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Anti-C5 monoclonal antibody therapy prevents collagen-induced arthritis and ameliorates established disease

(inflammation/complement/immune complex)

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ABSTRACT **Activated components of the complement system are potent mediators of inflammation that may play an important role in numerous disease states. For example, they have been implicated in the pathogenesis of inflammatory joint diseases including rheumatoid arthritis (RA). To target complement activation in immune-mediated joint inflammation, we have utilized monoclonal antibodies (mAbs) that inhibit the complement cascade at C5, blocking the generation of the major chemotactic and proinflammatory factors C5a and C5b-9. In this study, we demonstrate the efficacy of a mAb specific for murine C5 in the treatment of collagen-induced arthritis, an animal model for RA. We show that systemic administration of the anti-C5 mAb effectively inhibits terminal complement activation *in vivo* and prevents the onset of arthritis in immunized animals. Most important, anti-C5 mAb treatment is also highly effective in ameliorating established disease. These results demonstrate a critical role for activated terminal complement components not only in the induction but also in the progression of collagen-induced arthritis and suggest that C5 may be an attractive therapeutic target in RA.**

Among the various factors that contribute to joint inflammation in rheumatic diseases, activated components of the complement system may be considered to play a central role (1). Products of the complement cascade mediate a wide range of proinflammatory activities, including alterations in vascular permeability and tone, leukocyte chemotaxis, and the activation and lysis of multiple cell types (2).

For example, there is long-standing evidence for intraarticular complement activation in the pathogenesis of rheumatoid arthritis (RA) (3–9). Activated B cells and plasma cells are present in inflamed synovium, and in established disease, lymphoid follicles with germinal centers may be present as well (10). This results in high levels of local immunoglobulin production and the deposition of immune complexes (which may include IgG and IgM rheumatoid factors) in the synovium and in association with articular cartilage (11). Elevated levels of activated complement components, such as C3a, C5a, and C5b-9 (membrane attack complex), have been found within inflamed rheumatoid joints (3–9), and positive correlations have been established between the degree of complement activation and the severity of joint disease (5, 7).

The C5 component of complement is cleaved to form products with multiple proinflammatory effects and thus represents an attractive target for complement inhibition in immune-mediated inflammatory diseases. C5a is the most potent anaphylatoxin and a powerful chemotaxin for neutrophils and monocytes, with the ability to promote margination, extravasation, and activation of these cells (12). Cellular activation by C5a induces the release of multiple additional inflammatory mediators (13, 14). In addition, C5b-9 can also stimulate the release of multiple proinflammatory molecules (4, 6, 14) and may well play an important role in inflammation apart from its lytic function. Thus, blocking C5b-9 as well as C5a generation may be required for optimal inhibition of the inflammatory response. At the same time, inhibition of the complement cascade at C5 does not impair the generation of C3b through the classical and alternative pathways, preserving C3b-mediated opsonization of pathogenic microorganisms as well as opsonization and solubilization of immune complexes (2).

Until now, the role of activated terminal complement components in inflammation has not been examined using inhibitors that specifically target the complement cascade at the C5 molecule. To address this issue, we have undertaken an approach to complement inhibition employing C5-specific monoclonal antibodies (mAbs) that block C5a and C5b-9 generation.

In this study, we examine the role of terminal complement in the pathogenesis of type II collagen-induced arthritis (CIA), a model of autoimmune inflammatory joint disease that is histologically quite similar to RA, with inflammatory synovitis, pannus formation, and erosion of cartilage and bone (15). By using an inhibitory mAb specific for murine C5 (16), we show that systemic administration of the anti-C5 mAb effectively inhibits terminal complement activation *in vivo* and that anti-C5 mAb treatment prevents the onset of arthritis. Most important, mAb-mediated C5 inhibition is also highly effective in ameliorating established disease. These results demonstrate an important role for activated terminal complement components not only in the induction but also in the progression of disease in this model of immune-mediated joint inflammation.

MATERIALS AND METHODS

Mice. Eight- to-12-week-old male DBA/1LacJ mice were purchased from The Jackson Laboratory and were used in all experiments.

Antibodies and Antibody Administration. The anti-mouse C5 hybridoma BB5.1 was provided by B. Stockinger (National Institute for Medical Research, London). This is a murine antibody specific for the mouse C5 protein, raised by immunization of a C5-deficient mouse (16). The murine anti-human C8 hybridoma 135.8 was provided by P. Sims (Blood Research Institute, Milwaukee, WI). Both murine antibodies (mAbs) have a $\gamma 1$ isotype.

Induction and Clinical Evaluation of CIA. Bovine type II collagen (B-CII) (Elastin Products, Owensville, MO) was dissolved in 0.01 M acetic acid by stirring overnight at 4°C at

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Abbreviations: RA, rheumatoid arthritis; mAb, monoclonal antibody; CIA, collagen-induced arthritis; CFA, complete Freund's adjuvant; H&E, hematoxylin/eosin; PIP, proximal interphalangeal.

a concentration of 4 mg/ml. Complete Freund's adjuvant (CFA) was prepared by the addition of desiccated *Mycobacterium tuberculosis* H37RA (Difco) to incomplete Freund's adjuvant (Difco) at a concentration of 2 mg/ml. The solution of CII (4 mg/ml) was emulsified in an equal volume of CFA. A volume of 100 μl, containing 200 μg of CII and 100 μg of mycobacteria, was injected intradermally at the base of the tail. After 21 days, all mice were reimmunized using the identical protocol. The severity of arthritis in each affected paw was graded according to an established scoring system (17) as follows: 0, normal joint; 1, mild/moderate visible erythema and swelling; 2, severe erythema and swelling affecting an entire paw or joint; 3, deformed paw or joint with ankylosis. The sum of the scores for all four paws in each mouse was used as an arthritis index (maximum score per animal = 12) to represent overall disease severity and progression in an animal. To examine the effect of anti-C5 mAb on disease prevention, mice were randomly divided into treatment and control groups 3 weeks following the initial challenge with CII, at which time they were administered equal amounts of either the anti-mouse C5 mAb, BB5.1, or the isotype-matched anti-human C8 mAb, 135.8, respectively. Each animal received 750 μg i.p., twice weekly for 3 weeks. Mice were sacrificed 7 weeks following the initial immunization, at which time the joints were prepared for histologic evaluation. To evaluate the therapeutic effect of anti-C5 mAb treatment of established disease, anti-C5 or control mAb, 3 mg i.p. daily for a total of 10 days, was administered following the onset of clinical arthritis in one or more limbs. Mice were examined daily and the arthritis index was determined. The degree of swelling in the initially affected joint(s) was monitored by measuring paw thickness using calipers.

Hemolytic Assay. Mouse serum samples were diluted to 10% (vol/vol) with gelatin veronal buffer 2+ (Sigma) and added (50 μl per well) to 96-well microtiter plates containing 50 μl of human C5-deficient serum per well (Quidel, San Diego). The plates were incubated for 30 min at room temperature. Erythrocyte preparation and hemolytic assays were then performed as described (18).

Histopathology. Mice from anti-C5-treated and control groups were sacrificed and their limbs were fixed in 10% buffered formalin and decalcified in diluted RDO (Darlco, Oradell, NJ) solution for 1–3 days. The tissue was then processed and embedded in paraffin with a VIP tissue processor (Miles). Tissue sections (5 μm) were stained with hematoxylin/eosin (H&E) using standard methodology. To detect immunoglobulin deposition on joint surfaces, the limbs were demineralized, snap frozen, and cryostat sectioned. Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG, IgM, IgA (heavy and light chains) antibody (Zymed) at a 1:100 dilution was applied to the sections for direct fluorescence staining.

RESULTS

Effect of Anti-C5 mAb Therapy in Preventing CIA. To evaluate the efficacy of anti-C5 mAb treatment in the prevention of CIA, biweekly i.p. injections of anti-C5 or control mAb were initiated 3 weeks following the first immunization with native CII. The functional capacity of the mAb to block the generation of C5b-9 and C5a was directly demonstrated by its ability to inhibit complement-dependent hemolysis as well as C5a-dependent neutrophil chemotaxis (unpublished data). To confirm that the C5-specific mAb was effectively inhibiting complement *in vivo*, the hemolytic activity of sera from treated mice was compared to that of control mAb-treated animals. Sera obtained from anti-C5 mAb-treated animals 1 week after the initiation of therapy had significantly (≈60%) decreased hemolytic activity relative to control mAb-treated mice (Fig. 1*A*). Sera obtained from the treated mice at subsequent time points showed similar diminished hemolytic activity (unpublished data), indicating that serum complement was inhibited throughout the treatment period.

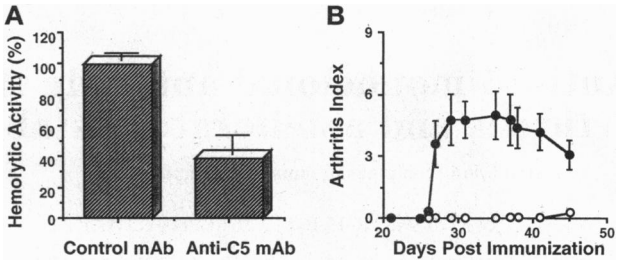

FIG. 1. Prevention of CIA with anti-C5 mAb therapy. Biweekly treatment with 750 μg of anti-C5 or control isotype-matched mAb was begun 3 weeks after the initial immunization. (*A*) Mice were bled at 4 weeks and serum hemolytic activity was measured. Anti C5-treated mice (n = 10) had reduced hemolytic complement activity compared to controls (n = 11). The diminished hemolytic activity was representative of multiple samples obtained during the course of treatment. (*B*) Arthritis index: severity of disease in each limb (1–3) times the number of limbs involved (1–4). ●, Control animals; ○, anti-C5-treated animals.

Mice were evaluated clinically for the presence of arthritis and the disease severity was quantitated by measurement of the arthritis index. Severe clinical disease, manifested by marked swelling and erythema of multiple limbs, was observed beginning 4–5 weeks after immunization in >90% of the animals treated with the control mAb (Fig. 1*B*). In contrast, all but one of the anti-C5-treated mice remained completely free of any clinical signs of inflammation during the 3-week period of treatment (Fig. 1*B*). Anti-C5 mAb therapy was shown to have no effect on humoral or cellular immune responses to CII (unpublished data), indicating that complement inhibition *per se* accounted for the antiinflammatory effects of the treatment.

Histologic analysis was performed to determine whether anti-C5 mAb therapy prevented morphologic as well as clinical signs of inflammation. Evaluation of diseased joints from control mAb-treated animals revealed proliferative fibrous synovium (pannus) invading the joint space, numerous inflammatory cells including neutrophils and monocytic cells, and severe to complete cartilage and bone erosion (Fig. 2*B*). In contrast, a representative joint from a C5-mAb-treated animal (Fig. 2*C*) manifested normal architecture with a normal synovial membrane and smooth cartilaginous surfaces indistinguishable from a normal joint (Fig. 2*A*). Immunostaining with FITC-conjugated antibody to mouse immunoglobulin demonstrated the presence of immunoglobulin localized within synovium and along the articular surface in the normal-appearing joints from anti-C5 mAb-treated animals and the inflamed joints from control mAb-treated mice (Fig. 2 *D* and *E*). This result confirmed that the anti-C5 mAb therapy had suppressed the inflammatory response despite immunoglobulin deposition.

Anti-C5 mAb Therapy of Established Arthritis. Having determined that activation of the complement cascade downstream of C5 was critical for the induction of CIA, we next examined whether activation of complement also had a role in sustaining the inflammatory response. To address this issue, anti-C5 or control mAb therapy was initiated only after clinical signs of inflammation had appeared in one or more joints. Systemic inhibition of complement was again confirmed by demonstrating reduced complement-dependent hemolytic activity in the sera of the anti-C5-treated mice throughout the course of therapy (Fig. 3*A*). The onset of disease in all animals was abrupt and severe and associated with a >50% increase in paw thickness (Fig. 3*B*). Treated and control animals were then monitored for 10 days with respect to the clinical signs of inflammation in the initially involved joints as well as by

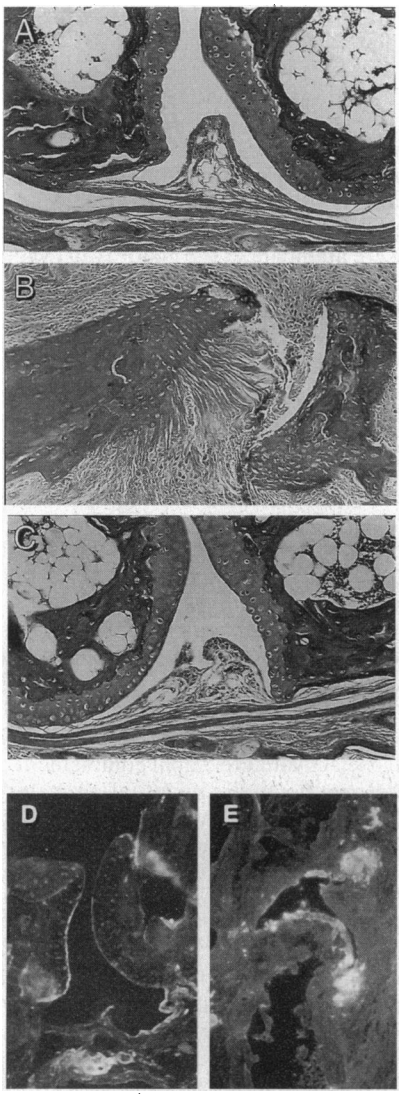

FIG. 2. Histopathologic analysis of joints from normal (*A*), control mAb-treated (*B*), and anti-C5 mAb-treated (*C*) mice. Animals were sacrificed and joints were prepared for histologic evaluation 7 weeks after the initial priming with CII. H&E-stained sections of joints of control animals (*A*) revealed normal joint architecture including normal bone, smooth articular surfaces, and a tag of synovial membrane comprised of a layer of synovial lining cells overlying a thin delicate stroma containing microvessels. In contrast, joints of animals treated with control mAb (*B*) showed destruction of normal architecture with complete loss of articular cartilage, bone erosion, and filling of the joint spaces with a fibrous synovium containing proliferated synovial cells, inflammatory cells, and deposited extracellular matrix. Animals treated with anti-C5 mAb (*C*) manifested histological features similar to those observed in normal animals, with normal bone and smooth cartilaginous articular surfaces and a normal synovial membrane and joint space. Immunofluorescence staining for immunoglobulin deposition revealed localization of antibody on the surface of cartilage and synovia from the anti-C5 mAb-treated (*D*) and control mAb-treated (*E*) mice. No fluorescence staining was observed in joints from normal nonimmunized mice (data not shown). (*A–C*: ×100, bar = 50 μM; *D* and *E*: ×50.)

measurement of overall clinical score. Therapy with anti-C5 mAb produced dramatic attenuation of the course of disease, as determined by reduction of swelling in the initially involved limb (Fig. 3*B*), as well as a markedly lower arthritis index (Fig. 3*C*), reflecting the attenuation of swelling in the limbs involved at onset as well as the prevention of disease spread to additional joints.

Recruitment of additional joints following initial presentation is not only characteristic of CIA but also an important

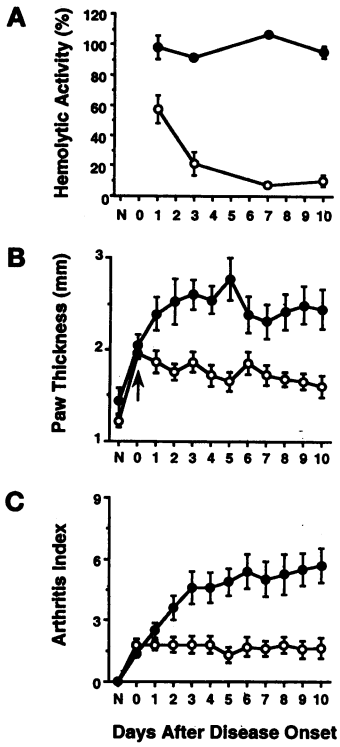

FIG. 3. Treatment of established CIA. Mice were begun on daily mAb therapy after onset of clinical disease. (*A*) Mice were bled at the indicated time points following the initiation of mAb treatment and their sera were assessed for complement-mediated hemolytic activity. Hemolytic activity of sera from anti-C5-treated mice (n = 10) was reduced to <20% of controls (n = 8). (*B*) The effect of C5 inhibition on the clinical course of inflammation in the initially involved limb was evaluated by daily measurement of paw thickness. ●, Control mAb-treated animals; ○, anti-C5-treated animals. The arrow indicates the time point at which mAb therapy was begun. N refers to the thickness of a normal appearing paw prior to the clinical onset of disease. The degree of paw swelling measured in the anti-C5 mAb-treated animals differed significantly from that of the control mAb-treated group (P < 0.001, two-factor ANOVA). (*C*) Measurement of arthritis index in treated (○) and control (●) animals.

measure of progression of disease in RA. We therefore determined the degree of new limb recruitment in anti-C5-treated versus control animals. The total number of limbs involved at onset of clinical disease, prior to the initiation of any mAb therapy, was determined in all animals, which were then randomized to either anti-C5 mAb or control mAb treatment. The animals were reevaluated 10 days later, at the end of the treatment period, for the total number of limbs involved with arthritis. The results show that the number of limbs manifesting clinical arthritis more than doubled in the control mAb-treated animals over 10 days, increasing from 8 to 18 (Table 1). In contrast, anti-C5 mAb treatment completely arrested the spread of disease to additional limbs, with one of the originally inflamed limbs resolving completely (Table 1). Moreover, the mean degree of paw swelling at day 10 in the 18 affected limbs from the control mAb-treated group was 2.4 ± 0.16 mm, in comparison to a mean paw thickness of 1.65 ± 0.13 mm in the 12 residually affected limbs from the anti-C5 mAb-treated group (P = 0.001). Thus, by two critical measures, the magnitude of clinical inflammation in involved joints and the prevention of disease spread, anti-C5 therapy markedly ameliorated the clinical course of established arthritis.

Histopathology Following Treatment of Established Disease. In concert with the clinical observations, histologic analysis demonstrated marked differences between anti-C5-treated and control animals (Fig. 4). The initially affected

Table 1. Anti-C5 mAb therapy of established CIA blocks disease progression

Treatment	*n*	No. of limbs affected		% increase/decrease of no. of limbs involved
		Day 0	Day 10	
Control mAb	8	8	18	+125
Anti-C5 mAb	10	13	12	−7.7

The anti-C5 or control mAb was instituted only after disease onset (day 0) and continued for 10 days. The number of limbs clinically involved at the onset of disease (day 0) versus 10 days following mAb treatment (day 10) was determined as a measure of disease progression. An initially involved limb was considered to be involved at the end of the study unless all clinical signs of inflammation had completely disappeared, even if the degree of swelling and redness had diminished considerably. The % increase or decrease in the number of limbs involved over the course of therapy is shown. *n*, Number of animals in each group.

proximal interphalangeal (PIP) joint from a control mAb-treated animal displayed marked synovial proliferation, an intense inflammatory cell infiltrate with a predominance of neutrophils, and significant erosion of articular cartilage (Fig. 4 *A* and *C*). In contrast, a representative joint from an anti-C5 mAb-treated mouse showed a more modest degree of synovial proliferation with a dramatic absence of neutrophil infiltration

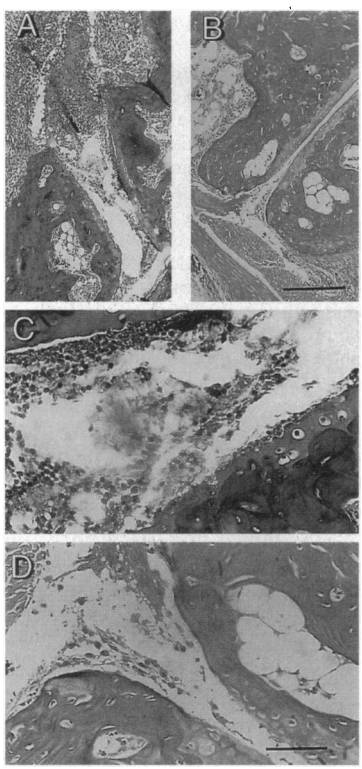

FIG. 4. Histopathology of anti-C5 treatment of established arthritis. Mice were treated with either control or anti-C5 mAb following onset of disease. After 10 days joints were prepared for histologic examination. H&E-stained sections of joints from control mAb-treated animals with established disease (*A* and *C*) revealed proliferative synovia with increased synovial cells and matrix and intense inflammatory cellular infiltrate rich in polymorphonuclear leukocytes filling the joint space. Pannus is noted overlying articular surfaces causing pit-like erosions in the articular surfaces (*A* and *C*). In contrast, sections of joints of anti-C5 mAb-treated animals (*B* and *D*) revealed a more moderate proliferative, fibrotic synovium (pannus) filling aspects of the joint space but essentially devoid of acute inflammatory cells. In addition, the articular surfaces of most anti-C5-treated joints appeared free of erosions (*B* and *D*). (*A* and *B*: ×50, bar = 100 μm; *C* and *D*: ×200, bar = 25 μm.)

and preservation of joint architecture with no significant cartilage or bone erosion (Fig. 4 *B* and *D*).

An analysis of the effects of anti-C5 therapy on two critical parameters of joint histopathology, neutrophil infiltration and cartilage and bone erosion, was performed on PIP joints from the initially involved limbs (Table 2). Erosions were observed in all joints from the controls but in only 4 of 11 (36%) from C5 mAb-treated animals ($P < 0.01$). Moreover, C5 inhibition had a dramatic effect on neutrophil infiltration. Whereas neutrophils were abundant in the majority of evaluable control joints, only 1 of the 11 anti-C5-treated joints had a discernible leukocyte infiltrate ($P < 0.01$).

DISCUSSION

The work presented in this study demonstrates a central role for activated terminal complement proteins in the pathogenesis of CIA, a model of immune-mediated joint inflammation with significant similarities to RA. We have shown that systemic administration of a C5-specific mAb can inhibit complement *in vivo*, prevent the development of CIA, and, when administered following disease onset, significantly ameliorate the course of established disease.

The prevention of disease by administration of anti-C5 mAb, after the induction of cellular and humoral immunity to CII but prior to onset of clinical signs of inflammation, demonstrates that complement activation is required for the initiation of disease. Moreover, disease suppression by C5 blockade is evidence that the activated terminal complement components C5a and C5b-9 are the predominant inflammatory mediators of the complement system in this setting. Interestingly, studies in which CIA susceptible mouse strains were bred to C5-deficient animals have also implicated complement in the pathogenesis of CIA (19–21). However, they have produced some conflicting results, leading some investigators to attribute only a minor role for C5 in the induction of CIA (20), or to conclude that T-cell receptor V_β haplotype plays a larger role in determining susceptibility to CIA (21). All of these experiments were performed in animals derived from F_1 or F_2 backcross matings and were thus characterized by a degree of genetic heterogeneity that made it difficult to distinguish the influence of C5 from that of multiple other linked and unlinked gene products.

Of greatest relevance for the treatment of inflammatory joint disease in humans, the data in this study also show that terminal complement components have an important role in the progression of established CIA. Previous studies on CIA induction have shown that results obtained from examination

Table 2. Anti-C5 mAb therapy of established CIA prevents bone erosion and neutrophil infiltration

Treatment	No. of joints with erosions/no. of PIP joints evaluated	No. of joints with neutrophil infiltration/no. of PIP joints evaluated
Control mAb	7/7 (100)	4/5 (80)*
Anti-C5 mAb	4/11 (36)	1/11 (9)

Mice were treated with either control or anti-C5 mAb for 10 days, following the onset of clinical arthritis. PIP joints from a limb involved at onset were then examined histopathologically for the presence of significant cartilage and bone erosion as well as for infiltration of neutrophils. χ^2 analysis demonstrated statistically significant differences between the anti-C5 mAb- and control mAb-treated mice in the percentage of joints with erosion ($P < 0.01$) and with neutrophil infiltration ($P < 0.01$). Values in parentheses are expressed as percentage.

*In several joints nuclear morphology was ambiguous secondary to the decalcification process. Neutrophil infiltration was evaluated only in joints with clear nuclear morphology.

of the mechanisms leading to the onset of inflammation cannot be extrapolated to the analysis of established inflammatory responses. This point is illustrated by the example of anti-CD4 mAb treatment of CIA, which prevents disease when administered at the time of challenge with type II collagen but is ineffective in altering the course of established arthritis (22, 23). In contrast, inhibition of the complement cascade at C5 was highly effective in preventing disease onset and in ameliorating the course of established arthritis, as evidenced by the prevention of recruitment of additional joints, marked attenuation of leukocyte infiltration, and significant reduction in the incidence of cartilage and bone erosion in affected joints.

These findings clearly implicate terminal complement, most likely C5a, as an important mediator of ongoing leukocyte accumulation within the joint. Moreover, C5a- and C5b-9-induced cellular activation has been shown to promote the release of multiple potent inflammatory molecules capable of mediating joint destruction, including prostaglandins, leukotrienes, reactive oxygen intermediates, proteolytic enzymes, and cytokines including interleukin 1 and tumor necrosis factor (TNF) (6, 12–14). Thus, the antiinflammatory effect of C5 blockade in established arthritis could be due in part to inhibition of the activity of downstream inflammatory mediators. Evidence in support of this concept has come from studies demonstrating synergy between complement and TNF in several models of systemic inflammation (24–27). It is possible that combined complement and cytokine inhibitor therapy of inflammatory disease could produce synergistic therapeutic efficacy.

There are several potential advantages to mAb-mediated complement inhibition *in vivo*. First, consistent with the prolonged serum half-lives of intact mAbs, we have found that significant inhibition of serum hemolytic activity is observed for as long as 1 week following a single i.v. administration of the anti-murine C5 mAb (Y.W. and S.A.R., unpublished data). In addition, primary C5 blockade by mAbs also has the possible advantage, relative to specific inhibition of complement convertases, of preventing the direct cleavage and activation of C5 by oxygen radicals or by tissue-derived enzymes such as kallikrein and plasmin that has been proposed to occur during inflammatory responses (28, 29). Finally, although hemolytic assays with DBA/1LacJ serum have shown that the anti-murine C5 mAb used in this study required a 20-fold molar excess of mAb relative to C5 to inhibit murine complement activation, we have recently derived a highly potent mAb specific for human C5 that blocks complement activation at a mAb:C5 molar ratio as low as 0.5:1 (E. Elliott, S.A.R., and L.A.M., unpublished data). The derivation of a potent anti-human C5-blocking mAb, together with the results presented here, provides persuasive evidence that C5-specific mAb therapy may be an effective approach to the treatment of inflammatory joint diseases.

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