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Research Article

Soluble Human Complement Receptor Type 1: In Vivo Inhibitor of Complement Suppressing Post-Ischemic Myocardial Inflammation and Necrosis

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The complement system is an important mediator of the acute inflammatory response, and an effective inhibitor would suppress tissue damage in many autoimmune and inflammatory diseases. Such an inhibitor might be found among the endogenous regulatory proteins of complement that block the enzymes that activate C3 and C5. Of these proteins, complement receptor type 1 (CR1; CD35) has the most inhibitory potential, but its restriction to a few cell types limits its function in vivo. This limitation was overcome by the recombinant, soluble human CR1, sCR1, which lacks the transmembrane and cytoplasmic domains. The sCR1 bivalently bound dimeric forms of its ligands, C3b and methylamine-treated C4 (C4-ma), and promoted their inactivation by factor I. In nanomolar concentrations, sCR1 blocked complement activation in human serum by the two pathways. The sCR1 had complement inhibitory and anti-inflammatory activities in a rat model of reperfusion injury of ischemic myocardium, reducing myocardial infarction size by 44 percent. These findings identify sCR1 as a potential agent for the suppression of complement-dependent tissue injury in autoimmune and inflammatory diseases.

ACTIVATION OF THE COMPLEMENT SYSTEM CAUSES TISSUE injury in animal models of autoimmune diseases, such as immune complex–induced vasculitis (*1*), glomerulonephritis (*2*), hemolytic anemia (*3*), myasthenia gravis (*4, 5*), type II collagen-induced arthritis (*6*), and experimental allergic neuritis (*7*), and in two nonimmunologically mediated forms of primary tissue damage, burn (*8*), and ischemia (*9, 10*). The pathobiologic effects of complement are mediated directly by C5b, C6, C7, C8, $C(9)_n$, the cytolytic membrane attack complex, and indirectly by the fragments of activated C3 and C5 that stimulate a range of proinflammatory responses from mast cells and leukocytes (*11*). Indeed, if animals are transiently depleted of C3 and C5 by treatment with cobra venom factor, they are protected from tissue injury in each of these disease models. Our studies provide evidence for the potential therapeutic

utility of interrupting complement activation at the C3 and C5 steps.

The complement system itself has five proteins that inhibit the proteolytic enzymes that activate C3 and C5, namely, the C3 and C5 convertases of the alternative and classical pathways. These regulatory components are the two plasma proteins, factor H (*12, 13*) and C4-binding protein (C4-bp) (*14, 15*), and three membrane proteins, complement receptor 1 (CR1) (*16–18*), decay-accelerating factor (DAF) (*19*), and membrane cofactor protein (MCP) (*20*) (Table 1). These proteins have structural and functional similarities and have been grouped as the regulator of complement activation (RCA) family. A sixth member of this family, complement receptor type 2, has limited regulatory function (*21*) and serves primarily as a receptor involved in activation of B lymphocytes. The RCA proteins are characterized by a repeating internal homology unit, the 60 to 70 amino acid short consensus repeat (SCR) (*22–29*), and are encoded by linked genes in the RCA cluster on human chromosome 1q32 (*30–32*). All proteins suppress complement activation by reversibly binding to the C3b or C4b (or both) subunits of bi- and trimolecular complexes that are the C3 and C5 convertases of the two complement pathways. Binding to the complexes causes either displacement of the catalytic subunits of the convertases, Bb and C2a, proteolytic inactivation of C3b and C4b by the plasma protease, factor I, or both (Table 1).

The molecular characterization of the RCA family offers a panel of proteins from which might be selected a candidate for effective in vivo inhibition of complement activation. Among the RCA members, CR1 has the greatest potential for this role because it has specificity for C3b and C4b, with distinct binding sites for both proteins (Fig. 1), a capacity for displacement of the catalytic subunits from the C3 or C5 convertases of both activating pathways, and cofactor function for the degradation of C3b and C4b by factor I (*16, 18*) (Table 1). In addition, the proteolysis of C3b and C4b releases CR1 and allows it to recycle in the inactivation process. Finally, and perhaps of critical importance, these functions of CR1 are not restricted by alternative pathway activating surfaces as are the inhibitory effects of factor H (*16, 33*), making the receptor especially suitable for blocking complement activation by nonimmunologic stimuli. Nevertheless, CR1 probably cannot participate extensively in the physiologic regulation of complement activation because it is not as widely distributed among different cell types, as are DAF and MCP, being present primarily on erythrocytes and leukocytes, and its plasma concentration is approximately 0.01 percent that of the soluble regulatory proteins, factor H and C4-bp (*34*).

We have overcome this limitation to the function of CR1 as a complement inhibitor by preparing a truncated, soluble form lacking the transmembrane and cytoplasmic domains. The soluble CR1 (sCR1) retains the C3b- and C4b-binding functions and factor I–cofactor activities of membrane-associated CR1, and inhibits activation of the classical and alternative pathways in vitro at concentrations that are 100 times less than those of the serum RCA proteins. Most important, sCR1 also suppresses complement activation in vivo and reduces inflammatory tissue damage in a model of myocardial infarction associated with reperfusion injury.

Recombinant sCR1 and analysis of its in vitro inhibitory functions. The cDNA sequence encoding the A allotype of CR1, which is contained in the construct pBSABCD (*35*), was modified by introducing a translational stop codon at the junction of the extracellular and transmembrane regions (Fig. 1) (*36*). Expression of the modified cDNA by transfected CHO cells led to secretion of a soluble molecule that was purified from culture supernatants of a hollow fiber bioreactor system by cation exchange high-performance liquid chromatography. The molecular weight (200,000) of sCR1 (Fig. 1) is similar to that of wild-type CR1, consistent with a deletion of only 67 of the 1998 residues of the full-length receptor. Electron microscopic analysis of sCR1 revealed flexible, filamentous structures as was predicted for membrane CR1 on the basis of the

Table 1. Inhibitory activities of the regulators of complement activation protein family.

Protein	Dissociation of C3 and C5 convertases		Factor I–cofactors		Restriction*
	Alternative	Classical	C3b	C4b	
Factor H	+	–	+	–	Yes
C4-bp	–	+	–	+	Not applicable
DAF	+	+	–	–	Not known
MCP	–	–	+	+	Not known
CR1	+	+	+	+	No

*By alternative pathway activators.

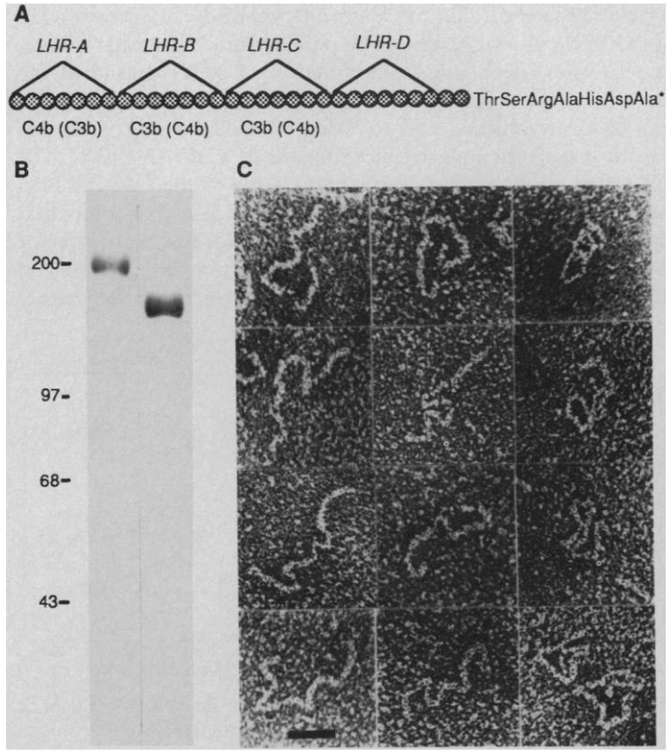

Fig. 1. The preparation, purification, and visualization of sCR1. (**A**) Model of the proposed structure of sCR1 demonstrating the 30 tandemly aligned SCR's. Every eighth SCR is a highly homologous repeat, forming four long homologous repeats (LHR's), one of which, LHR-A, has a site that preferentially binds C4b and two of which, LHR-B and -C, preferentially bind C3b. The sCR1 differs from cellular CR1 only by the absence of the transmembrane and cytoplasmic domains, having been truncated at alanine at position 1931, the first amino acid of the transmembrane segment of the receptor. (**B**) Analysis of purified, recombinant sCR1 by SDS-PAGE (*60*) in its reduced (left lane) and unreduced (right lane) forms. (**C**) Electron micrographs of negatively stained sCR1 obtained with a JEOL 1200 CX (*61*). The bar represents 40 nm.

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presence of 30 tandemly repeated SCR's (*35*) (Fig. 1).

To assess the integrity of the C3b- and C4b-binding sites in sCR1 we determined the capacity of the soluble receptor to compete with CR1 on human erythrocytes for homodimers of C3b and C4-ma, a C4b-like form of the protein in which the thiolester of the alpha chain has been hydrolyzed (*37*). The concentrations of sCR1 required for 50 percent inhibition of the binding of dimeric C3b and C4-ma to erythrocytes were 1.3 nM and 12 nM, respectively (Fig. 2, A and B). Similar concentrations of unlabeled dimers of C3b and C4-ma were inhibitory, whereas higher concentrations (approximately 100 times) of the monomeric forms of these ligands were required to compete with the binding of the labeled dimers. Since the concentrations of sCR1 that interacted with the labeled dimers in the fluid phase were comparable to the concentrations at which the unlabeled dimers interacted with cellular CR1, sCR1 may be considered to bind dimeric C3b and C4-ma bivalently. Therefore, at least two of the three binding sites in sCR1 (Fig. 1) may be engaged simultaneously to create high avidity interactions with polymeric C3b and C4-ma; the bivalent binding of dimeric C4-ma indicates that one or both of the sites with primary specificity for C3b must also bind C4-ma, as has been shown (*35*). This capability suggests that sCR1 would be effective for inhibiting the C5 convertases of complement, which contain heterodimeric complexes of C4b and C3b in the classical pathway (*38*) and homodimers of C3b in the alternative pathway (*39*).

To assay the factor I–cofactor activity of sCR1, the recombinant protein was incubated with C3b and C4-ma, respectively, in the presence of factor I, and the fragmentation of the α chains of C3b and C4-ma was analyzed by SDS–polyacrylamide gel electrophoresis (PAGE). The highest concentration of sCR1 promoted the factor I–mediated cleavage of C3b to iC3b, characterized by the 68- and 43-kD fragments, and to C3dg of 40 kD; only iC3b was generated with the lowest concentrations of sCR1 (Fig. 3A). The sCR1 also promoted the cleavage of C4-ma to the C4c and C4d fragments (Fig. 3B). Therefore, sCR1, like detergent-solubilized membrane CR1 (*16, 18*), combines the cofactor functions of factor H and C4-bp and can irreversibly inactivate the classical and alternative C3 and C5 convertases. These reactions also release sCR1, permitting its interaction with additional C3b and C4-ma.

The capacity of sCR1 to suppress complement activation was assessed in 20 percent human serum in which the alternative and classical pathways were triggered by the prototypical activators, namely, zymosan, an insoluble yeast cell wall extract, and aggregated rabbit–immunoglobulin G (IgG), respectively. The sCR1 caused dose-dependent inhibition of the generation of the C3a-desArg and C5a-desArg cleavage fragments of C3 and C5 in both activation reactions (Fig. 4). The alternative pathway C3 and C5 convertases were equally susceptible to the inhibitory effect of sCR1, while the classical pathway C5 convertase was more readily suppressed by sCR1 than was the classical C3 convertase in this and other experiments. The differential inhibition by sCR1 of the classical pathway C3 and C5 convertases probably reflects (i) lower affinity, monovalent binding to the C4b subunit of the C3 convertase and (ii) higher avidity, bivalent binding to the C4b-C3b subunit of the C5 convertase. The equivalent effectiveness of sCR1 in blocking C3 and C5 activation in the alternative pathway may indicate that the critical C3 convertase sites in this reaction are contained within clusters of C3b molecules where the enzymatic sites are stabilized by properdin (*40*). Consistent with bivalent interactions of sCR1 with the C3 or C5 convertases are the nanomolar concentrations of the recombinant protein at which 50 percent inhibition was achieved; these concentrations are similar to those at which sCR1 bound the dimers of C3b and C4-ma (Fig. 2). These inhibitory concentrations of sCR1 are 100 times lower than the concentrations of factor H and C4-bp in 20 percent serum, demonstrating the remarkable inhibitory activity that has been achieved by releasing CR1 from its normal membrane location.

Suppression by sCR1 of complement activation and myocardial reperfusion injury in rats. To determine whether the rat was a suitable species for an in vivo analysis of sCR1, we assessed the effects of the recombinant protein on complement-dependent lysis of antibody-sensitized sheep erythrocytes (EA) by rat and human serum, respectively. Although suppression of EA lysis by rat complement required twice the concentration of sCR1 as that necessary

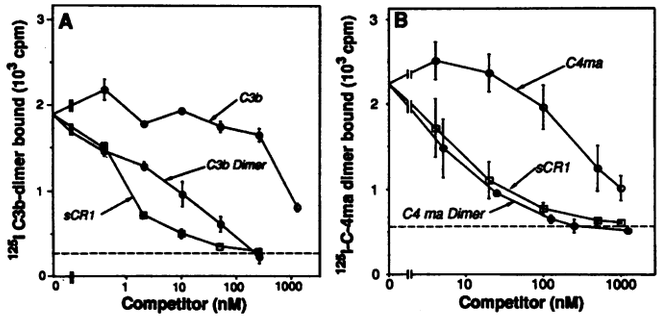

Fig. 2. Analysis of the binding of sCR1 to fluid phase ^{125}I-labeled dimeric C3b and dimeric C4-ma by competition with erythrocyte CR1. (**A**) Samples of 6.8×10^7 human erythrocytes were incubated in a solution of phosphate-buffer saline (PBS) and 0.1 percent bovine serum albumin for 60 minutes at 0°C with 3.5 ng of ^{125}I-labeled C3b dimer in the absence or presence of incremental amounts of sCR1, C3b dimer, or C3b monomer; the cell-bound ligand was then separated from the free ligand by centrifugation of the erythrocytes through dibutylphthalate (*62*). The dashed line indicates the binding of ^{125}I-labeled C3b dimer to erythrocytes in the presence of excess rabbit antibody (IgG) to CR1. (**B**) Replicate samples of erythrocytes were incubated with 80 ng of ^{125}I-labeled C4-ma dimer alone or in the presence of sCR1, C4-ma dimer, or C4-ma monomer, the bound was then separated from free labeled ligand. The dashed line indicates the binding of ^{125}I-labeled C4-ma dimer to erythrocytes in the presence of rabbit antiserum to CR1. The error bars indicate ranges, from two determinations.

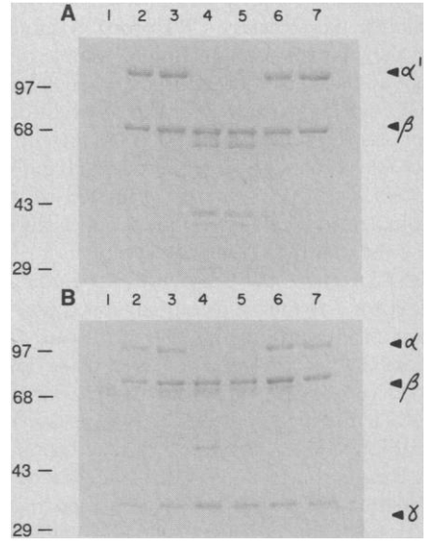

Fig. 3. The promotion by sCR1 of the cleavage of C3b and C4-ma by factor I. (**A**) C3b (370 μg/ml) was incubated for 15 minutes at 20°C in PBS alone (lane 2), with factor I (lane 3), or with factor I in the presence of sCR1: at 80 μg/ml (lane 4), 8 μg/ml (lane 5), or 0.8 μg/ml (lane 6), or with 80 μg of sCR1 alone (lane 7); the samples were then reduced with 0.1 M dithiothreitol (DTT) and analyzed by SDS-PAGE (*34*). The factor I, which was functionally purified by sequential chromatography on DEAE Sephacel and S-Sepharose (Pharmacia, Piscataway, New Jersey), was also assessed (lane 1). The positions of the standards are shown on the left, and of the α′ and β chains of C3b on the right. (**B**) C4-ma (477 μg/ml) was incubated in PBS alone (lane 2), with factor I (lane 3), or with factor I in the presence of sCR1 at 80 μg/ml (lane 4), at 8 μg/ml (lane 5), or at 0.8 μg/ml (lane 6), after which the samples were reduced and assessed by SDS-PAGE. Factor I alone also was assessed (lane 1). The positions of the molecular standards (kilodaltons) are shown on the left, and of the α, β, and γ chains of C4-ma on the right.

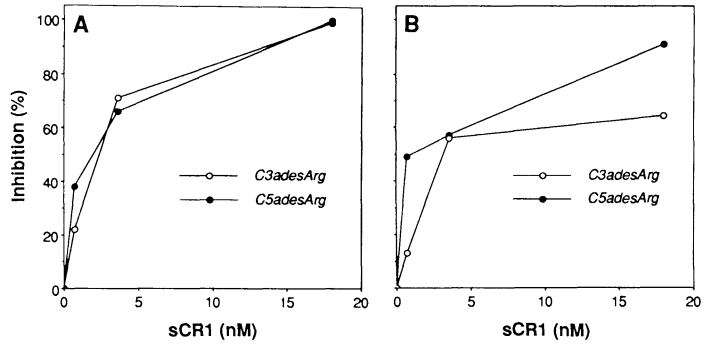

Fig. 4. Inhibition by sCR1 of complement activation by the classical and alternative pathways. (**A**) The alternative pathway was activated in normal human serum diluted 1/5 with barbital-buffered saline containing 2 mM Mg^{2+}, 8 mM EGTA by addition of zymosan (1 mg/ml) in the absence and presence of incremental concentrations of sCR1. After incubation for 20 minutes at 37°C, the reactions were stopped by addition of an equal volume of 0.01 M EDTA, and the concentrations of C3a-desArg and C5a-desArg were measured by radioimmunoassay (Amersham, Chicago, IL). (**B**) Activation of the classical pathway was assessed in an identical manner except that heat aggregated rabbit IgG (60 μg/ml) was substituted for zymosan and the barbital-buffered saline in which the serum was diluted lacked magnesium EGTA. Each point represents the mean of two determinations, which differed by less than 2 percent.

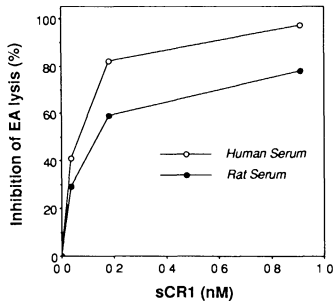

Fig. 5. Inhibition by sCR1 of the complement-dependent lysis of sheep erythrocytes (E) sensitized with rabbit immunoglobulin M (EA). The EA (1×10^8 per milliliter) mixtures were incubated for 30 minutes at 37°C with human serum diluted 1 to 222 and rat serum diluted 1 to 256, respectively, in the presence of incremental concentrations of sCR1, after which lysis was assessed by spectrophotometric measurement of hemoglobin in the supernatant. In the absence of sCR1, there was 91 and 79 percent lysis of EA in the human and rat serums, respectively.

for comparable inhibition of lysis by human complement (Fig. 5), the effectiveness of sCR1 in this assay indicated at least that it acted on the rat classical C5 convertase.

We administered sCR1 to rats subjected to transient myocardial ischemia with subsequent reperfusion. The mechanism of injury to ischemic, but not irreversibly damaged, reperfused myocardium involves a leukocyte-dependent inflammatory reaction (*41–45*) that may require complement activation (*9, 10*). Animals were divided into groups at random and were given bolus intravenous injections of phosphate-buffered saline alone ($n = 29$) or containing 1 mg of sCR1 ($n = 31$) immediately before occlusion of the left coronary artery by suture ligation. After 35 minutes, the sutures were released, the thorax was closed, and the animals were returned to their cages for 7 days, when they were killed and the myocardial infarct was measured (*46*); the 7-day interval provided a reliable assessment of infarct size and permitted analysis of possible adverse effects of sCR1 on infarct healing. Survival rates in the group given buffer alone (24 of 29) and the sCR1-treated group (25 of 31) were similar, with deaths occurring immediately after coronary artery ligation in all but one of the control rats. Ligation of the coronary artery was judged to be successful in 22 animals in each group that met all of the following criteria: immediate electrocardiographic changes compatible with ischemia, cyanosis of the anterior left ventricular wall, and histologic evidence of myocardial necrosis post mortem. The suture was released successfully in all rats except two of the sCR1-treated animals. Analysis of all survivors, including the two rats in whom reperfusion was not achieved, demonstrated that treatment with sCR1 reduced the size of myocardial infarction from a mean of 16 ± 2 percent of the left ventricular mass in the control rats to 9 ± 2 percent in the sCR1 group ($P < 0.01$) (Fig. 6A). The frequency of transmural infarction also was lower in the sCR1-treated (6 of 25) than in the control rats (12 of 24) ($P < 0.04$) (Fig. 6B).

The infarct segment thickness summed over four sections of hearts from all rats treated with sCR1 was 7.8 ± 0.4 mm, which was not significantly different from that of all untreated animals, 7.3 ± 0.4 mm, but slightly less than that of the remote, uninfarcted interventricular septum from the hearts of these two groups of rats (sCR1 rats, 9.3 ± 0.2 mm; untreated rats, 9.4 ± 0.2 mm). There was also no difference in the intraventricular cavity size of these two groups (sCR1 rats, 64.9 ± 3.6 mm^3; untreated rats, 68.9 ± 2.6 mm^3). Therefore, sCR1 suppresses myocardial infarct size but does not interfere with healing in a manner that causes ventricular dilatation and left ventricular wall thinning, as judged from observation of hearts one week after infarction.

To determine whether suppression of tissue damage by sCR1 was associated with inhibition of complement activation by ischemic myocardium, another group of buffer-treated ($n = 7$) and sCR1-treated rats ($n = 8$) was subjected to the same ischemia-reperfusion protocol, and the animals were killed 3 hours after reperfusion. The hearts were assessed by nitroblue tetrazolium (NBT) staining (*47*) to delineate regions of irreversible injury from viable myocardium, and by immunoperoxidase staining (*48*) with a mouse monoclonal antibody to the rat C5b-9 membrane attack complex (*49*) (Fig. 7). In the NBT-negative, infarcted areas of the control rats ($n = 7$), the C5b-9 complex was present primarily along the endothelium of capillaries and venules, but not in the myocardial fibers (Fig. 7). In contrast, in rats that had received sCR1 ($n = 8$), the NBT-negative areas were consistently reduced in size, and little or no C5b-9 complex was detectable in these regions, as exemplified by the representative sections shown.

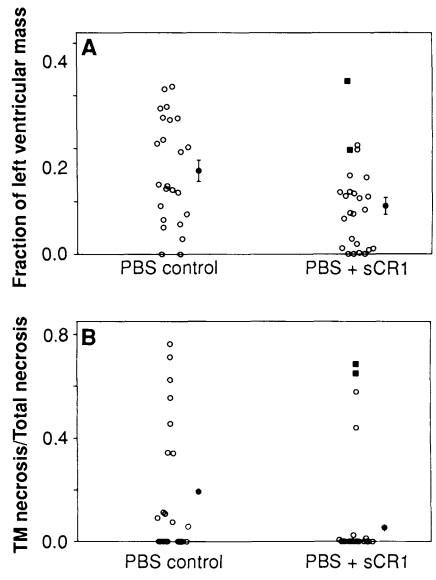

Fig. 6. Distribution of (**A**) the fraction of the left ventricular mass demonstrating myocardial infarction and (**B**) the ratio of transmural necrosis to total necrosis in hearts taken from individual rats 1 week after a 35-minute period of transient ischemia caused by temporary ligation of the left coronary artery. Immediately before the ligation, 1 ml of PBS alone or containing 1 mg of sCR1 was administered intravenously to the rats. The two closed squares represent the results of sCR1-treated animals in which release of the coronary artery ligation was unsuccessful. The closed circles and error bars represent the mean and standard error of the mean for each group.

Quantitation of leukocytes (*45*) in these serial sections revealed that within infarct zones of control rats there were 195 ± 28 leukocytes per square millimeter (150 high-power fields; $n = 3$), which were almost exclusively within capillaries and venules. Corresponding sections from the hearts of rats that had received sCR1 had only 83 ± 3 leukocytes per square millimeter ($n = 4$; $P = 0.006$), indicating that suppression of complement activation was associated with decreased accumulation of this inflammatory cell type.

The localization of C5b-9 complexes along endothelial surfaces suggests that these cells may be the primary site of complement activation in the pathogenesis of reflow injury to ischemic myocardium, and contrasts with the more diffuse distribution of complement proteins throughout infarcted myocardium 24 hours following coronary artery occlusion (*50*). While the latter may simply reflect the capacity of necrotic tissue to activate complement, the former may indicate that ischemically stressed endothelium acquires a complement-activating function. Complement activation by endothelial cells would be an especially potent stimulus for the early localization of neutrophils to ischemic myocardium, with C5a activating intravascular leukocytes and causing their rapid upregulation of cellular receptors, including CR1 and CR3 (*51, 52*). The latter receptor has been shown to promote the attachment of neutrophils to complement-activating endothelial cells bearing the ligand for CR3, iC3b (*53*). Therefore, suppression of complement activation by sCR1 may account for the decreased numbers of neutrophils apparently adherent to endothelial cells.

Reperfusion of ischemic myocardium by thrombolytic agents reduces infarct size, improves left ventricular function, and reduces mortality if established within a few hours of coronary artery occlusion (*54–56*). However, the potential benefits of reperfusion may not be fully achieved because reflow into myocardium that is severely ischemic, but not irreversibly injured, may induce necrosis (*57, 58*). Two events thought to be causally related to necrosis are intravascular accumulation of neutrophils and microvascular endothelial cell injury (*59*), both of which may be a consequence of complement activation. The present finding of a myocardial protective effect of sCR1 supports the possibility of a central role for complement, extending earlier studies in which complement was depleted with cobra venom factor, and offers a means by which the tissue-sparing potential of thrombolytic therapy may be enhanced.

Two major thrusts in complement research have been the definition of its molecular reactions and of its role in the pathogenesis of autoimmune and inflammatory diseases, the common goal for both

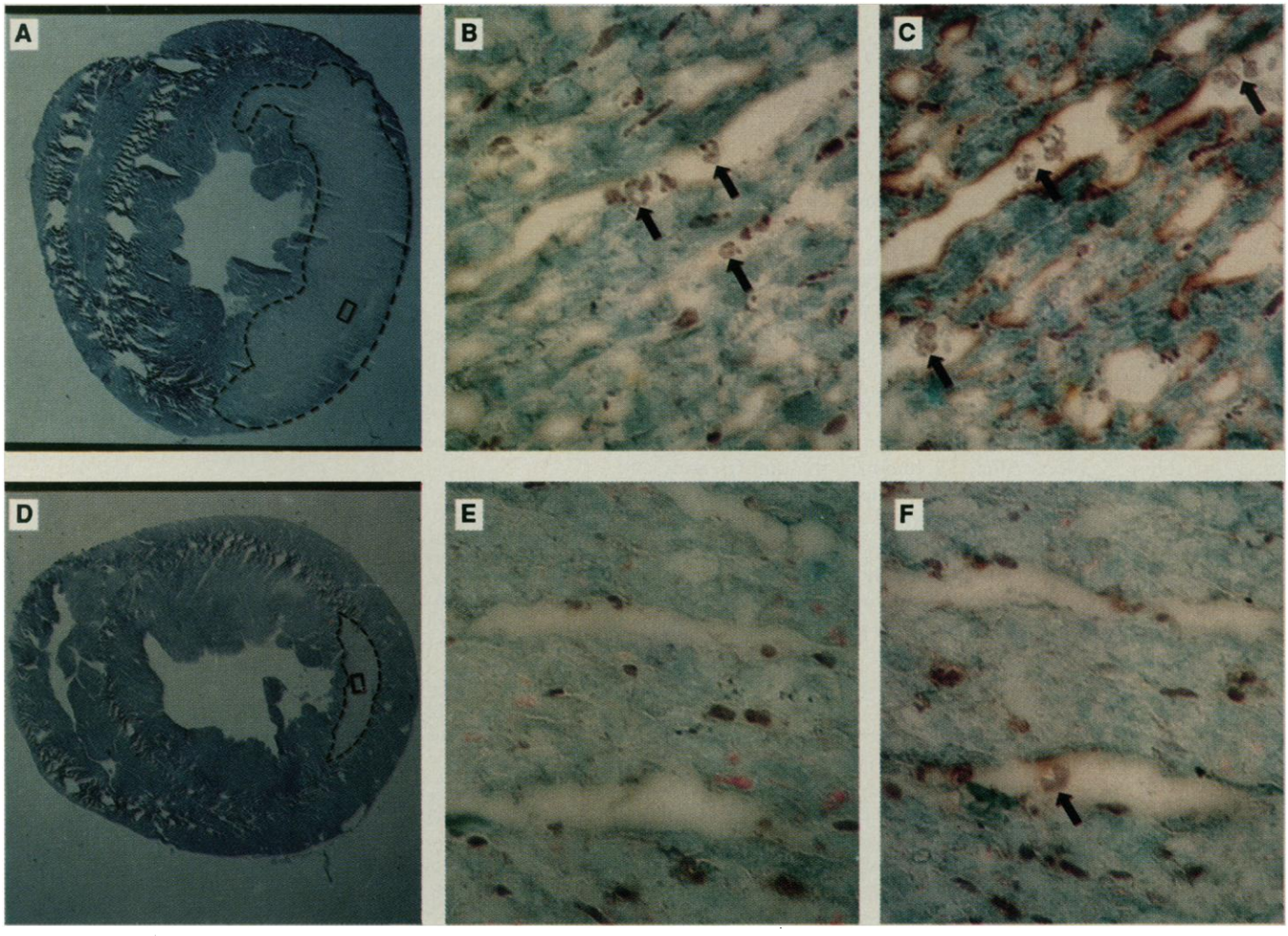

Fig. 7. Demonstration of infarct zones by nitro blue tetrazolium (NBT) staining (**A** and **D**; original magnification, ×10) and of complement activation by immunoperoxidase staining with a control, isotype-matched, irrelevant antibody (**B**, and **E**) and with monoclonal antibody to the C5b-9 complex (**C** and **F**; original magnification ×500) in consecutive 4-μm sections of hearts taken from buffer-treated (A, B, and C) and sCR1-treated rats (D, E, and F) 3 hours after a 35-minute period of left-coronary-artery ligation. The immunoperoxidase sections were counterstained with Gill's hematoxylin 3. The regions not stained with NBT and encompassed by dashed lines indicate the infarct zones (A and D). The boxes show the locations in the infarct zones that correspond to the regions in the serial sections that are shown in panels B, C, E, and F; arrowheads in these panels indicate leukocytes.

being the development of a means for interrupting tissue injury in these diseases. By releasing CR1 from its restricting membrane anchor to take advantage of an inhibitory activity that exceeds by two orders of magnitude that of the plasma regulatory proteins, the present study has attempted directly to approach this goal. The capacity of sCR1 to inhibit complement activation and cellular necrosis in a quantitative in vivo model of nonimmune complement activation provides the necessary rationale for assessing its effects in more complex models of autoimmune diseases in future studies. Finally, sCR1 may serve as a prototype of a series of genetically engineered inhibitors containing the active sites of the receptor, which constitute less than 20 percent of its total linear sequence (*35*), an approach whose feasibility has been shown by the transfer of the active site SCR's of the CR1 homologue, CR2, to CR1 with conservation of function (*60*).

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36. The plasmid, pBSABCD, containing the entire cDNA coding sequence of CR1 was digested with Bal I at a site 29 bp 5′ of the transmembrane sequence. The resulting linear fragment was ligated to a synthetic double-stranded oligonucleotide.

    5′-CCAAATGTACCTCTCGTGCACATGATGCTTAACTCGAG-3′
    3′-GGTTTACATGGAGAGCACGTGTACTACGAATTGAGCTC-5′

    that restored the extracytoplasmic sequence, introduced a stop codon (underlined) after the alanine codon at the start of the transmembrane segment, and placed an Xho I restriction site at the 3′ end. The modified cDNA was excised with Xho I and ligated to the Xho I–digested mammalian expression vector, pTCSgpt (T Cell Sciences, Cambridge, MA). After linearization by Fsp I restriction, the vector was cotransfected with pSV2dhfr into CHO cells deficient in dihydrofolate reductase [L. Chasin and G. Urlaub, *Proc. Natl. Acad. Sci. U.S.A.* 77, 4216 (1980)] and high expressing transformants were selected by growth in media containing methotrexate. Large quantities of sCR1 were produced in a hollow fiber bioreactor system (Cell-Pharm Cell Culture System I, CD Medical, Miami Lakes, FL). The sCR1 was purified by cation exchange high-performance liquid chromatography on a Hydropore-SCX column (Rainin, Woburn, MA), and quantified by BCA assay (Pierce, Rockford, IL) with bovine serum albumin as a standard. The sCR1 has been assigned the T Cell Sciences number, TP1-HD.
37. C3 and C4 were purified [C. H. Hammer *et al.*, *J Biol. Chem* **256**, 3995 (1981)], and the C3 was converted to C3b by treatment with trypsin, dimerized by incubation with a 1:4 molar ratio of bismaleimidohexane (Pierce, Rockford, IL) for 30 minutes at 0°C, and labeled with ^{125}I [P. J. Fraker and J. C. Speck, *Biochem. Biophy. Res Commun.* **80**, 849 (1978)]. C4 was treated with methylamine [(S. K. Law and R. P. Levine, *Proc. Natl. Acad Sci. U.S.A.* 77, 7194 (1980)], and spontaneously generated dimers were isolated by gel filtration and labeled with ^{125}I.
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46. For analysis of infarct size [H. F. Weisman, D. E. Bush, J. A. Mannisi, M. L. Weisfeldt, B. Healy, *ibid* **78**, 186 (1988)], the aortas of hearts that had been excised from rats under methoxyflurane anesthesia were cannulated, and the coronary arteries were perfused first with Krebs Henseleit solution and then with 30 mM KCl for diastolic arrest. After fixation by intracoronary perfusion and immersion in 10 percent buffered formalin, 2-mm transverse sections of the hearts were histologically analyzed by digitizing the areas of the normal, total infarcted, and transmurally infarcted left ventricular myocardium. The summed values of the fractional areas of all slices were used to calculate myocardial infarct size and the ratio of transmural necrosis to total necrosis. All procedures and care of the animals were in accordance with institutional guidelines.
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