3 and C5 convertase inhibitory activity of factor H. The remainder of the factor H SCR-containing structure is comprised of binding sites for in vivo ligands. CR2-fH has recently been shown to demonstrate a protective effect in the intestinal ischemia-reperfusion injury model and to exhibit the same targeting and tissue-binding characteristics as CR2-Crry (31). The objectives of these studies were to examine the effects of CR2-fH in the development of CAIA in wild-type (WT) mice in vivo and in immune complex (IC)-induced complement activation in vitro. The results of recent studies from our laboratory have established that adherent IC and surrogate IC in the form of adherent IgG are capable of activating the complement system through the AP (25).

Materials and Methods

Animals

Ten-week-old C57BL/6 male WT mice were obtained from The Jackson Laboratory. All mice in different treatment groups were age-matched. Filter top cages were used with 3 mice in each cage, kept in a barrier animal facility with a climate-controlled environment with 12 h light/dark cycles. All mice were fed breeder's chow provided by the Center for Laboratory Animal Care (University of Colorado Denver). Sera from WT, $C3^{-/-}$,$C4^{-/-}$, $Bf^{-/-}$, $MBL^{-/-}Df^{-/-}$ (CP only), $C1q^{-/-}/Df^{-/-}$ (LP only), and $MBL^{-/-}/C1q^{-/-}$ (AP only) mice were used, with all genetically deficient mice bred onto the C57BL/6 background for at least seven generations.

Plasmid expression vector and recombinant protein purification

A cDNA construct was made of a CR2-fH fusion protein consisting of the 4 N-terminal SCRs of CR2 linked to the 5 N-terminal SCRs of mouse factor H. The recombinant mouse CR2-fH protein was produced by amplification of the DNA plasmid in DH5α competent cells (Invitrogen) and purification of the plasmid by Maxi-Prep (Qiagen), according to the manufacturers' instructions. The plasmid was used to transfect Freestyle 293 cells (Invitrogen), and CR2-fH was purified from the cell supernatant by affinity chromatography. An affinity column was created by conjugating polyclonal goat anti-human factor H antisera (Quidel) to CNBr-activated Sepharose 4B (GE Healthcare) according to the manufacturer's instructions. Supernatant from cells transfected with the plasmid for CR2-fH was passed over the column at physiologic pH, washed with PBS, and eluted with 0.1 M sodium glycine (pH 2.7). The

eluted protein was collected into 1 M Tris (pH 8), and the buffer was exchanged with PBS (pH 7.4). The purity of the protein was verified by electrophoresis on a 10% Bis-Tris polyacrylamide gel (Invitrogen) and staining with Coomassie blue (m.w. 70 kDa). Confirmation of the identity of the purified factor H was made by Western blot analysis by subjecting the protein to electrophoresis on a 10% Bis-Tris gel, followed by transfer to nitrocellulose. The membrane was then probed with a goat anti-human factor H antiserum (Quidel) (diluted 1/1000) followed by HRP-conjugated rabbit anti-goat IgG (Fc) Ab (diluted to 1 μg/ml), then visualized using a chemiluminescence detection kit (Amersham Biosciences). After purification, each batch of recombinant CR2-fH was also tested for retention of activity in inhibition of the AP in vitro using sera from $C4^{-/-}$ mice, as previously described (26).

Recombinant CR2-Crry used in some experiments was also purified by similar methods as described above. Other inhibitors of the AP complement activation including the mouse anti-mouse factor B mAb 1379 (32) and mouse anti-mouse C5 mAb BB5.1 were used in some assays for comparison with CR2-fH (14).

Induction of CAIA and treatment protocol

CAIA was induced by using a cocktail of four mAb to bovine CII (Arthrogen-CIA; Chondrex) resuspended in sterile Dulbecco's PBS. All four (three IgG2a, one IgG2b) mAb in this cocktail recognize the conserved epitopes (CB11) shared by various species of CII. All mice received i.p. injections of 8 mg/mouse of Arthrogen on day 0 and 50 μg/mouse of LPS from *Escherichia coli* strain 0111B4 on day 3 to synchronize the development of arthritis.

Three separate experiments with WT mice were used for studies on the effects of CR2-fH on CAIA. In the first experiment, two groups of 4 mice were used. The first group was injected with 200 μl of PBS 15 min after the injection of 8 mg of Arthrogen and again 15 min after the injection of LPS. The second group was injected with 250 μg of CR2-fH at the same two times as the PBS (total of 500 μg/mouse). Second and third experiments were conducted to examine different doses of CR2-fH. In the second experiment, three groups of mice were used. The first group received 200 μl of PBS injected at the two times, as described above. The second and third groups were injected with 250 or 500 μg, respectively, of CR2-fH at the same times as the PBS (total of 500 or 1000 μg/mouse). In the third experiment, two groups of 4 WT mice were used, injected with PBS as described above. WT mice were also injected i.p. twice with 250 μg of CR2-Crry (total of 500 μg CR2-Crry) in an identical fashion. All mice treated with PBS started to develop arthritis on day 4 and were sacrificed on day 10. Blood was drawn retro-orbitally from all mice at day 0 before injection of mAb to CII, at day 3 before LPS injection, and at day 10 before sacrifice. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Colorado Denver.

Examination for clinical disease activity

The severity of clinical disease activity in all groups of WT mice was determined every day by two trained laboratory personnel acting independently and blinded to the treatment. The clinical disease activity was scored on a 3-point scale per paw: 0 = normal joint; 1 = slight inflammation and redness; 2 = severe erythema and swelling affecting the entire paw with inhibition of use; and 3 = deformed paw or joint with ankylosis, joint rigidity and loss of function. The total score for clinical disease activity (DAS) was based on all four paws with a maximum score of 12 for each mouse.

Histopathology of knee joints

At day 10, both forepaws and the entire right hind limb, including the paw, ankle and knee, were surgically removed from all mice treated either with PBS, CR2-fH or CR2-Crry and fixed immediately in 10% neutral buffered formalin (NBF) (Biochemical Sciences). The preparation

of tissue samples and histological analyses were performed as previously described (16). All sections were read by a trained observer who was also blinded to the treatment and to the DAS of each mouse. The joint sections were scored for changes in inflammation, pannus, cartilage damage, and bone damage, on a scale of 0–5. Details of the Histopathology Scoring Criteria can be found in the Extended Methods.[4] The overall score was calculated as the total of the four individual parameters with each parameter represented as the mean value for 5 joints per mouse (front right and left paws, and hind right limb knee, ankle, and paw).

Immunohistochemistry for C3 and factor H deposition

In initial experiments, mice were sacrificed at 1, 8, and 24 h after injection of the mAb to CII with knees excised and fixed in formalin. In the experiments using treatment with CR2-fH, after sacrifice on day 10 both forepaws and the right hind limb were excised and fixed in 10% formalin. C3 deposition in the joints (synovium and cartilage) was localized with a polyclonal goat anti-mouse C3 antiserum (ICN Pharmaceuticals), and factor H localization was examined with a polyclonal goat antiserum specific for human factor H but cross-reactive with mouse factor H (Quidel) (26). Immmunohistochemical stain scoring was performed in a blinded fashion using a 3-point system for the synovium and surrounding tissue where 0 represented no staining and 3 represented 3+ staining. The scoring system for the cartilage was similar: 0 = no staining present; 1 = one area of moderate staining of chondrocytes in one joint; 2 = multiple areas of moderate staining of chondrocytes in multiple joints; 3 = multiple areas of intense staining of chondrocytes and/or diffuse multifocal staining of articular cartilage lesions. For each animal, the synovium and cartilage scores were determined separately for each of the five joints summarized above. The synovium and cartilage scores for all animals in an experiment were then calculated separately as mean ± SEM.

Effect of CR2-fH on C3 deposition, C3a levels and C5 activation in vitro

The in vitro effect of CR2-fH on C3 deposition induced by adherent immune complexes (IC) of CII and mAb to CII was examined. The source of C3 included sera from WT mice or sera from $C3^{-/-}$, $C4^{-/-}$, $MBL^{-/-}/Df^{-/-}$ (CP only), $C1q^{-/-}/Df^{-/-}$ (LP only), and $C1q^{-/-}MBL^{-/-}$ (AP only) mice. Preparation of the adherent IC was conducted as previously described (26). Serum samples were incubated with different concentrations of CR2-fH (0, 0.125, 0.25, 0.5, 1, 2, 4, 8, or 10 μg/10 μl of serum) for 30 min at 4°C. Sera without or with CR2-fH were then diluted 1/10 in Veronal saline buffer (VSB) plus 1 mM $MgCl_2$ and 2 mM $CaCl_2$ and added to the wells with incubation at 37°C for 1 h. Following washes in PBS/0.5% Tween, HRP-conjugated goat anti-mouse C3 Ab (MP Biomedicals) was added to the wells. The anti-C3 Ab was diluted 1/4000 in freshly made PBS/0.05% Tween to reduce the background to minimum levels. Plates were incubated at room temperature for another 1 h. After six more washes, the color reaction and absorbance were determined at 450 nm subtracting the background absorbance at 550 nm. In all experiments, C3 deposition was also measured under similar conditions by using plates coated with CII alone. Data were expressed by using the following formula: mean OD with IC minus OD of CII alone. Minimal background was observed with controls of IC or CII alone incubated with VSB plus 1 mM $MgCl_2$ and 2 mM $CaCl_2$ in the absence of sera.

To examine the effects of complement inhibitors on C3 deposition and C5a activation in vitro, Costar 96-well ELISA plates were coated with anti-CII mAb only (arthrogen; 25 μg/well) overnight, followed by washing and blocking with 1% BSA for 2 h. Serum samples diluted 1/50 in 1% BSA/PBS were incubated on the plate for 2 h. Following washing, anti-human C3 polyclonal biotinylated detector Ab at 1 μg/ml in 1% BSA/PBS was added and incubated for 1 h. Peroxidase-conjugated streptavidin diluted 1/1000 in 1% BSA/PBS was added to the plate, followed by the addition of 100 μl of 2,2′-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-

[4]The online version of this article contains Extended Methods.

diammonium salt to each well. When the reaction reached completion, 1% SDS was added. The ELISA plates were then read at 450 nm subtracting the background absorbance at 550 nm. The levels of C5a were determined in the assay supernatant using a specific C5a ELISA kit (BD Biosciences). CII-IC could not be used for this experiment because adherent CII alone induced some C5 activation. Different dilutions of the pooled sera from WT and *C3*$^{-/-}$ mice were diluted and treated with inhibitors for 30 min at 4°C in calcium sufficient VSB. The levels of C3 deposition on the plate and of C5a in the supernatants were measured as described above. In some studies the levels of C3a in the supernatants were also measured using C3a ELISA kit (BD Biosciences) with sera from WT and *C4*$^{-/-}$ mice incubated on plates coated with anti-CII mAb only.

Serum levels of C3 and factor B

Serum levels of C3 were measured by ELISA, as previously described (26). Levels of factor B in the serum samples were measured by using modifications of a previously described ELISA for factor B (33). Briefly, 96-well ELISA plates were coated for 24 h at 4°C with 3 μg/ml polyclonal goat anti-human factor B Ab diluted in sodium bicarbonate buffer (pH 9.5) (Diasorin). After washing the wells 4 times with PBS/0.05% Tween 20, the plate was blocked with 2% milk (instant nonfat dry, pasteurized extra grade; Nestle USA) in PBS for 1 h at 37° C. All serum samples for this ELISA were diluted 1/100 in PBS with 2% milk. After adding 100 μl of diluted serum samples to the wells, plates were incubated for 2 h at 37°C followed by 4 washings with PBS/0.5% Tween. One hundred microliters of a biotinylated mAb to mouse factor B diluted 1/9000 in PBS/0.5% Tween 20 was added to each well followed by incubation at 37°C for 1 h. Again, after four washings, 100 μl of HRP-conjugated streptavidin (R&D Systems) diluted 1/1000 in 1× PBS/0.5% Tween 20 was added to each well and incubated at 37°C for 1 h. After four more washes, the color reaction was developed by adding 100 μl/well of tetramethylbenzidine (TMB) substrate reagent mix (1/1) (BD Pharmingen). The reaction was stopped after 25 min at room temperature in the dark by adding 50 μl/well of 2 N H_2SO_4 solution followed by absorbance at 450 nm correcting for background absorbance at 550 nm. In these studies, sera from WT mice were used as a positive control and sera from *C3*$^{-/-}$ and *fB*$^{-/-}$ mice were used as negative controls.

Cytokine mRNA levels in knee joints

Cytokine mRNA levels were determined in knee joints at day 10 from WT mice treated with PBS alone and from WT mice treated with low and high doses of CR2-fH. Total RNA was extracted by pulverizing the frozen individual knee joints (synovium, bones, and some adjacent muscles and skin) using Tri-reagent (Sigma-Aldrich), following the manufacturer's instructions. Joint mRNA levels for TNF-α, IL-1α, IL-1β, IFN-γ, IL-4, IL-10, and IL-1Ra were measured by real time RT-PCR using appropriate primers and probes as previously published (16). Analysis of mRNA levels was performed on an ABI Prism 7700 sequence detector system located at the University of Colorado Cancer Center RT-PCR Core (PE Applied Bio-systems); 18 S rRNA was also measured as an internal control for loading of equal amounts of each sample. Quantitative RT-PCR was performed in duplicate for each cytokine from every mRNA sample. The results were expressed as mRNA of specific cytokine (picogram)/18 S rRNA (nanogram).

Statistics

All mice were included in the final analysis of arthritis incidence, disease severity, histology, immunohistochemistry, and quantitative RT-PCR of cytokine mRNA. An unpaired two-tailed *t* test was used to analyze these data. All data were expressed as the mean ± SEM with $p < 0.05$ considered significant. Preliminary analyses using a null hypothesis for w-statistics indicated that the data were normally distributed.

Results

Inhibitory effect of CR2-fH and of CR2-Crry on CAIA

To assess the effects of two different doses of CR2-fH and a single dose of CR2-Crry on CAIA, WT C57BL/6 mice were injected with a mixture of mAb to CII (8 mg/mouse). All mice in the control group were injected with PBS whereas treated mice received 250 or 500 μg of CR2-fH or 250 μg of CR2-Crry administered on days 0 and 3 (total of 500 or 1,000 μg of CR2-fH and 500 μg of CR2-Crry per animal). Mice treated with PBS developed disease on day 4 (Fig. 1*A*). A 24 h delay in the development of CAIA was noted in the mice treated with the lower dose of CR2-fH and a 48 h delay with CR2-Crry and the higher dose of CR2-fH. At day 10, the incidence of disease was 100% in all treatment groups (Fig. 1*A*). No adverse effect of CR2-fH or CR2-Crry treatment was seen with either dose of CR2-fH or with a single dose of CR2-Crry.

Clinical disease activity was significantly reduced by CR2-fH in a dose-dependent manner, beginning on day 6 (Fig. 1*B*). The average DAS on day 10 was 10.9 ± 0.4, 6.38 ± 1.03 and 2.0 ± 0.34 in mice treated with PBS, and with a low or high dose of CR2-fH, respectively (mean ± SEM with $n = 15$, 8, and 7). Mice treated with a low dose of CR2-fH showed a 41% reduction in the DAS on day 10 compared with the mice treated with PBS ($p < 0.001$). Mice treated with a high dose of CR2-fH showed an 81% reduction in DAS on day 10 compared with mice treated with PBS ($p < 0.001$). This shows the marked inhibitory effect of CR2-fH on CAIA. Mice treated with a total of 500 μg/mouse of CR2-Crry developed significantly less disease compared with mice treated with PBS (Fig. 1*B*). Overall there was a 53% decrease in the DAS in the mice treated with CR2-Crry at day 10 ($p < 0.001$).

Effects of CR2-fH on histological changes and C3 deposition in CAIA

Histopathological analysis was performed on joints from mice treated with PBS or CR2-fH (Table I). Marked and significant decreases were observed in the total score as well as in the individual scores for pannus, cartilage damage and bone damage in mice treated with both doses of CR2-fH compared with mice treated with PBS; the score for inflammation was significantly different from PBS using the higher dose of CR2-fH. Histopathological analysis was also performed on joints mice treated with CR2-Crry; a significant decrease ($p < 0.05$) was observed in the total score (Table I).

The levels of C3 deposition in the synovium and cartilage at day 10 were significantly reduced in the mice treated with CR2-fH in a dose-dependent fashion (all $p < 0.01$) (Table II). These results indicate that mice treated with both doses of CR2-fH exhibit reduced C3 deposition in their joints. Representative tissue sections of C3 deposition in knee joints are shown in Fig. 2. The knee joint sections were also analyzed for C3 deposition in the synovium and on the cartilage in the mice treated with CR2-Crry (Table II). There was a significant decrease ($p < 0.01$) in the levels of C3 deposition on the surface of the cartilage (Table II).

Localization of factor H and C3 in joint tissues

We also determined the localization of factor H and C3 in the knee joints of mice at early time points after the i.p. injection of anti-CII mAb or PBS. Both C3 and factor H were present on the cartilage surface and in the synovial tissues. The levels of C3 and fH increased significantly ($p < 0.05$) over the first 24 h after injection of the mAb to CII (Table III). There was a 3.5 fold increase in total C3 deposition in the knee joints at 24 h compared with PBS treated mice. There was a 2.4 fold increase in the levels of total fH in the knee joints at 24 h compared with PBS treated mice, although the accumulation was relatively delayed as compared with C3. Although elevated levels of factor H are present, whether the deposited factor H is bound to the cartilage or synovium at sufficiently high levels to inhibit the AP is not known.

Serum levels of C3 and factor B

Serum levels of C3 and factor B at days 0, 3, and 10 were determined by ELISA and expressed in OD units because of a lack of high quality purified proteins for use as standards. There were no differences in the levels of C3 and factor B in the sera of mice on each day between the 3 treatment groups, PBS vs 500 or 1000 μg total of CR2-fH (data not shown).

Effect of CR2-fH on levels of cytokine mRNA in the knee joints of mice with CAIA

To explore the effects of CR2-fH on mediators in the joints of mice with CAIA, mRNA levels of the cytokines TNF-α, IFN-γ, IL-1α, IL-1β, IL-4, IL-10, and IL-1Ra were measured by RT-PCR. There were no significant differences in cytokine mRNA levels in the knee joints of mice treated with a low dose of CR2-fH compared with mice treated with PBS (Table IV). However, a 3-fold decrease in the level of TNF-α mRNA was seen in the knee joints of mice treated with a high dose of CR2-fH compared with mice treated with PBS ($p < 0.05$). In addition, a 2-fold increase in the mRNA levels of IL-10 was also seen in the knee joints of mice treated with a high dose of CR2-fH ($p < 0.05$). Thus, inhibition of the AP by CR2-fH resulted in a decrease in the mRNA level of the pro-inflammatory cytokine TNF-α in the joints of mice with CAIA as well as an increase in the mRNA level of the anti-inflammatory cytokine IL-10.

Effect of CR2-fH on immune complex-induced C3 deposition mediated by different complement pathways in vitro

We next examined the effects of CR2-fH on different complement pathways in vitro using sera from various gene deleted mice where only the CP, AP or LP was intact. Complete inhibition of IC-induced C3 deposition by CR2-fH was observed in the sera from mice possessing only an intact AP. No C3 deposition was observed in the sera from mice where only the LP was intact, in the presence or absence of CR2-fH (Fig. 3). CR2-fH at 10 μg/10 μl serum led to a small but non-significant decrease in C3 deposition using sera from mice possessing only an intact CP and a modest significant decrease was seen using sera from WT mice. Control experiments with murine CR2 and factor H alone showed no inhibitory effects (data not shown), as reported previously using zymosan AP activation assays (31).

Effect of CR2-fH on immune complex-induced C3 deposition in vitro

We next examined the effect of CR2-fH on inhibition of IC-induced C3 deposition in vitro using sera from WT and complement deficient mice. Different concentrations of CR2-fH were used with 1/10 dilutions of sera from WT, $MBL^{-/-}/Df^{-/-}$ (CP only), $C1q^{-/-}/MBL^{-/-}$ (AP only) and $C4^{-/-}$ (AP only) mice. Full inhibition of C3b deposition was observed with 0.5 μg CR2-fH/10 μl serum and with higher concentrations of CR2-fH using sera from those two strains of mice possessing only an intact AP (Fig. 4*A*). In contrast, concentrations of CR2-fH up to 8 μg/10 μl serum led to no decreases in C3b deposition using sera from mice with only an intact CP as well as with sera from WT mice. Significance testing could not be performed on these data because the volume of sera required was large for this experiment and serum was pooled from three different mice with performance of one replicate. Control experiments with CR2 or factor H (2 μg/10 μl serum) alone gave no inhibition of C3 deposition using sera from WT or $C4^{-/-}$ mice (data not shown). These data indicate that CR2-fH effectively inhibits only the AP of complement.

Since CR2-fH specifically inhibits C3 activation by the AP, it was unclear why CR2-fH exhibited little to no effect on CII-IC-induced-C3 deposition using sera from WT mice. The CP has a critical requirement for Ca^{2+}. To examine the role of the CP, IC-induced C3 deposition was conducted using sera from WT mice in the presence or absence of Ca^{2+}. In the absence of Ca^{2+}, full inhibition of CII-IC-induced C3 deposition was observed with 0.125 μg/10 μl serum and with higher amounts of CR2-fH, whereas no inhibition was observed with any

concentration of CR2-fH up to 8 μg/10 μl serum in the presence of Ca^{2+} (Fig. 4*B*). No C3 deposition was observed with sera from $C3^{-/-}$ mice. These data show that the CP predominates in intact sera but when the CP is inactive, CR2-fH specifically inhibits the AP.

Effect of CR2-fH on anti-CII mAb-induced C5a generation in vitro

Alternative pathway C5 convertase-mediated C5a generation by ICs was also inhibited by CR2-fH. Adherent CII-ICs were not used in this experiment because CII alone led to some background C5 activation. CR2-fH (2 μg) was incubated with 10 μl of serial dilutions of WT sera. The mixture of CR2-fH and sera was then incubated on an ELISA plate coated with anti-CII mAb. The levels of C3 deposition on the plate and C5a in the supernatants were determined by ELISA. The baseline levels of C5a in the serum alone were low. CR2-fH led to significant ($p < 0.05$) decrease in the amount of C3 deposited on the plate at all serum dilutions (Fig. 5*A*). Moreover, CR2-fH completely inhibited C5a production ($p < 0.001$) at all serum dilutions (Fig. 5*B*). No C3 deposition or C5a generation was seen using sera from $C3^{-/-}$ mice used as a negative control (data not shown). These results suggest that in WT sera where all 3 pathways of complement are intact, and using adherent anti-CII mAb to activate complement, C3 convertase activity is induced primarily by the CP, whereas the AP is responsible for the majority of C5 convertase activity.

Effects of CR2-fH, anti-factor B mAb and anti-C5 mAb on anti-CII mAb induced-C3 deposition, C3a and C5a generation in vitro

The relative concentration-dependent effects of CR2-fH on the deposition of C3 as well as the generation of C3a and C5a were compared with anti-factor B mAb 1379 and anti-C5 mAb BB5.1 (Fig. 6). Varying concentrations of CR2-fH, anti-factor B mAb and anti-C5 mAb all up to 8 μg/10 μl serum were incubated at 4°C for 30 min with 10 μl sera from WT (Fig. 6, *A–C*) and $C4^{-/-}$ mice (Fig. 6, *D–F*). The mixtures of sera incubated with CR2-fH, anti-factor B mAb, or anti-C5 mAb were added to wells coated with anti-CII mAbs. The levels of C3 deposition on the plate and of C3a and C5a in the supernatants were determined by ELISA. The baseline levels of C3 deposition on the plate as well as C3a and C5a generation in the serum using uncoated plates were found to be less than 10% of the levels observed after stimulation with anti-CII mAb.

None of the three inhibitors exhibited a major affect on C3 deposition using WT sera, where the CP plays a major role, although CR2-fH showed an inconsistent decrease in C3 deposition (Fig. 6*A*). However, a decrease in the levels of C3a was found with CR2-fH compared with other two inhibitors, beginning at low concentrations (Fig. 6*B*). A modest decrease in C3a generation was observed with anti-factor B mAb only at high concentrations. Although all three inhibitors reduced the generation of C5a from WT sera, the concentration of each inhibitor required was different as CR2-fH was substantially more potent at lower concentrations (Fig. 6*C*).

To confirm the specific effects of these inhibitors, sera from $C4^{-/-}$ mice was used; in the absence of C4, only the AP can activate the complement system. CR2-fH at low concentrations led to a significant ($p < 0.001$) decrease in the amount of C3 deposited as compared with the other inhibitors (Fig. 6*D*). A significant ($p < 0.001$) decrease was also found when comparing anti-factor B mAb to anti-C5 mAb but only at high concentrations (Fig. 6*D*). CR2-fH also demonstrated an enhanced ability to block the generation of C3a and C5a as compared with anti-factor B mAb and anti-C5 mAb (Fig. 6, *E* and *F*). These results show that CR2-fH possesses a superior dose-dependent inhibition of AP activity in comparison to the two other inhibitors examined.

Discussion

These data demonstrate that CR2-fH treatment led to a marked reduction in disease activity in CAIA, an animal model of the effector phase of IC-induced arthritis. In addition, CR2-fH specifically inhibited the AP in vitro, with no significant effects on the CP or LP, after activation of complement by adherent mAb to CII, a surrogate for adherent IC. CR2-fH was also more potent at inhibiting C3a and C5a generation in vitro, induced by adherent mAb to CII, in comparison with mAbs to factor B and C5. CR2-fH, that inhibits only the AP, demonstrated similar inhibition of CAIA as CR2-Crry, a molecule that inhibits all three complement activation pathways. These results confirm the dependency of CAIA on the AP and indicate the potential potency of AP inhibitors that specifically localize to inflammatory sites.

CR2-fH at both lower and higher doses significantly decreased disease activity in CAIA. A similar decrease was seen in the histological measures of inflammation, pannus formation, cartilage damage and bone damage in knee joints of mice treated with CR2-fH. Furthermore, C3 deposition on the synovium and cartilage in the knee joints was decreased significantly in both CR2-fH treatment groups. These histological results suggest that CR2-fH functions in the local environment of the joint.

In our studies, the first injection of CR2-fH was given i.p. 15 min after the injection of anti-collagen Abs and the second injection of CR2-fH was given i.p. 15 min after the injection of LPS. Complement activation in the joint is necessary to generate the C3 fragments iC3b and C3dg to which CR2-fH binds. It is notable from the results in Table III that there are increased levels of C3 on the cartilage and in the synovium shortly after the injection of anti-CII mAbs, which would provide these binding sites for CR2-fH.

In contrast to the effects of CR2-fH and CR2-Crry in the current studies, we have shown previously that Crry-Ig, the soluble untargeted counterpart of CR2-Crry, has no effect on clinical disease activity in CIA (14). However, transgenic mice over-expressing Crry were protected from CIA indicating the importance of this membrane bound complement inhibitor (15). Thus, CR2-targeted inhibitors appear to be more effective at reducing disease activity as compared with non-targeted C3 convertase inhibitors such as soluble Crry-Ig. This difference is likely to be due to pharmaco-dynamic limitations of soluble Crry-Ig as well as a potential relative lack of access to the joint. In addition, as a potential therapeutic agent CR2-fH may have advantages over CR2-Crry since inhibition of only the AP may be safer than inhibition of all three complement activation pathways.

This present study also demonstrates that CR2-fH is superior to anti-factor B mAb and anti-C5 mAb in blocking generation of C3a and C5a in vitro. This enhanced potency is likely due to the specific targeting of CR2-fH to the sites of C3 activation on the adherent mAb to CII, whereas the mAb to factor B and C5 do not specifically target to surface-bound C3 and C5 convertases.

We found a 3-fold decrease in the level of mRNA for the pro-inflammatory cytokine TNF-α in the knee joints of mice treated with the high dose of CR2-fH. In addition, a 2-fold increase in the level of mRNA for the anti-inflammatory cytokine IL-10 was seen in the knee joints of mice treated with the high dose of CR2-fH. These quantitative RT-PCR studies in the knee joints of CAIA mice showed that the administration of CR2-fH has a local effect on the production of cytokines. Interestingly, these changes in pro-inflammatory and anti-inflammatory cytokine profiles of mice treated with high dose of CR2-fH are not seen in mice treated with low dose of CR2-fH. These data suggest that there might be a minimum threshold level of CR2-fH required to affect local changes in cytokine levels in the joints.

Many different mouse models of disease show a dependency on the AP to develop tissue injury, with or without the requirement to engage the CP or LP for the initial C3 fixation necessary to initiate the amplification loop. We have shown that the AP is sufficient and necessary for the development of CAIA (16). Similarly, MRL/*lpr* mice deficient in factor B or factor D showed protection against the development of glomerulonephritis compared with wild type MRL/*lpr* mice (34). We have also shown that renal ischemia-reperfusion injury is dependent on the AP (35) as mice treated with anti-factor B Ab are protected from this experimental disease (32). Recently, it has also been reported that factor B deficient mice develop a delayed and significantly less severe cutaneous blistering disease compared with WT mice (36). Previous studies have shown that a soluble form of human complement receptor of the Ig superfamily reversed inflammation and bone loss in two experimental models of arthritis by specifically inhibiting the AP of complement in the joints (37).

C3a and C5a are the two potent pro inflammatory chemoattractants for neutrophils at the site of inflammation. Neutrophils are the most abundant type of inflammatory cells in the circulation and their half-life is short. In the absence of survival factors, neutrophils undergo spontaneous apoptosis, a process critical to the resolution of inflammation. C5a treatment has been described to delay neutrophil apoptosis in healthy donors; therefore, C5a is a survival factor for neutrophils (38). The decrease in the clinical disease activity of CAIA in WT mice in vivo by the administration of CR2-fH is likely due to the direct effects of CR2-fH on C3a and C5a generation by the AP. Factor H is known to regulate the AP by three different mechanisms: inhibition of AP C3 convertase formation by binding to C3b and inhibiting the binding of factor B (39); accelerating the dissociation of already assembled AP C3 convertases (40); and functioning as a cofactor for factor I in the proteolytic cleavage of C3b to iC3b (41). CR2-fH retains each of these enzymatic activities of factor H.

It is possible that a component of the observed effect of CR2-fH or CR2-Crry in some animal models of disease in vivo may be due to the CR2 portion of these inhibitors acting on endogenous CR2 receptor function. CR2 also binds multiple ligands including C3d, gp350, CD23 and IFN-α (42) and the use of CR2-Crry in the CIA model led to decreased levels of anti-CII Abs (30). However, we have provided evidence that CR2-fH specifically reduces injury during the effector phase of CAIA in mice, and it is unlikely that the other CR2 ligands play a role in the CAIA model.

In conclusion, previous clinical and experimental studies have shown the predominant role of complement in the pathogenesis of many inflammatory diseases (43). The current studies have focused on the role of the AP in the CAIA model and have confirmed its key role in pathogenesis. Future studies should explore how the AP is dysregulated in this model, which might be due to inadequate fH access or binding to the cartilage and/or synovium, to altered function of membrane complement regulatory proteins, to increased local complement synthesis, or to a combination of these effects. Further dissection of the role of the AP in this model should allow an increase in our understanding of the mechanisms by which this pathway promotes inflammatory joint destruction.

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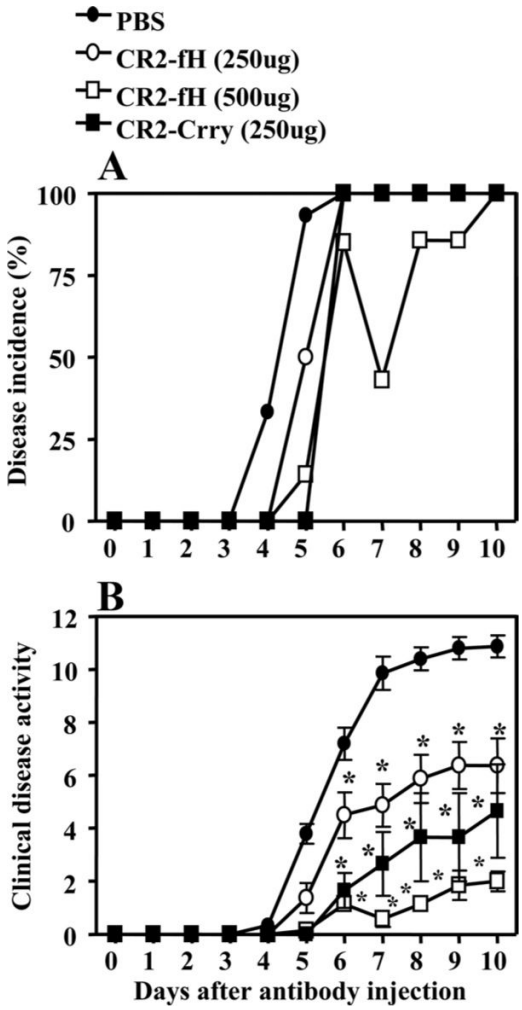

FIGURE 1.

Effect of CR2-fH and CR2-Crry on CAIA. A mixture of four mAbs to CII were injected i.p. on day 0 followed by an i.p. injection of LPS on day 3. The recombinant fusion protein CR2-fH at 250 or 500 μg was injected i.p. twice: 15 min before the mAb injection and 15 min before the LPS injection (total of 500 and 1000 μg of CR2-fH). CR2-Crry recombinant fusion protein was injected at 250 μg i.p. twice: 15 min before the mAb injection and 15 min before the LPS injection (total of 500 μg of CR2-Crry). Incidence of disease and DAS were determined daily by an observer blinded to the treatment of each mouse. The data are expressed as the incidence (%) of arthritis (*A*) and clinical disease activity score (*B*) vs days after injection of the mAb. The data represent the mean ± SEM based on: PBS, $n = 15$; CR2-fH 250 μg/injection, $n = 8$; CR2-fH 500 μg/injection, $n = 7$; and CR2-Crry 250 μg/injection, $n = 3$. *, $p < 0.001$ in comparison with PBS treatment.

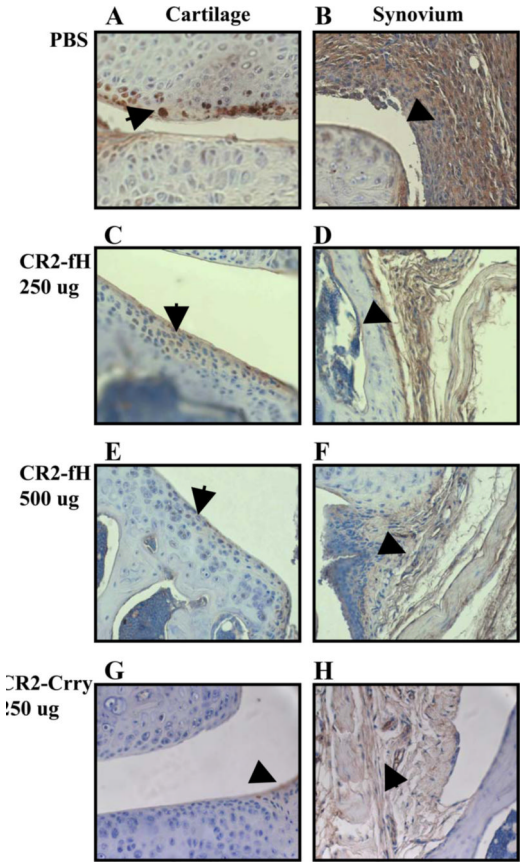

FIGURE 2.

C3 deposition in the cartilage and synovium of knee joints from mice with CAIA treated with PBS, CR2-fH (250 μg/injection given twice and 500 μg/mouse given twice) and CR2-Crry (250 μg/injection given twice). *A* and *B*, Mice treated with PBS showed marked deposition of C3 (brown staining shown by black arrows) on the cartilage surface and in the synovium. *C* and *D*, Mice treated with 500 μg CR2-fH showed minimal C3 staining of the cartilage surface, slight staining of the chondrocytes, and moderate staining of synovial tissue. *E* and *F*, Mice treated with 1000 μg CR2-fH showed minimal C3 staining on the surface of the cartilage, scattered brown staining of the chondrocytes, and minimal staining of the synovium (black arrows). *G* and *H*, Mice treated with 500 μg CR2-Crry showed minimal C3 staining on surface of the cartilage, scattered brown staining of the chondrocytes, and minimal staining of synovium (black arrows). Mice having the highest DAS were selected in each treatment group. Magnification of cartilage surface and synovium was ×40.

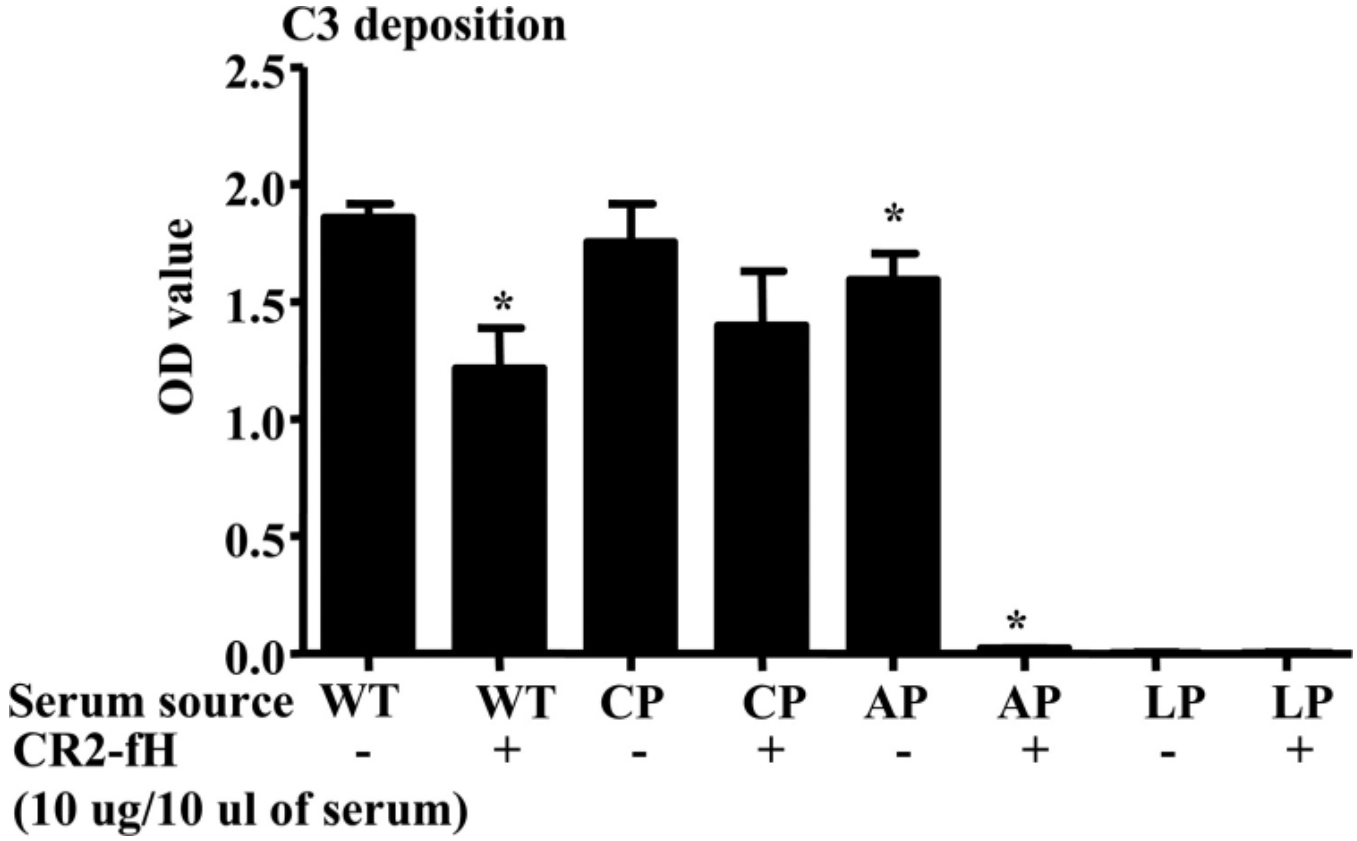

FIGURE 3.

Effect of CR2-fH on the three different complement activation pathways. Sera from WT mice, mice with only an intact CP ($MBL^{-/-}/Df^{-/-}$), mice with only an intact LP ($C1q^{-/-}/Df^{-/-}$), and mice with only an intact AP ($C1q^{-/-}/MBL^{-/-}$) were either pre-incubated or not pre-incubated with CR2-fH (10 μg/10 μl of serum) for 30 min at 4°C in VSB containing 1 mM Ca^{2+}. C3 deposition was measured after incubating 1/10 dilutions of sera on plates with adherent CII-IC. The data are expressed as OD values, mean ± SEM based on $n = 3$ for each serum. *, $p < 0.0001$ for C3 deposition using sera with only an active AP in the presence vs absence of CR2-fH. C3 deposition without or with CR2-fH using sera from WT mice were also significantly different ($p < 0.05$) and from mice with only an intact CP were not significantly different. As previously shown, sera with only an intact LP failed to induce any detectable C3 deposition.

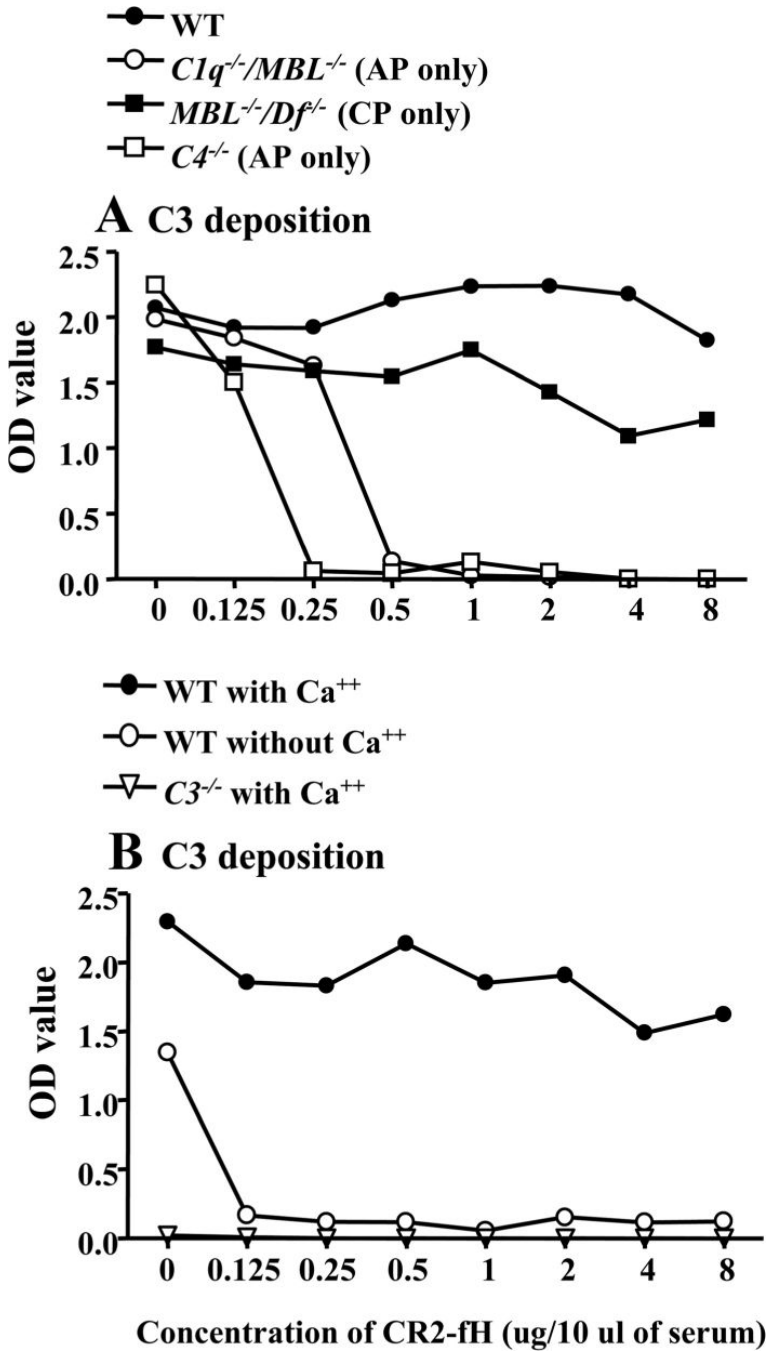

FIGURE 4.

Effects of CR2-fH on C3 deposition induced by CII-IC in vitro. Sera from three different mice were pooled and diluted (1/10) in VSB with 1 mM Ca^{2+} before pre-incubation without CR2-fH or with seven different amounts of CR2-fH. The data are expressed as C3 deposition (OD units) vs the amount of CR2-fH (μg). *A*, C3 deposition using sera from WT mice, mice with only an intact AP ($C1q^{-/-}/MBL^{-/-}$ or $C4^{-/-}$), and mice with only an intact CP ($MBL^{-/-}/Df^{-/-}$). *B*, C3 deposition with sera from WT mice, with or without Ca^{2+} (MG/EGTA), or from $C3^{-/-}$ mice in the presence of Ca^{2+}. Activation of the CP is dependent on the presence of Ca^{2+}. Sera from three different WT mice were pooled and diluted 1/10 in VSB with or without Ca^{2+} before

preincubation with different concentration of CR2-fH for 20 min at 4°C. Sera from $C3^{-/-}$ mice were used as a negative control to show the specificity of the ELISA.

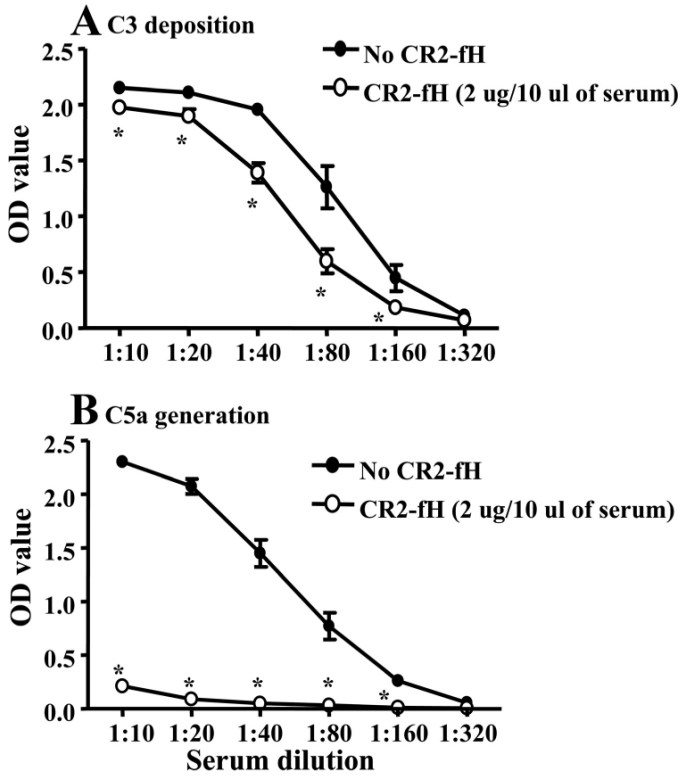

FIGURE 5.

Effects of CR2-fH on C3 deposition (*A*) and C5a generation (*B*) in vitro by WT sera incubated with adherent mAb to CII. Serum of CR2-fH (2 μg/10 μl) was preincubated with serial 2-fold dilutions of WT sera, starting at a 1/10 dilution, before addition to a plate coated with mAb to CII. Adherent CII-IC were not used because CII alone induced some C5 activation. The levels of C3 deposition on the plate and of C5a in the supernatants were determined by ELISA. The baseline levels of C5a in the sera before incubation on the mAb to CII were subtracted from the measured total C5a at the end of the experiment. *, $p < 0.005$ or *, $p < 0.001$ for C3 deposition and C5a generation respectively using sera from WT mice in the presence vs absence of CR2-fH. The data are expressed as C3 deposition or C5a levels, both expressed in OD units (mean ± SEM based on $n = 5$), vs the serum dilution.

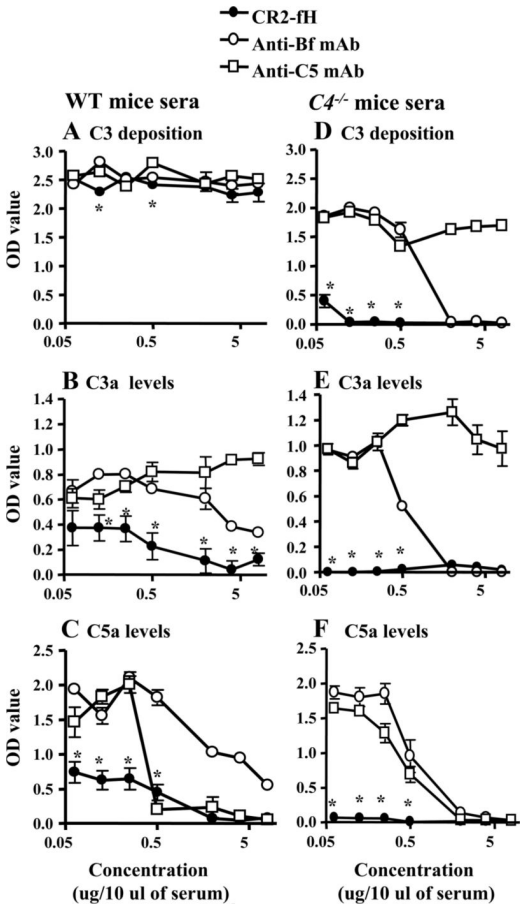

FIGURE 6.

The effect of CR2-fH, anti-factor B mAb, and anti-C5 mAb on C3 deposition, and C3a and C5a generation induced by anti-CII Abs in vitro. Sera from WT mice (*A*–*C*) and sera from $C4^{-/-}$ (*D*–*F*) mice were diluted 1/10 in VSB buffer. Diluted sera were preincubated for 30 min. at 4°C with different concentrations (0, 0.0625, 0.125, 0.250, 0.5, 2, 4, 8 μg/10 μl of serum) of CR2-fH, anti-factor B mAb and anti-C5 mAb (BB5.1) before addition to a plate coated with mAb to CII. Adherent CII-ICs were not used because CII alone induced some C5 activation. The levels of C3 deposition on the plate and of C3a and C5a in the supernatants were determined by ELISA. The baseline levels of C3a and C5a in the sera before incubation on the mAb to CII were subtracted from the measured total C3a and C5a at the end of the experiment. No differences were seen in the absence of treatment on C3 deposition, C3a levels, and C5a levels using sera from WT and $C4^{-/-}$ mice (data not shown). The data are expressed as C3 deposition, C3a levels, or C5a levels, expressed in OD units (mean ± SEM based on $n = 3$). *, $p < 0.05$ showing that treatment with different concentrations of CR2-fH are significantly different vs treatment with anti-factor B mAb or with anti-C5 mAb.

Table I

Histopathology scores in the joints of mice with and without treatment with CR2-Crry or CR2-fH[a]

Treatment	Inflammation	Pannus	Cartilage Damage	Bone Damage	Total
PBS (n = 11)	2.65 ± 0.18	2.62 ± 0.14	2.64 ± 0.14	2.56 ± 0.14	10.5 ± 0.55
CR2-fH, 250 μg[b] (n = 8)	2.17 ± 0.19	2.0 ± 0.16	2.25 ± 0.14	1.97 ± 0.18	8.4 ± 0.65
p	<0.06	<0.004	<0.05	<0.009	<0.01
CR2-fH, 500 μg[b] (n = 7)	0.85 ± 0.14	1.2 ± 0.12	0.97 ± 0.16	0.97 ± 0.11	4.0 ± 0.48
p	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
CR2-Crry, 250 μg[b] (n = 3)	1.47 ± 0.57	1.60 ± 0.40	1.33 ± 0.33	1.47 ± 0.37	5.87 ± 1.67
p	<0.13	<0.06	<0.03	<0.04	<0.05

[a]Histopathology scores in joints are expressed on a scale of 0–5; the data are expressed as mean ± SEM based on 5 joints per mouse. Details of the scoring methods can be found in *Materials and Methods* and in the Extended Methods online.

[b]Each injection of CR2-fH and CR2-Crry was given twice.

Table II

C3 deposition in the joints at day 10 with and without treatment with CR2-Crry or CR2-fH[a]

Treatment	Synovium	Cartilage	Total
PBS (*n* = 11)	2.47 ± 0.11	2.21 ± 0.13	4.69 ± 0.23
CR2-fH, 250 μg[b] (*n* = 8)	1.72 ± 0.35	1.62 ± 0.33	3.35 ± 0.68
p	<0.0005	<0.01	<0.002
CR2-fH, 500 μg[b] (*n* = 7)	1.34 ± 0.16	1.42 ± 0.14	2.48 ± 0.29
p	<0.0001	<0.0001	<0.0001
CR2-Crry, 250 μg[b] (*n* = 3)	1.80 ± 0.25	1.07 ± 0.18	2.87 ± 0.52
p	<0.07	<0.005	<0.01

[a] Five joints were examined in each animal and the results are expressed as mean ± SEM. Details of the scoring methods can be found in *Materials and Methods.*

[b] Each injection of CR2-fH and CR2-Crry was given twice.

Table III

Deposition of C3 and factor H in the knee joints of mice at early times after injection of mAb to CII[a]

	mAb to CII			
	1 h[b]	8 h[b]	24 h[b]	PBS 24 h[b]
C3 deposition				
Cartilage	0.58 ± 0.15[c]	0.83 ± 0.21[c]	0.75 ± 0.11[c]	0.08 ± 0.12
Synovium	1.25 ± 0.17[c]	1.50 ± 0.18[c]	1.58 ± 0.15[c]	0.58 ± 0.35
Total joint	1.83 ± 0.31[c]	2.33 ± 0.36[c]	2.33 ± 0.25[c]	0.67 ± 0.35
Factor H deposition				
Cartilage	1.42 ± 0.37	1.92 ± 0.20[c]	1.58 ± 0.20[c]	0.75 ± 0.35
Synovium	0.67 ± 0.33	0.75 ± 0.17	1.25 ± 0.17[c]	0.42 ± 0.17
Total joint	2.08 ± 0.69	2.67 ± 0.36[c]	2.83 ± 0.28[c]	1.17 ± 0.47

[a] The data are expressed as mean ± SEM based on $n = 6$. Details of the scoring methods can be found in *Materials and Methods.*

[b] The times represent the hours after i.p. injection of the mAb to CII or of PBS as a control.

[c] $p < 0.05$ compared with PBS-treated mice.

Table IV

Cytokine mRNA levels in the joints at day 10 with and without treatment with CR2-fH[a]

	Treatment_Groups[b]		
Cytokines	**PBS (*n* = 11)**	**CR2-fH 250 μg[b] (*n* = 8)**	**CR2-fH 500 μg[c] (*n* = 7)**
TNF-α	19.9 ± 4.3	21.1 ± 7.4	7.63 ± 0.9[c]
IFN-γ	767.9 ± 79.9	733.5 ± 156.6	548.7 ± 214.6
IL-1α	22.9 ± 4.6	28.74 ± 8.2	14.4 ± 4.1
IL-1β	63.4 ± 15.7	52.79 ± 8.0	60.5 ± 20.2
IL-4	25.7 ± 5.3	11.6 ± 5.5	18.84 ± 5.0
IL-10	425.9 ± 76.4	364.3 ± 55.5	934.5 ± 195.3[c]
IL-1Ra	739.9 ± 56.3	839.8 ± 116.4	836.8 ± 60.2

[a] The data are expressed as level of mRNA pg/ng rRNA with mean ± SEM based on the number of mice in each treatment group.

[b] Each injection of CR2-fH was given twice.

[c] Values of p were significant ($p < 0.05$) in comparison with mice treated with only PBS.