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A Novel Targeted Inhibitor of the Alternative Pathway of Complement and Its Therapeutic Application in Ischemia/Reperfusion Injury[1]

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Bioavailability and therapeutic efficacy of soluble Crry, a mouse inhibitor of all complement activation pathways, is significantly enhanced when linked to a fragment of complement receptor 2 (CR2), a receptor that targets C3 activation products. In this study, we characterize alternative pathway-specific inhibitors consisting of a single or dimeric N-terminal region of mouse factor H (fH; short consensus repeats 1–5) linked to the same CR2 fragment (CR2-fH and CR2-fHfH). Both CR2-fH and CR2-fHfH were highly effective at inhibiting the alternative pathway in vitro and demonstrated a higher specific activity than CR2-Crry. CR2-fH was also more effective than endogenous serum fH in blocking target deposition of C3. Target binding and complement inhibitory activity of CR2-fH/CR2-fHfH was dependent on CR2- and C3-mediated interactions. The alternative pathway of complement plays a role in intestine ischemia/reperfusion injury. However, serum fH fails to provide protection against intestine ischemia/reperfusion injury although it can bind to and provide cell surfaces with protection from complement and is present in plasma at a high concentration. In a mouse model, CR2-fH and CR2-fHfH provided complete protection from local (intestine) and remote (lung) injury. CR2-fH targeted to the site of local injury and greatly reduced levels of tissue C3 deposition. Thus, the targeting mechanism significantly enhances alternative pathway-specific complement inhibitory activity of the N-terminal domain of fH and has the potential to reduce side effects that may be associated with systemic complement blockade. The data further indicate alternative pathway dependence for local and remote injury following intestinal ischemia/reperfusion in a clinically relevant therapeutic paradigm. ***The Journal of Immunology,*** **2008, 181: 8068–8076.**

Complement provides an important host defense system and also plays an important role in immunoregulatory mechanisms such as the modulation of humoral and cellular immunity, the regulation tolerance to self-Ags, the catabolism of immune complexes, and the clearance of apoptotic cells. However, under certain conditions, inappropriate or excessive complement activation occurs and complement plays a key role in causing tissue injury resulting from inflammatory and ischemic conditions. There are three pathways of complement activation; the classical, lectin, and alternative pathways. The alternative pathway can be spontaneously activated on certain surfaces (in a process known as "tickover"), but also provides an amplification loop when complement activation is initiated by the classical or lectin pathways (for review of complement system, see Ref. 1). Although the great majority of in vitro immune complex initiated complement activation assays show little alternative pathway influence, in one recently described in vitro system it has been shown that 80% of complement activation products generated following classical pathway activation can be the result of alternative pathway amplification (2). It has been shown that the classical and/or lectin pathways can variously play an essential role in autoimmune, inflammatory, and ischemic disease models, but there is also strong evidence that in many, if not all, disease models in mice, presumably through the amplification loop, the alternative pathway is required for full in vivo expression of injury (for review, see Refs. 3 and 4).

Due to the ability of the alternative pathway to spontaneously activate and to amplify the other complement pathways, regulatory mechanisms are necessary to prevent host cell injury and to maintain the normal physiological functions of complement. A key regulator of the alternative pathway is factor H (fH),[4] a specific soluble inhibitor present in human and rodent serum at concentrations between 300 and 600 μg/ml. fH is a monomeric glycoprotein of ~155 kDa and is composed of 20 repetitive short consensus repeats (SCRs) of ~60 aa in length and can regulate the alternative pathway both in the fluid phase and on cell surfaces following its attachment via a C-terminal binding domain (5–7). fH associates with C3b and functions by preventing the formation of, or causing the dissociation or inactivation of, the alternative pathway C3 convertase, the central enzymatic complex of the alternative pathway. There are three C3b binding sites within the fH molecule located within SCR1–4, SCR8–15 and SCR19–20, and SCR19–20 also binds C3d (8). Only SCR1–4 has cofactor activity for factor I, a serine protease that cleaves and inactivates C3b. fH also contains

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[4] Abbreviations used in this paper: fH, factor H; SCR, short consensus repeat; CR2, complement receptor 2; IRI, ischemia/reperfusion injury; CHO, Chinese hamster ovary; fB, factor B; I/R, ischemia/reperfusion; fD, factor D; MBL, mannan-binding lectin.

heparin and polyanion binding sites located in SCR7, SCR9–17, and SCR19–20 (for reviews on the structure and function of fH, refer to Refs. 9 and 10). The recombinant truncated fH-based inhibitors characterized in the current study contain only SCR1–5.

The important role of the alternative pathway in inflammatory injury, even in conditions initiated by the other complement pathways, makes it an attractive target for therapeutic intervention. Since complement also has an important role in host defense and immune homeostasis, the selective blockade of the alternative pathway may minimize side effects associated with inhibitors that block all pathways at the C3 or C5 step. Furthermore, based on previous studies on the targeting of a pan C3 convertase inhibitor (Crry) (11), the targeting of alternative pathway inhibition to sites of complement activation offers the possibility of significantly increasing inhibitor efficacy and further reducing systemic side effects that may be associated with systemic inhibition.

In this study, we have developed novel targeted inhibitors of the alternative pathway consisting of a fragment of complement receptor 2 (CR2) linked to N-terminal domains of fH (CR2-fH and CR2-fHfH). The CR2 targeting strategy has been described previously for the targeting of Crry, a mouse inhibitor of all complement pathways (11). The CR2 moiety binds to iC3b, C3dg, and C3d cell and target-bound breakdown fragments of C3 generated at sites of complement activation. In this study, the targeted alternative pathway inhibitors are characterized in vitro, along with an untargeted fH (SCR1–5) fragment, and in the presence of endogenous fH found in the mouse serum and blood used for in vitro as well as in vivo models, respectively. For in vivo characterization, we used a mouse model of intestinal ischemia/reperfusion injury (IRI). Using this model, previous studies with complement-deficient mice have shown both lectin pathway dependence for complement activation (12) and an essential role for the alternative pathway in the development of tissue injury (13). Herein, we investigate the specific contribution of the alternative pathway to intestine injury in the clinically relevant setting of complement inhibition. In addition, we previously characterized the pan inhibitor CR2-Crry in this model of intestine IRI, thus affording the opportunity for comparative studies.

Materials and Methods

Reagents, Abs, and sera

Reagents for DNA manipulation and plasmid construction were obtained from Invitrogen, Stratagene, and New England Biolabs. FuGENE-HD Transfection Reagent for preparation of stably transfected cell lines was from Roche Applied Science. Rat anti-mouse CR2 mAb 7G6 (14) and anti-mouse Crry mAb 5D5 (15) were purified from hybridoma supernatant by protein G affinity chromatography (Amersham Biosciences). Sheep anti-human fH polyclonal Ab was obtained from Abcam and FITC-conjugated anti-mouse C3 from ICN Biomedicals. Rabbit anti-mouse C3d polyclonal Ab was from MP Biomedicals and goat anti-human C3 polyclonal (cross-reacts with mouse C3) from DakoCytomation. All secondary Abs were obtained from Sigma-Aldrich. Rat serum was purchased from AbD Serotec and mouse serum was prepared from blood collected from healthy BALB/c mice in the laboratory. cDNA encoding mouse fH and purified mouse fH were provided by Drs. R. Quigg (University of Chicago, Chicago, IL) and J. Thurman (University of Colorado, Denver, CO), respectively.

Plasmid construction, protein expression, and protein purification

For construction of expression plasmids encoding CR2-fH, a sequence containing the 4 N-terminal SCRs of mouse CR2 (residues 1–257 of mature protein; GenBank accession number M35684) was linked to a sequence encoding the five N-terminal SCRs of mouse fH (residues 1–303 of mature protein; GenBank accession number NM009888). Constructs were prepared either with a linker sequence between CR2 and fH encoding either $(G_4S)_2$ or KEIL, the latter representing a portion of the natural linker between SCRs 4 and 5 of mouse CR2. For the CR2-fHfH construct, a repeat fH(SCR1–5) encoding sequence was linked to the 3′ end of the CR2-fH construct with a linking sequence encoding TED. Plasmid constructs encoding each component of the fusion protein were also prepared, i.e., mouse CR2(SCR1–4) and mouse fH(SCR1–5). All constructs were prepared by standard PCR methods and were cloned into the previously described PBM vector with a CD5 signal peptide sequence for expression (16). Construction of the expression plasmid for CR2-Crry has been described previously (11). Expression plasmids were transfected into Chinese hamster ovary (CHO) cells with FuGene-HD transfection reagent according to the manufacturer's instructions (Roche Applied Science). Positive clones were selected by limiting dilution as previously described (16) and protein expression was quantified by ELISA and dot blot.

For protein purification, CHO cells expressing each recombinant protein were cultured in flasks (375 cm^2) and supernatant was harvested at confluence. Culture supernatant was centrifuged at 2000 × *g* for 20 min and filtered through a 0.22-μm filter. CR2(SCR1–4), CR2-Crry, CR2-fH, and CR2-fHfH were purified from filtered supernatants by anti-mouse CR2 (mAb 7G6) affinity chromatography as described previously for CR2-Crry (16). For purification of fH (SCR1–5), filtered supernatant was concentrated and exchanged with PBS using Millipore Centricon Plus-20 concentrators. Cold saturated ammonium sulfate was then added to make a final ammonium sulfate saturation of 40% and the mixture was left at 4°C overnight. The mixture was then centrifuged (2000 × *g*/2 h), the pellet resuspended in PBS (0.5 g/1.5 ml of PBS), and applied to a Superdex-75 FPLC Gel filtration column. Positive fractions were identified by dot blot using cross-reactive anti-human fH polyclonal Ab (Abcam). Positive fractions were pooled and protein purity was analyzed by both SDS-PAGE and Western blot. Each purified protein gave a single band of appropriate molecular mass.

In vitro-binding and complement inhibition assays

For binding assays, zymosan particles were activated by boiling with 0.15 mol/L NaCl, washed twice with PBS, and incubated with 10% BALB/c mouse serum in DMEM at 37°C for 20 min. The C3-opsonized particles were then washed twice and resuspended in PBS containing 0.1% BSA (PBSB). For inhibitor-binding assays, aliquots of activated zymosan particles (2×10^7 per assay reaction) were incubated with 500 nM recombinant complement inhibitor (CR2-fH, CR2-fHH, or CR2-Crry) in the absence or presence of varying concentrations (0–2 μM) of anti-mouse CR2 mAb 7G6 at 4°C for 1 h. Particles were then washed with PBSB and incubated with 10 μg/ml sheep anti-mouse fH polyclonal Ab or rat anti-mouse Crry mAb 5D5 in PBSB (4°C/1 h). Particles were then washed again, incubated with FITC-conjugated secondary anti-sheep or anti-rat Ab in PBSB (4°C/1 h), and finally washed and analyzed by flow cytometry. In a separate binding experiment, we determined whether polyclonal anti-C3 and anti-C3d Abs (0–2 μM)) blocked the binding of CR2-fH to C3-opsonized zymosan beads with the same conditions and protocol described above.

Analysis of alternative pathway inhibition was conducted by measuring C3 deposition on zymosan particles as previously described (17). In brief, each reaction tube contained 2×10^7 activated zymosan particles in PBSB and a final concentration of 10 mM EGTA and 5 mM $MgCl_2$. To each reaction tube was then added CR2-fH, CR2-fHfH, CR2-Crry, fH (SCR1–5), CR2, or purified mouse fH to final concentrations of between 0 and 100 nM. Next, 10 μl of BALB/c mouse serum was added to each tube and the sample volume of each tube was brought to 100 μl with PBSB. Samples were then incubated at 37°C for 20 min, after which EDTA (10 mM final concentration) was added to stop the reactions. Particles were then washed twice with PBSB and incubated with FITC-conjugated anti-mouse C3 in PBSB at 4°C for 1 h and washed again before analysis by flow cytometry. Percent inhibition of C3 deposition was calculated using the formula [1 − [sample mean channel fluorescence − background (10 mM EDTA condition)/positive control mean channel fluorescence (no recombinant protein) − background]] × 100. To determine the effect of 7G6 mAb on the complement inhibitory activity of recombinant proteins, zymosan assays were performed as described above using a fixed concentration of each recombinant inhibitor (25 nM final concentration) and varying concentrations of 7G6 (0–200 nM final concentration).

Analysis of classical pathway inhibition was conducted by measuring C3 deposition on Ab-sensitized CHO cells as described elsewhere (11). Briefly, CHO cells were opsonized with 2% CHO cell membrane antiserum and aliquots were incubated with CR2-fH or CR2-Crry at a final concentration of 0, 50, 100, and 200 nm in 5% normal or $fB^{-/-}$ mouse serum (final volume, 100 μl). Heat-inactivated mouse serum was used as negative control. Following incubation at 37°C for 30 min, 10 mM EDTA was added to stop further complement activation and C3 deposition on CHO cells was analyzed by flow cytometry (anti-mouse C3 Ab). Percentage of inhibition of C3 deposition was calculated using the formula

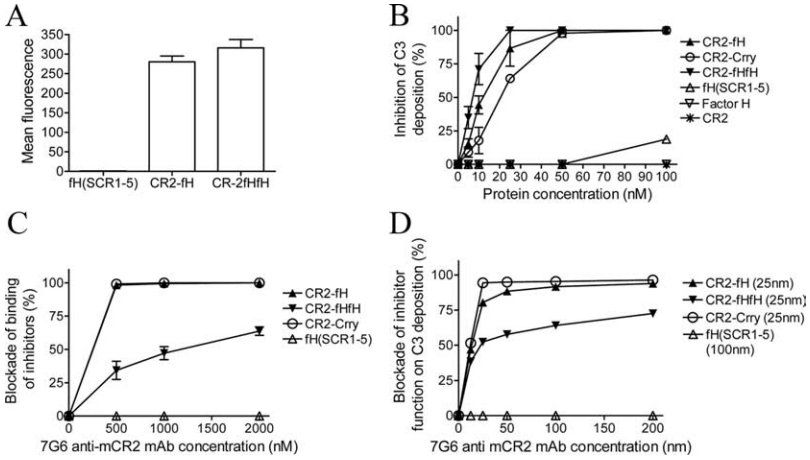

FIGURE 1. Binding and functional activity of recombinant proteins. *A,* Flow cytometric analysis of recombinant protein binding to C3-opsonized zymosan particles (500 nM inhibitor; mean ± SD, $n = 3$). *B,* Inhibition of C3 deposition on zymosan particles assayed by anti-C3 flow cytometry (mean ± SD, $n = 3$). *C,* Inhibition of recombinant protein binding (500 nM) to C3-opsonized zymosan particles in the presence of anti-CR2 mAb 7G6 (mean ± SD, $n = 3$). *D,* Inhibition of complement inhibitor activity in the presence of anti-CR2 mAb 7G6 as measured by C3 deposition on zymosan particles (mean ± SD, $n = 3$). Where not shown, error bars are contained within symbol.

[1 − [sample mean channel fluorescence − background (heat-inactivated serum)/positive control mean channel fluorescence (no recombinant protein) − background]] × 100.

Intestinal IRI

The same intestinal IRI model and procedures were used as described in our previous characterization of CR2-Crry (11). Briefly, BALB/c mice (The Jackson Laboratory) were randomly divided into five treatment groups (PBS, CR2-fH, CR2-fHfH, CR2-Crry, and sham operated with no treatment) and subjected to 30 min of ischemia followed by 90 min of reperfusion. Inhibitors were administered i.v. by tail vein injection 30 min after reperfusion. All animal procedures were approved by the Medical University of South Carolina Animal Care and Use Committee.

Histology and injury assessment

Tissue samples for histological staining were taken from the intestine and either fixed in 10% formalin at 4°C overnight and processed to paraffin or were frozen in liquid nitrogen for immunofluorescence analysis. Lungs were inflated by instillation of formalin at 15 mm Hg and allowed to fix. Sections of intestine from each animal were stained with H&E and scored for mucosal damage and villi height as previously described (11). Injury was assessed according to the following scale: 0, normal villi; 1, villi with tip distortion; 2, villi lacking goblet cells and containing Gugenheim spaces (edematous spaces within the epithelial cells); 3, villi with patchy disruption of the epithelial cells; 4, villi with exposed but intact lamina propria and epithelial cell sloughing; 5, villi in which lamina propria were exuding; and 6, denuded villi or villi displaying hemorrhage. All histological evaluations were conducted in a blinded fashion. For morphometric analysis of damage, intestine and lung H&E-stained sections were analyzed after ischemia reperfusion injury. For intestine damage, the villi in samples from each group were measured using computerized image analysis software (AxioVision 4.6; Zeiss Microsystems). The extent of remote lung injury was assessed by measuring the expansion of the parenchymal wall caused by the infiltration of inflammatory cells as previously described (11). Parenchymal wall expansion was measured using the AxioVision image analysis system (Zeiss Microsystems). In brief, slides were screened in a blinded fashion and six images per lung recorded in fields not containing bronchi or major pulmonary vasculature. Twenty random measurements of parenchymal wall thickness were recorded in each field. The results are expressed as a mean wall thickness per section ± SD.

Immunofluorescence microscopy

Frozen sections were prepared from samples of intestine removed 2 h after reperfusion, fixed in cold acetone for 5 min, washed in running water, and then equilibrated in PBS (11). Sections were incubated for 1 h at room temperature with cross-reactive anti-human C3d-FITC (DakoCytomation) and then washed with PBS. Sections were coverslipped with VectaMount (Vector Laboratories) and visualized by confocal microscopy. Specificity of immunostaining was confirmed by both the use of isotype control Abs and by the omission of primary Abs. The presence of CR2-fH in vivo binding was assessed in frozen sections of intestines harvested at 2 h after reperfusion by the same procedure outlined above, using rat anti-mouse CR2 mAb 7G6 and a secondary anti-rat IgG-Alexia Fluor 488 Ab (Molecular Probes). To assess the affects of complement inhibition with CR2-fH on the deposition of C3d in vivo, a time course analysis was performed after IRI. Animals were either treated with PBS or CR2-fH and intestine was harvested at 30 min, 1 h, and 2 h after reperfusion. CR2-fH and PBS were administered as described above 30 min after reperfusion. C3d deposition and CR2 localization were assessed as outlined above.

In vivo kinetics of CR2-fH

Blood was collected from BALB/c mice before i.v. injection of 0.2 mg of CR2-fH and at various time points thereafter (0.5, 1, 2, 4, 12, 24, and 48 h). Serum samples were prepared as previously described (17) and serum concentrations of CR2-fH were assessed by ELISA as previously described (11) using anti-CR2 7G6 as capture Ab and anti-human fH polyclonal Ab for detection. In a separate analysis, serum levels of CR2-fH were assessed by Western blot followed by densitometric analysis of scanned blot (Bio-Rad Gel Doc XR). Serum proteins were separated by nonreducing 4–15% SDS-PAGE and following transfer CR2-fH was detected with a combination of sheep anti-human fH polyclonal Ab and peroxidase-conjugated goat anti-sheep IgG secondary Ab. The membrane was developed with an ECL detection kit (Amersham Biosciences).

Statistical analysis

Data are shown as means ± SD. StatView Statistical Analysis Software (version 5; SAS Institute) was used for analyses of all data. A $p < 0.05$ was considered significant.

Results

In vitro characterization of recombinant proteins

The recombinant soluble proteins CR2-fH, CR2-fHfH, CR2(SCR1-4), and fH(SCR1–5) were isolated from supernatants of stably expressing CHO cell clones, and all purified proteins gave a single band of appropriate molecular mass when analyzed by SDS-PAGE (data not shown). Preparation and characterization of CR2-Crry has been described previously (11). The C3 targeting activity of the CR2 domain of the fH-containing proteins was assessed by flow cytometry; both CR2-fH and CR2-fHfH, but not fH(SCR1–5), bound to zymosan particles first opsonized with mouse C3 (Fig. 1*A*).

The alternative pathway complement inhibitory activity of the recombinant proteins was then assayed by coincubations during alternative pathway-specific zymosan particle activation of mouse serum. The effect of the recombinant inhibitors was quantified by flow cytometric analysis of the resulting C3 deposition on the zymosan particles (Fig. 1*B*). The CR2-linked complement inhibitors all provided dose-dependent inhibition of C3 deposition, with CR2-fHfH being the most effective inhibitor and CR2-Crry the least. Protein concentrations providing 50% inhibition of C3 deposition were 7.5 nM (CR2-fHfH), 12 nM (CR2-fH), and 20 nM (CR2-Crry). There was no difference between the activities of CR2-fH containing either a $(G_4S)_2$ linker or a short "natural" linker (see *Materials and*

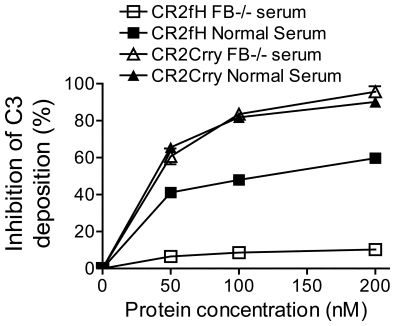

FIGURE 2. Classical pathway activity of CR2-Crry and CR2-fH. Ab-sensitized CHO cells were incubated with the indicated concentration of inhibitor in either normal or fB-deficient serum. Inhibition of C3 deposition on CHO cells was assayed by flow cytometry (mean ± SD, $n = 3$). Mean fluorescence intensity at percent inhibition was 454 ± 10 (normal serum) and 609 ± 32 (fB $^{-/-}$ serum). Where not shown, error bars are contained within symbol.

Methods) and data shown is for the latter construct. fH-(SCR1–5) provided 50% inhibition of C3 deposition at 250 nM (20-fold higher concentration than CR2-fH), whereas purified fH provided <10% inhibition at 2 μM. Of further note, ~350 nM of endogenous mouse serum fH was present in the assay systems at the concentration of serum used. CR2 (1–4) had no complement inhibitory activity at concentrations up to 2 μM.

The importance of the CR2 targeting domain for CR2-fH/fHfH complement inhibitory efficacy was further investigated in assays using the function blocking anti-mouse CR2 mAb 7G6 (14), which interacts with the iC3b/C3d-binding region of CR2. The mAb 7G6 inhibited both the binding and complement inhibitory activity of CR2-fH and CR2-fHfH in a dose-dependent manner (Fig. 1, *C* and *D*), demonstrating the critical importance of CR2-mediated targeting for the complement inhibitory activity of these proteins. Note that in the binding assays, higher relative concentrations of both inhibitor and mAb 7G6 were used to obtain readily readable fluorescence signals by anti-fH flow cytometry. The activity of CR2-Crry was also blocked by mAb 7G6. The complement inhibitory activity of fH(SCR1–5), which provided ~20% inhibition of C3 deposition on zymosan particles at the concentration used in the blocking assays, was unaffected by mAb 7G6. Of interest, mAb 7G6 was slightly less effective at blocking CR2-fHfH binding than CR2-fH binding, but was significantly less effective at blocking the C3 inhibitory activity (measured as C3 deposition) of CR2-fHfH compared with CR2-fH. This may be due to an increased avidity of the fH dimer for deposited C3b and loose binding that is not retained in the flow cytometric binding assay. In a separate experiment, we investigated whether a polyclonal Ab to either C3 or C3d interfered with the binding of CR2-fH to C3-opsonized zymosan particles. Under the same conditions used in the 7G6-blocking experiments, CR2-fH (200 nM) was incubated with varying concentrations of anti-C3 Ab. Binding of CR2-fH to opsonized zymosan particles was completely blocked by both anti-C3 and anti-C3d at a concentration of 200 nM and 1 μM, respectively (data not shown). Together, the results from these binding experiments demonstrate the binding of constructs via a CR2-C3 interaction.

In contrast to the inhibitory effect of CR2-fH in the zymosan-activated alternative pathway assay, CR2-fH did not cause any significant reduction in C3 deposition in a classical pathway assay utilizing Ab-sensitized CHO cells and factor B (fB)-deficient serum (no alternative pathway activity) (Fig. 2). In contrast, CR2-Crry effectively inhibited C3 deposition in this classical pathway assay. The intermediate effect of CR2-fH on C3 deposition in the presence of normal mouse serum is likely a reflection of the role of the alternative pathway in the complement amplification loop.

Effect of alternative pathway inhibition on intestinal IRI

Native fH contains C3b, C3d, and polyanion binding sites by which it can bind to cell surfaces and provide protection from complement activation in vitro. Nevertheless, fH (as well as the membrane-bound complement inhibitors) fail to provide protection from complement-mediated injury in vivo during several pathological conditions. We hypothesized that CR2-linked fH proteins might augment the normally ineffective function of endogenous fH in vivo. To test this hypothesis, we investigated the ability of CR2-fH/fHfH to provide protection from complement in a relevant in vivo model involving cell injury. We previously demonstrated that CR2-Crry, a similarly targeted inhibitor, but of all three complement pathways, provides protection against intestine IRI in a mouse model (11). In this study, we investigated the effect of specifically inhibiting the alternative pathway on intestine IRI using CR2-fH and CR2-fHfH and further compared the relative efficacies of the alternative pathway inhibitors with CR2-Crry. The intestine IRI model consisted of 40 min of ischemia followed by 2-h reperfusion, with i.v. administration of complement inhibitors 30 min after reperfusion. In each treatment group, intestine injury was assessed by histologic grading on a scale of 0–6 (18) and by measurement of villi height. Sham-operated animals had no obvious evidence of villi damage with an injury score of <1. Animals subjected to ischemia and reperfusion and treated with PBS exhibited extensive damage of the normal intestinal villus structure, with marked epithelial denudation resulting in a reduction in villi height, hemorrhage, and capillary dilatation. In contrast, animals treated with either CR2-fH, CR2-fHfH, or CR2-Crry demonstrated a dose-dependent protection profile with intestinal villus structure comparable to sham-operated controls at higher doses of inhibitor (Fig. 3*A*). Although CR2-fHfH was a more potent inhibitor than CR2-fH in vitro, there was no difference in the efficacy of the two inhibitors in the intestine IRI model. Significantly, the alternative pathway inhibitors were able to provide almost complete protection from injury, although an approximate 2-fold lower dose of CR2-Crry was required to provide an equivalent level of protection (in terms of molarity, CR2-Crry and CR2-fH have similar molecular weights). Representative images of H&E-stained intestine sections are shown in Fig. 4, and the images highlight the similar levels of protection provided by CR2-fH, CR2-fHfH, and CR2-Crry at maximally protective doses.

To further assess the degree of intestinal damage in control and treated groups, intestinal villi height was assessed by computerized image analysis (Fig. 3*B*). Intestinal reperfusion injury is characterized by the loss of villi epithelial cells, which is associated with a concomitant decrease in villi height. In keeping with the histological score, animals subjected to intestinal IRI demonstrated a significant and marked decrease in villi height when compared with sham-operated controls. Treatment with the targeted complement inhibitors at therapeutic doses resulted in protection from epithelial denudation and villi heights were no different from those seen in sham-operated controls.

Effect of alternative pathway inhibition on remote organ injury

Complement is known to play a role in remote organ injury following ischemia and reperfusion of the intestine, although there is evidence that different complement-dependent mechanisms may be involved in local and remote injury (19–21). We therefore investigated the effect of the targeted alternative pathway inhibitors

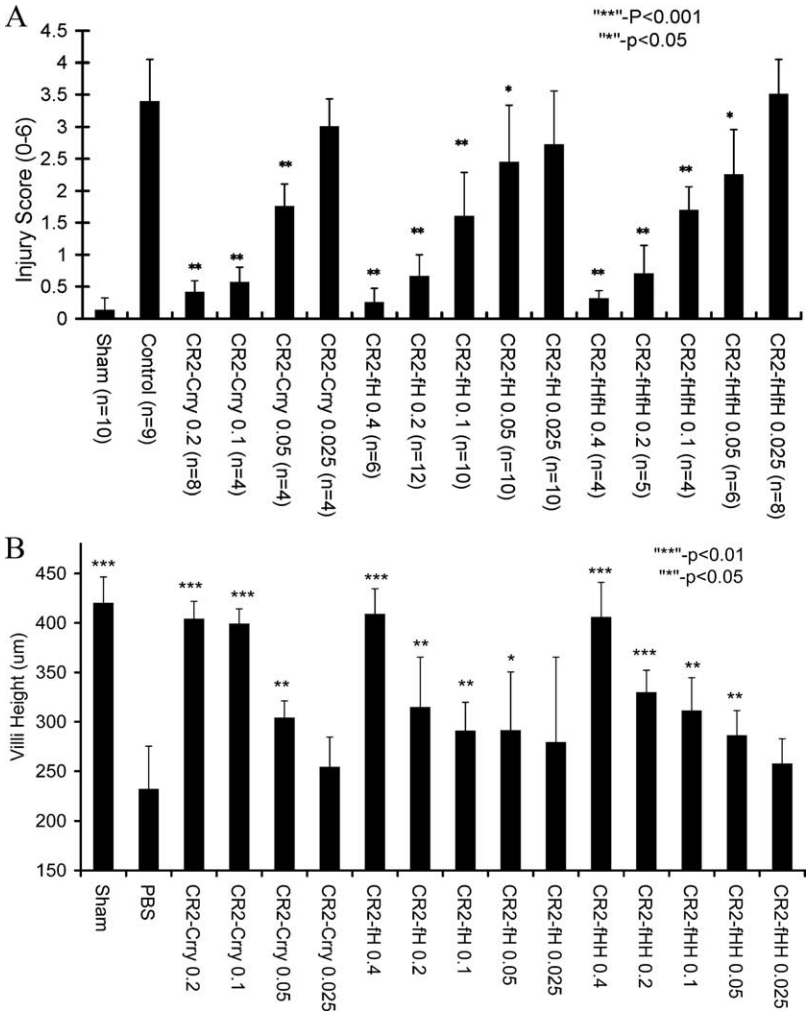

FIGURE 3. Quantitative evaluation of intestinal IRI with different doses (mg) of CR2-Crry, CR2-fH, and CR2-fHH. H&E-stained sections of intestine were assessed for mucosal injury (*A*) and villi height (*B*). All measurements were obtained at ×100 magnification. Mean ± SD; $n = 4–12$ mice/group for injury score (as indicated) and $n = 5$ mice/group for villi height.

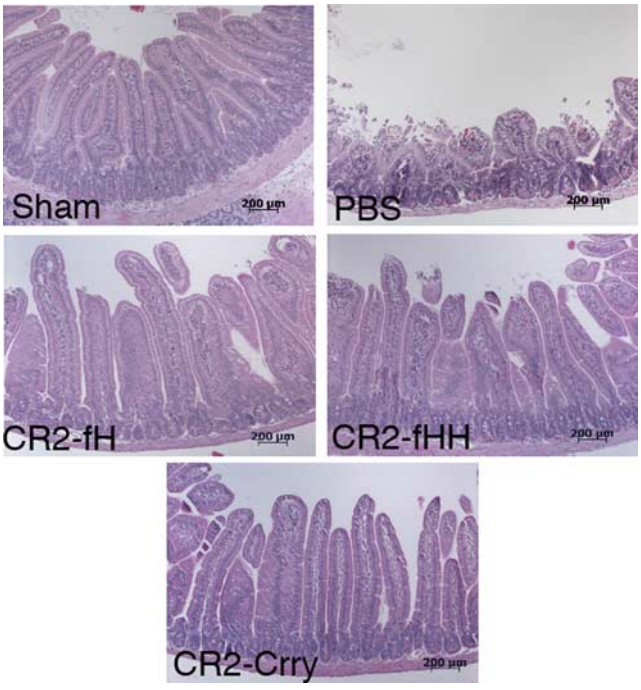

FIGURE 4. Representative H&E-stained intestine sections from mice treated with a therapeutic dose of CR2-Crry (0.2 mg), CR2-fH (0.4 mg), and CR2-fHH (0.4 mg). Sham-operated and PBS controls are included for comparison. Photomicrograph magnification is ×200. Each panel is representative of 6–10 mice/group.

on lung injury following intestine ischemia/reperfusion (I/R). Remote lung injury is characterized by the infiltration of inflammatory cells and expansion of the alveolar walls, and injury was assessed in lung sections by morphometric analysis of alveolar wall thickness. In keeping with previous reports (11, 22), PBS animals subjected to ischemia reperfusion showed evidence of lung damage, marked by the presence of infiltrating inflammatory cells, pneumocyte swelling, fibrin deposition, and expansion of the alveolar walls (Fig. 5*A*). Treatment with CR2-fH and CR2-fHfH was protective against lung injury as demonstrated by the absence of alveolar wall expansion and alveolar wall thickening and was comparable to that seen in sham-operated animals (Fig. 5*B*). The dose of CR2-fH and CR2-fHfH that provided almost complete protection from local intestine injury (0.4 mg) was completely protective against lung injury, with no significant difference between sham controls and inhibitor-treated mice ($p < 0.01$). In agreement with previous data, CR2-Crry was also completely protective at doses paralleling maximum protection from local injury (0.1–0.2 mg). Thus, in this therapeutically relevant paradigm, remote injury is alternative pathway dependent.

In vivo targeting of CR2-fH and effect of complement inhibitors on C3 deposition

Cellular localization and dynamics of C3d deposition and CR2-fH binding after intestine I/R were investigated by immunofluorescence microscopy. Animals subjected to intestine I/R

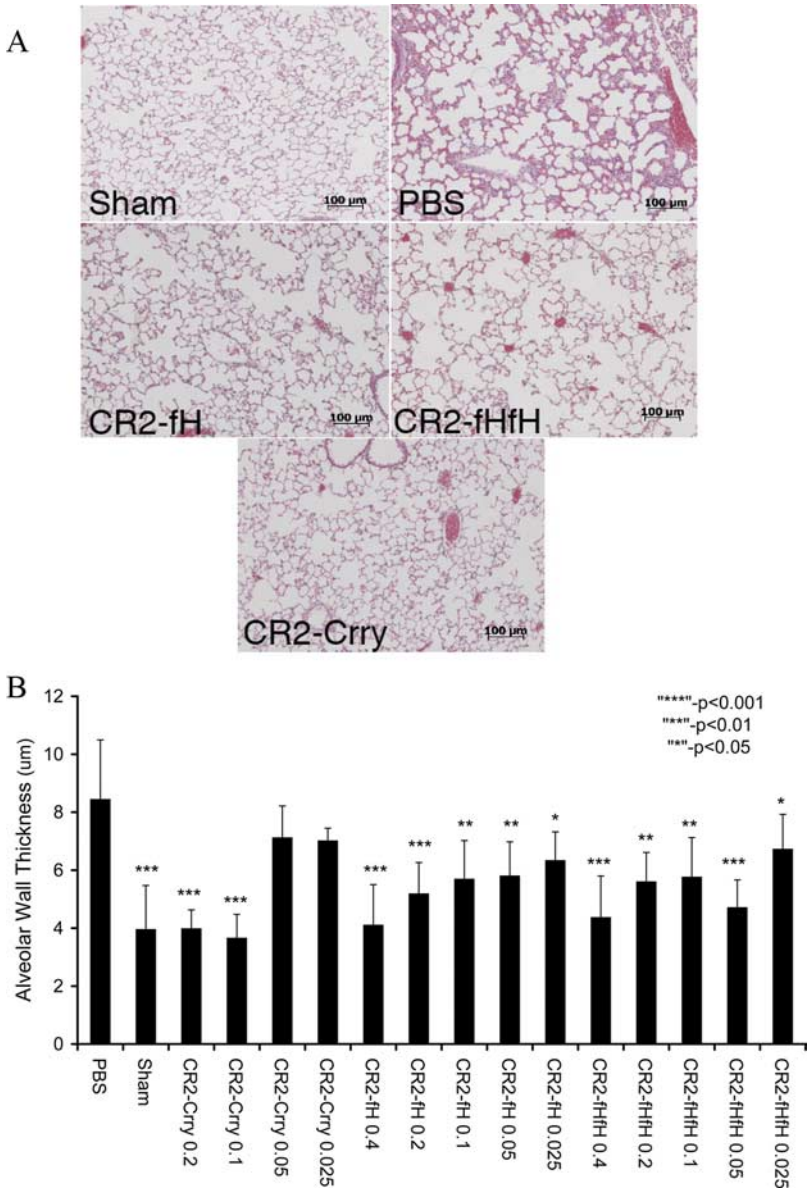

FIGURE 5. Evaluation of remote organ injury of the lung following intestinal IRI. *A*, H&E-stained sections of lungs from mice treated with a therapeutic dose of CR2-fH (0.4 mg), CR2-fHfH (0.4 mg), and CR2-Crry (0.2 mg). Sham-operated and PBS controls are shown for comparison. Note the absence of parenchymal wall thickening in sham, CR2-fH, CR2-fHfH, and CR2-Crry when compared with PBS-treated animals. Each panel is representative of 6–10 mice/group. *B*, Quantitative evaluation of the degree of alveolar wall expansion in the lungs, measured as described in *Materials and Methods*. Mean ± SD, n = 5–12 mice/group.

were treated with either PBS or CR2-fH (at 30 min after reperfusion, as per therapeutic protocol), and intestines were harvested for analysis at 30 min, 1 h, and 2 h after reperfusion. Intense C3d staining was seen in intestine samples of PBS-treated animals at all time points, with peak C3d deposition at 2 h after reperfusion. C3d was deposited within damaged denuded villi and on surrounding capillary structures within the villi (Fig. 6). Furthermore, intestines from PBS-treated animals displayed marked changes in villi structure at 1 and 2 h after reperfusion, correlating with C3d deposition. In CR2-fH-treated mice, the level of C3d deposition at 30 min after reperfusion was similar to that seen in PBS-treated mice at 30 min after reperfusion (as expected since intestines were harvested immediately after CR2-fH administration). However, at 1 and 2 h after reperfusion, C3d deposition was barely detectable in CR2-fH-treated animals. Furthermore, villi structure was largely maintained at all time points, indicating protection from damage. In agreement with our previous data, C3d deposition was also almost undetectable in CR2-Crry-treated mice (data not shown) (11).

As would be expected, CR2-fH was absent from intestine sections harvested at 30 min after reperfusion (immediately after CR2-fH administration). At 1 and 2 h after reperfusion, CR2-fH was detected on endothelial surfaces in the submucosa and on capillaries within the intestinal villi and was also seen on epithelial cells and diffusely throughout the villi structure (Fig. 6). There was no apparent difference in CR2 staining intensity between 1- and 2-h samples, likely reflecting the dynamics of C3d (CR2 ligand) deposition, which is effectively inhibited upon initial binding of CR2-fH. There was no CR2-positive staining seen on the epithelium or endothelium in sections from sham (data not shown) or PBS-treated mice. CR2 staining was also observed on B cells present within the lamina propria between the villi in PBS controls and in sham-operated mice, as would be expected due to endogenous expression of CR2 by B cells.

Circulatory half-life

A short circulatory half-life is a potential benefit for a targeted complement inhibitor due to decreased impact on host defense and

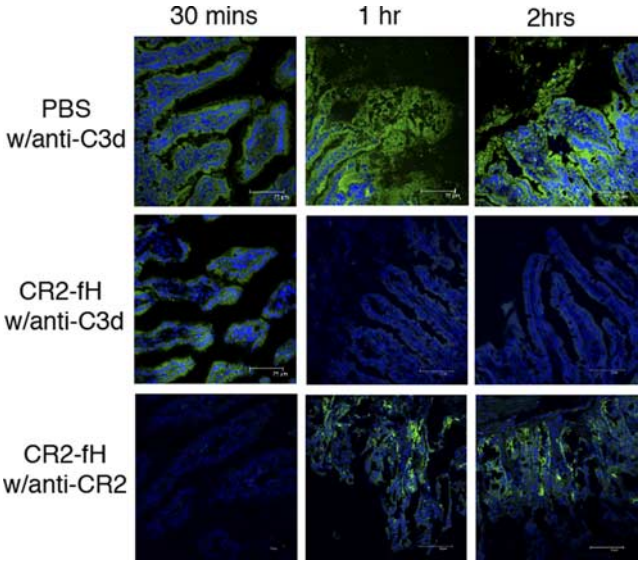

FIGURE 6. Dynamics of C3d deposition and CR2-fH binding following intestine I/R. Intestine sections harvested at 30 min, 1 h, or 2 h after reperfusion from animals treated with either PBS or CR2-fH (at 30 min after reperfusion) were stained for C3d or CR2 (green) and counterstained with ToPro-3 (blue). Representative images are shown ($n = 3$ mice/group).

normal physiological functions of complement. The circulatory half-life of CR2-fH was 8.8 h as determined by ELISA (Fig. 7) and is similar to the previously determined circulatory half-life of CR2-Crry (8.7 h) (11). Analysis of serum CR2-fH levels by Western blot and densitometric analysis gave a similar result for circulatory half-life and also demonstrated no apparent degradation of the fusion protein (data not shown). Note that these results also confirm that circulating CR2-fH levels (maximum = 20 μg/ml) were substantially lower than endogenous fH levels (300–600 μg/ml).

Discussion

There is increasing recognition that the alternative pathway of complement plays a key role in causing tissue injury in a variety of inflammatory and ischemic conditions, even when there is dependence on the classical or lectin pathways for initiating complement activation. The reason for this may be a reflection of the importance of the alternative pathway in amplifying complement activation, but other possibilities discussed in a recent review (4) include the modulation of complement regulatory proteins induced by initial activation and the provision of additional alternative pathway proteins by infiltrating neutrophils. The classical and lectin pathways are, however, known to provide important host defense function against some pathogens

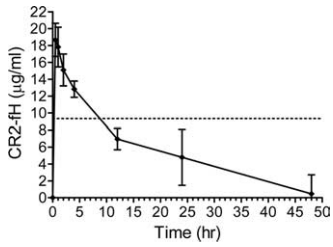

FIGURE 7. Mean serum circulatory levels of CR2fH. The serum levels of CR2fH were measured by ELISA in serum prepared from blood collected at the indicated times following i.v. injection (0.2 mg). A $t_{½}$ of 8.8 h was calculated. Mean ± SD, $n = 5–6$ mice/group.

and to play a role in some of the normal immune regulatory and homeostatic functions of complement. Therefore, the selective inhibition of the alternative pathway offers potential benefit with regard to reducing adverse side effects that may be encountered with total complement blockade. Furthermore, the targeting of alternative pathway inhibition to the site of tissue injury in pathological conditions enhances these potential benefits by reducing systemic complement inhibition and, as has been shown for the pan C3 convertase inhibitor Crry, targeting has the additional benefit of significantly increasing relative efficacy (11).

C1q-deficient mice are not protected from intestine IRI, but C2/fB- and mannan-binding lectin (MBL)-deficient mice are protected, and local injury can be restored by the administration of C2 and MBL, respectively (12). These data indicate lectin pathway dependence for intestine IRI. However, an important role for the alternative pathway in the same model of intestine IRI has also been shown by the demonstration that factor D (fD)-deficient mice are protected and that injury can be restored by the administration of fD (13). In the current study, CR2-fH is protective against intestine IRI, although an approximate 2-fold lower concentration of CR2-Crry provides equivalent protection. In vitro, CR2-fH is a more potent inhibitor of the alternative pathway than CR2-Crry. The lower efficacy of CR2-fH compared with CR2-Crry in vivo may be a reflection of a contribution of the lectin pathway to injury, but it is difficult to reconcile all of the data obtained using complement-deficient mice with the data obtained here using a complement inhibitor. A recent in vitro study demonstrated that MBL can activate the alternative pathway without the involvement of C2 (23), but mouse intestine injury in the study reported above was shown to be dependent on both MBL and C2 (12). Nevertheless, it is significant that CR2-fH can provide almost complete protection from intestine injury and almost completely abolish detectable intestinal C3 deposition, an apparent demonstration of alternative pathway dependence in a clinically relevant setting. Also of note, the therapeutic dose of CR2-fH is still much lower than the dose of Crry-Ig (untargeted systemic inhibitor) necessary to provide a similar level of protection from both intestine and lung injury (17, 24).

Alternative pathway dependence under therapeutic conditions was also demonstrated for remote lung injury following intestine IRI. This is another apparent discrepancy with previous data using complement-deficient mice showing that fD-deficient mice are only partially protected from lung injury following intestine IRI (13). Collective evidence indicates that remote lung injury is dependent on local (intestine) complement activation, C5a generation, and the systemic activation and pulmonary infiltration of polymorphonuclear neutrophils (12, 13, 21, 25). Lung injury involves complement activation in the lung, and data from Hart et al. (12) indicate different mechanisms of activation, since local (intestine) injury is C2 and MBL dependent, whereas lung injury is C2 dependent but MBL independent. These authors suggest a role for ficolins, proteins that can also serve as lectin pathway recognition molecules, in the activation of complement in the lung. However, although different complement activation mechanisms may be involved in local and remote injury, alternative pathway inhibition by CR2-fH was completely protective against lung injury following intestine IRI.

The importance of CR2-mediated targeting for complement inhibitory activity of fH(SCR1–5) at the surface of zymosan particles was demonstrated by the blockade of CR2-fH activity by anti-CR2 mAb 7G6. Native fH contains C3, heparin, and other polyanion binding sites and can be considered to have its

own "targeting" domains. The C-terminal domains SCR19–20 have been shown to be particularly important for binding to cell-bound C3b/C3d and for cell surface complement regulatory function (5–7). It is therefore intriguing that C3-targeted CR2-fH provides protection from intestine IRI, whereas endogenous fH, present at a much higher concentration and with a similarly reported C3d binding site (CCR19–20), does not. The concentration of fH in mouse and human serum is 3–4 μmol/ml and the serum concentration of CR2-fH immediately after injection of 0.2 mg is 0.32 μmol/ml (see Fig. 7). For efficient binding of fH (at least in vitro), cellular targets must contain both C3b and polyanions, a requirement that should in principle be fulfilled by intestine endothelial cells following ischemia and reperfusion. We were unable to directly show fH binding to the intestine in our model by immunohistology with Abs available to us because of technical difficulties, but the binding of both mouse and human fH to mouse and human endothelial cells, respectively, has been demonstrated in vitro (26–28). So why is there an apparent discrepancy in the in vivo activities of CR2-fH and fH? Structural studies on fH have resulted in different models of how fH functions in the fluid phase and at surfaces. One recent model proposes that the long linker between SCR18–19 may allow cell surface binding while permitting SCR1–18 to orientate freely in solution phase to facilitate contact with C3b (29). Perhaps CR2-fH allows less flexibility/movement in the fluid phase and orientates fH1–5 in a more favorable orientation for interaction with C3. Another possibility that may account for the activity of CR2-fH is different ligand-binding affinities of CR2-fH compared with native fH relative to C3b and iC3b-C3d interactions. Also, available evidence indicates a complex relationship between polyanion binding, C3 binding, and functional activity of fH. There are multiple C3 and polyanion binding sites in the fH molecule, and fH binds significantly better to C3b on cells containing polyanions than to cells without polyanions (5). All of the complement inhibitory activities of fH are contained within the SCR1–4 C3- binding region, but SCR19–20, which can bind both C3d and polyanions, appears to be essential for the binding of fH to cell-bound C3b and for effective complement inhibitory activity (5, 7). Nevertheless, an important role for polyanion binding by SCR19–20 is indicated by the fact that fH mutations associated with atypical hemolytic uremic syndrome congregate in the polyanion binding site of SCR19–20 (30), and polyanions have been shown to weaken in vitro interactions between SCR20 and C3d (27). It is therefore intriguing that CR2-fH, which contains only a C3d and C3b binding site (the latter via fH component), is a more effective complement inhibitor than native fH. Additional work is necessary to better understand the significant and unanticipated differences between CR2-fH and fH complement inhibitory activity.

In summary, we describe a novel inhibitor of the alternative pathway that utilizes CR2-mediated targeting to significantly enhance the complement inhibitory activity of an N-terminal fragment of fH. Surprisingly, the inhibitor is significantly more effective than native fH in an in vivo model of complement-dependent injury and has potential advantages over Ab-mediated systemic inhibition of complement in terms of dosing and potential systemic side effects of complete fluid-phase inhibition. Finally, complement-deficient animals have provided important insights into complement-associated disease mechanisms, but data obtained can sometimes be in disagreement with data obtained from studies in which complement has been temporarily inhibited. Complement deficiency can influence various immune functions, with the possible modulation of compensatory pathways, that may impact pathogenesis in ways different to temporary inhibition. In this study, we demonstrate the importance of the alternative pathway of complement in both local and remote injury following intestine I/R in a clinically relevant therapeutic paradigm.

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Disclosures

Dr. V. M. Holers is co-founder of Taligen Therapeutics Inc, which develops complement inhibitors for therapeutic use.

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