Alternative Complement Pathway Activation Is Essential for Inflammation and Joint Destruction in the Passive Transfer Model of Collagen-Induced Arthritis[1]

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Activation of each complement initiation pathway (classical, alternative, and lectin) can lead to the generation of bioactive fragments with resulting inflammation in target organs. The objective of the current study was to determine the role of specific complement activation pathways in the pathogenesis of experimental anti-type II collagen mAb-passive transfer arthritis. C57BL/6 mice were used that were genetically deficient in either the alternative pathway protein factor B ($Bf^{-/-}$) or in the classical pathway component C4 ($C4^{-/-}$). Clinical disease activity was markedly decreased in $Bf^{-/-}$ compared with wild-type (WT) mice (0.5 ± 0.22 ($n = 6$) in $Bf^{-/-}$ vs 8.83 ± 0.41 ($n = 6$) in WT mice ($p < 0.0001$)). Disease activity scores were not different between $C4^{-/-}$ and WT mice. Analyses of joints showed that C3 deposition, inflammation, pannus, cartilage, and bone damage scores were all significantly less in $Bf^{-/-}$ as compared with WT mice. There were significant decreases in mRNA levels of C3, C4, CR2, CR3, C3aR, and C5aR in the knees of $Bf^{-/-}$ as compared with $C4^{-/-}$ and WT mice with arthritis; mRNA levels for complement regulatory proteins did not differ between the three strains. These results indicate that the alternative pathway is absolutely required for the induction of arthritis following injection of anti-collagen Abs. The mechanisms by which these target organ-specific mAbs bypass the requirements for engagement of the classical pathway remain to be defined but do not appear to involve a lack of alternative pathway regulatory proteins. *The Journal of Immunology,* **2006, 177: 1904–1912.**

The pathogenesis of rheumatoid arthritis (RA)[3] can be divided into three phases: initiation, perpetuation, and chronic inflammation. Innate immune mechanisms may be involved in all three stages (1, 2). The processes that initiate the rheumatoid disease process largely remain unknown, are probably active during a long subclinical phase of the disease, and may include multiple mechanisms. One possible scenario is that RA is initiated by non-Ag-specific mechanisms involving innate immune cells and mediators, with the subsequent development of Ag-specific responses (1, 2). This theory proposes that a variety of nonspecific inflammatory insults to the synovium lead to cytokine-mediated maturation of resident APCs, primarily dendritic cells that migrate to regional lymph nodes. Support for this hypothesis is found in the collagen-induced arthritis (CIA) model where immunization of $Rag1^{-/-}$ DBA/1j mice with type II collagen leads to moderate synovial inflammation in the complete absence of lymphocytes (3).

Autoantibodies such as anti-cyclic citrullinated peptide Abs and rheumatoid factors are present for at least 4–5 years in the serum of many asymptomatic individuals who ultimately develop clinically active RA (4). It is very likely that anti-cyclic citrullinated peptide Abs are pathogenic at some point during the disease course (5). How these autoantibodies develop early in the disease course and whether they are then able to induce joint inflammation and ultimately lead to joint destruction is a matter of ongoing study (6).

Regardless of the specific mechanisms by which RA develops, the innate immune system likely plays important roles at each point in the disease evolution. Cells and cytokines of the innate immune system play important roles in the induction and perpetuation of inflammation and tissue damage in RA. Complement is a key component of the innate immune system (7), and C3 and C5 activation in inflamed joints has been previously shown to characterize active disease in patients with RA (8–10), as well as to be essential to the development of CIA in mice and other similar rodent models (9, 11–18). However, despite these studies, there is relatively little understanding of the specific mechanisms by which C3 and C5 are activated in this disease process and how complement activation fragments themselves intersect with other innate immune mechanisms.

The alternative pathway of complement, which is arguably among the most evolutionarily ancient members of this system (19), has been increasingly shown to play important roles in the initiation of complement-dependent tissue injury (20, 21). This is true in both Ab-mediated diseases such as anti-phospholipid Ab-mediated fetal loss (22), lupus nephritis (23, 24), intestinal ischemia-reperfusion injury (25), and anti-glucose-6-phosphate isomerase (GPI) Ab-mediated arthritis (26), as well as in Ab-independent diseases such as renal ischemia-reperfusion injury (27).

Previous studies of the anti-GPI Ab serum transfer model of RA have shown that the alternative, but not the classical, complement pathway is required for the development of inflammation and joint destruction (26). Subsequently, important roles for neutrophils

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[3] Abbreviations used in this paper: RA, rheumatoid arthritis; CIA, collagen-induced arthritis; GPI, glucose-6-phosphate isomerase; CII, type II collagen; WT, wild type.

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(28), mast cells (29), immune complexes, and vascular permeability (30) have been identified in this passive serum transfer arthritis model. Although it is clear that the alternative pathway is essential for the anti-GPI serum transfer model, the mechanism(s) by which the alternative pathway is activated have not been defined. Possibilities suggested have included the predominant IgG1 subclass of the anti-GPI Abs, a type of Ab that has been described as potentially having alternative pathway activating capacity (31) or a relative lack of complement regulatory proteins on the cartilage surface (26), although this issue has not been formally studied.

In other studies, Hietala et al. (15) assessed the development of CIA in DBA/1j mice in which gene-targeted deficiency states of factor B as compared with C3 were backcrossed onto the DBA/1j strain, as well as evaluated the effects of passive transfer of anti-type II collagen (CII) mAbs in the same backcrossed mice (18). These authors concluded that both the classical and alternative pathways of complement were involved in each model. However, the role of the classical pathway could not be accurately evaluated because C4-deficient mice were not studied.

Herein we have explored the role of the alternative and classical pathways in the anti-CII model of passive transfer arthritis in the C57BL/6 strain wherein $Bf^{-/-}$, $C4^{-/-}$, and wild-type (WT) mice were all of the H-2^b allotype. Using a mixture of four IgG2a/IgG2b anti-CII mAbs, we have found that it is the alternative, but not the classical, complement pathway that is required for the development of inflammation and joint destruction following transfer of anti-CII mAbs. Several mechanisms to account for this phenotype are subsequently explored.

Materials and Methods

Animals

Eight- to 10-wk-old C57BL/6 male mice homozygous for gene deletions of factor B ($Bf^{-/-}$) or C4 ($C4^{-/-}$) were used. Targeted deletion of mouse factor B and mouse C4, respectively, was accomplished as described previously (32, 33). C4-deficient mice backcrossed to the F17 generation onto C57BL/6 and then intercrossed to create a $C4^{-/-}$ strain were used. F7 $Bf^{-/-}$ C57BL/6 homozygous mice were also used. Simultaneously created F7 $Bf^{+/+}$ C57BL/6 homozygous mice and C57BL/6 (The Jackson Laboratory) were used as control strains for $Bf^{-/-}$ and $C4^{-/-}$ mice, respectively.

The absence of factor B and C4 in the respective gene-deficient mice was established by analyzing serum obtained from mice without any induction of arthritis or from anesthetized experimental mice by cardiac puncture on day 10. The blood was allowed to coagulate on ice, and serum was collected by centrifugation at 4°C. The presence or absence of factor B or C4 in the serum was determined using Western blot analysis. Each serum sample was diluted 1/30 in Laemmli sample buffer and subjected to SDS-PAGE. Serum proteins from $Bf^{-/-}$ and $C4^{-/-}$ mice were separated on 8% Tris-glycine and 10% Bis-Tris gels, respectively, followed by electrophoretic transfer onto Immobilon membrane (Millipore). After blocking with 5% dry milk in PBS containing 0.1% Tween 20 for 1 h at room temperature, the membrane was incubated overnight at 4°C with murine anti-mouse factor B mAb (1/1000 dilution of clone 1379 (22)) or with rat anti-mouse C4 mAb (clone 16D2; Cell Sciences), respectively. Following washing, the membranes were incubated with goat anti-mouse peroxidase-conjugated secondary Ab (1/40,000 dilution; Jackson ImmunoResearch Laboratories) or with goat anti-rat peroxidase-conjugated secondary Ab (1/80,000 dilution; Cell Sciences), respectively, for 1 h at room temperature and then were developed using ECL (Supersignal West Pico Chemiluminescent Substrate kit; Pierce). Factor B and C4 were seen consistently in the sera of WT mice and the converse-deficient mice but were absent in all the sera of specifically gene-targeted mice, with or without disease.

All animals were kept in a barrier animal facility with a climate-controlled environment having 12-h light/dark cycles. Filter top cages were used with three mice in each cage. During the course of this study, all experimental mice were fed breeder's chow provided by the Center for Laboratory Animal Care, University of Colorado Health Sciences Center. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Colorado Health Sciences Center.

Passive transfer model of CIA

Arthrogen-CIA is an arthritis-inducing mixture of four murine anti-CII mAbs suspended in sterile Dulbecco's phosphate-buffered saline (Chemicon International). Three of the four anti-collagen mAbs in this mixture are IgG2a (clones F10-21, A2-10, and D8-6) and one is IgG2b (clone D1-2G);

Table I. *Mouse oligonucleotides for quantitative RT-PCR*

mRNA	Primer	Oligonucleotides (5′ to 3′)
TNF-α	FP[a]	GGACAGTGACCTGGACTGTGG
	RP	AGTGAATTCGGAAAGCCCATT
	TaqMan probe	CCTCTCATGCACCACCATCAAGGACTC
IL-1β	FP	TCGCTCAGGGTCACAAGAAA
	RP	CCATCAGAGGCAAGGAGGAA
	TaqMan probe	CATGGCACATTCTGTTCAAAGAGAGCCTG
IFN-γ	FP	AGCTCATCCGAGTGGTCCAC
	RP	AGCAGCGACTCCTTTTCCG
	TaqMan probe	TTGCCGGAATCCAGCCTCAGGA
IL-4	FP	GGCATTTTGAACGAGGTCACA
	RP	AGGACGTTTGGCACATCCA
	TaqMan probe	AGAAGGGACGCCATGCACGGAG
IL-10	FP	TGCAGCAGCTCAGAGGGTT
	RP	TGGCCACAGTTTTCAGGGAT
	TaqMan probe	CCTACTGTCATCCCCCAGCCGCTT
IL-1Ra	FP	TGTTTAGCTCACCCATGGCTT
	RP	TCTTGCAGGGTCTTTTCCCA
	TaqMan probe	AGAGGCAGCCTGCCGCCCTT
MIP-1α	FP	CCAAGTCTTCTCAGCGCCAT
	RP	TCCGGCTGTAGGAGAAGCAG
	TaqMan probe	TGGAGCTGACACCCCGACTGCCT
C3	FP	GATTTTGATGAGTACACCATGACCA
	RP	GCTGCCCTGCCTGCAC
	TaqMan probe	CCAGCAGGTCATCAAGTCAGGCTCAGA
C4	FP	TGCTGAAGATTCTGAGTTTGGC
	RP	CAGCTAGCCGTCTCCTGCA
	TaqMan probe	AACAGGTGGGCAACTCCCCGG
CR3	FP	CTGCATGTCCGGAGGAAATT
	RP	TCGTCTGTGGCAAACACCA
	TaqMan probe	TGGCGCAATGTCACGAGGCTG
C3aR	FP	TCATCGAAACGTGAGAACCG
	RP	AATACGGGCACACACATCACA
	TaqMan probe	TCTGTGGATGTGTCTGGGTGGTAGCCT
C5aR	FP	TGTGGGTGACAGCCTTCGA
	RP	CCGCCAGATTCAGAAACCAG
	TaqMan probe	AGACGGGCCGTCAAACGCCA
fB	FP	GGCAACAGCTGGTACCCTCTT
	RP	CCTTTAGCCAGGGCAGCAC
	TaqMan probe	CGGGACTTCCACATCAACCTCTTCCA
C4Bp	FP	GCTGTATTTGGCAAATGTGGTC
	RP	GTCCGATTCACGTCACTTGCT
	TaqMan probe	CCACCTGCTATACCCAATGCCCTGC
fH	FP	TCACCACCTTCTGGGTATTCCT
	RP	TCCTGACGCATGGGACTTC
	TaqMan probe	CTTCGTTGCACAGCACAAGGGTG
Crry	FP	TCAAAGCCAGTGCCAGTCTG
	RF	CACTGATTGAGCGAGATACACATTT
	TaqMan probe	TGGCAGCTGGAATCCTCTTCTGGC
CD59	FP	CCAGGATTCCTGTCTCTATGCTG
	RP	CTCACCATGACAATCTGATTGTTTC
	TaqMan probe	AGCCGGAATGCAAGTGTATCAAAGGTGTT
MCP	FP	AAGGTGATGATGAAGCATACTGGA
	RP	TTTTTATTTTTGGAGGTGGTAAACAA
	TaqMan probe	TGGCTATCCCCCACATTGTGAAAAGATTT
DAF1	FP	TGCTGCTGTCCCCAACTGT
	RP	CCTGGCATTAGGAATGTCTGG
	TaqMan probe	CGCGGAGACTGCGGCCCA

[a] FP, forward primer; RF, reverse primer. The final concentration of FP, RP, and probes for all the cytokine mRNA was 900, 900, and 200 nM, respectively. All TaqMan fluorogenic probes were HPLC purified oligonucleotides with a 5′-label: fluorescent (reporter dye; FAM-6 carboxy-fluorescein) and a 3′-label: quencher (quencher dye; TAMRA-6 carboxy-tetramethyl-rhodamine). These primers were purchased from Applied Biosystems. IL-1Ra = IL-1 receptor antagonist; fB, factor B; fH, factor H; MCP or CD46, membrane coreceptor protein; and DAF1 or CD55, decay accelerating factor protein.

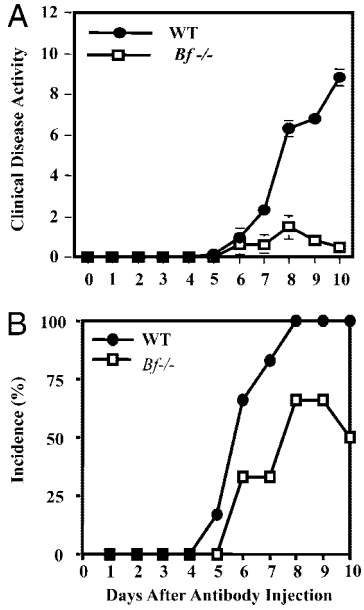

FIGURE 1. Clinical disease activity of anti-CII mAb-induced arthritis in WT and $Bf^{-/-}$ mice. Mice were given a mixture of anti-CII mAbs at day 0. All mice were injected with LPS at day 3. The clinical disease activity was assessed every day using a 3-point score for each paw with four paws per animal (maximum score of 12). *A*, The data are expressed as the clinical disease activity score of WT (●) and $Bf^{-/-}$ (□) mice vs days after the initial injection of the anti-CII mAb mixture. *B*, Overall disease incidence of anti-CII mAb-induced arthritis in WT (●) and $Bf^{-/-}$ (□) mice. These data are represented as mean ± SEM (n = 6 in each group).

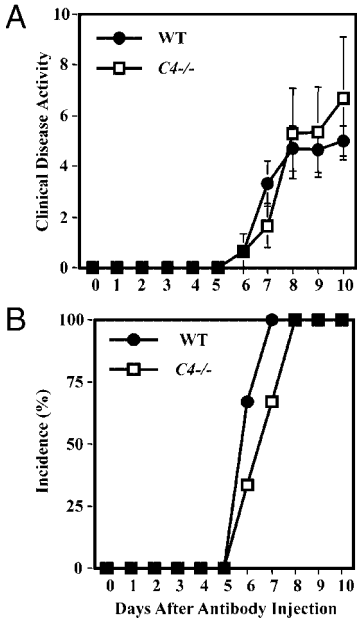

FIGURE 2. *A*, Clinical disease activity of Ab-induced arthritis in WT and $C4^{-/-}$ mice. The data are expressed as the clinical disease activity score of WT (●) and $C4^{-/-}$ (□) mice vs days after the initial injection of the anti-collagen mAb mixture. *B*, Disease incidence of anti-CII mAb-induced arthritis in WT (●) and $C4^{-/-}$ (□) mice. These data are represented as mean ± SEM (n = 3 in each group).

all recognize conserved epitopes clustered within an 83-aa peptide fragment CB11 of CII. These epitopes share common amino acid sequences with many different species of CII, including chicken, mouse, rat, bovine, porcine, monkey, and human. Two groups consisting each of three $Bf^{-/-}$ and three $Bf^{+/+}$ mice were given an i.p. injection at day 0 with 8 mg/mouse Arthrogen-CIA (800 μl/mouse/injection). At day 3, all mice were injected i.p. with 50 μg/mouse bacterial LPS (LPS from *Escherichia coli* strain 0111B4, 50 μl/mouse/injection) to synchronize the time of onset of the arthritis. One group consisting of three $C4^{-/-}$ and three C57BL/6 WT mice were also injected i.p. with a mixture of Arthrogen-CIA, as described above. No toxicity of Arthrogen-CIA mAbs or of LPS was observed during the course of this experiment. Mice started to develop arthritis at day 4 and were sacrificed at day 10. Additional mice studied included four age-matched WT $Bf^{-/-}$ and $C4^{-/-}$ mice, and their respective controls, all not treated with Arthrogen-CIA or with LPS.

Assessment of clinical disease activity

The severity of clinical disease activity was determined every day by two trained laboratory personnel acting independently and blinded to the mouse type (12). Clinical disease activity was scored on a 3-point scale per paw: 0 = normal joint; 1 = slight inflammation and redness; 2 = severe erythema and swelling affecting the entire paw with inhibition of use; and 3 = deformed paw or joint with ankylosis, joint rigidity, and loss of function. The total score for clinical disease activity was based on all 4 paws and was a maximum of 12 for each mouse.

Histopathology of knee joints

At day 10, both forepaws and the entire right hind limb (including the paw, ankle and knee) were removed surgically from all mice and fixed immediately in 10% buffered formalin (Biochemical Sciences). The preparation of tissue samples and histological analysis were performed as described previously (12). All sections were read by a trained observer who was blinded to the mouse types and to the clinical disease activity score of each mouse. The joint sections were scored for inflammation, pannus, cartilage damage, and bone damage, using a scale for each parameter of 0–5. Each histological damage parameter was calculated as the mean value for five joints per mouse, and the overall histological damage score was calculated as the total of the four individual parameters.

Immunohistological assessment of macrophages and neutrophils in the synovium

Formalin-fixed tissue sections of the two wrists, as well as a knee, ankle, and paw were cut at 6 μm from WT, $Bf^{-/-}$, and $C4^{-/-}$ mice with and without disease. One slide was stained for the mouse F4/80 molecule, a 160-kDa glycoprotein expressed by murine macrophages. This Ag is not expressed by lymphocytes or polymorphonuclear neutrophils. Before incubation with primary monoclonal rat anti-mouse F4/80 (Serotech), all sections were treated with proteinase K (DakoCytomation). Sections were

Table II. *Histopathology scores*

	WT and $Bf^{-/-}$ Mice (n = 6 for each)			WT and $C4^{-/-}$ Mice (n = 3 for each)		
Histology Parameters[a]	WT	$Bf^{-/-}$	p value	WT	$C4^{-/-}$	p value
Inflammation	2.36 ± 0.65	0.37 ± 0.02	<0.02	1.20 ± 0.31	2.07 ± 1.05	0.5
Pannus	0.84 ± 0.31	0.03 ± 0.03	<0.03	0.33 ± 0.13	0.67 ± 0.35	0.4
Cartilage damage	1.24 ± 0.37	0.17 ± 0.11	<0.02	0.93 ± 0.13	1.33 ± 0.681	0.6
Bone damage	0.84 ± 0.31	0.03 ± 0.03	<0.03	0.07 ± 0.07	0.53 ± 0.29	0.2
Total	5.28 ± 1.60	0.60 ± 0.41	<0.02	2.53 ± 0.55	4.60 ± 2.36	0.4

[a] Histopathological changes in joints are scored on a scale of 0–5; the data are expressed as mean ± SEM.

Table III. *Macrophages and neutrophils in the synovium of mice with arthritis*[a]

WT and $Bf^{-/-}$ Mice ($n = 6$ for each)				WT and $C4^{-/-}$ Mice ($n = 3$ for each)			
Macrophages		Neutrophils		Macrophages		Neutrophils	
WT	$Bf^{-/-}$	WT	$Bf^{-/-}$	WT	$C4^{-/-}$	WT	$C4^{-/-}$
4.81 ± 1.45	2.17 ± 0.81	5.12 ± 1.85	0.51 ± 0.03[b]	2.68 ± 1.03	9.96 ± 2.95	0.29 ± 0.09	3.89 ± 2.13

[a] Percentages of total cells in the synovium staining for F4/80 (macrophages) and for neutrophils; mean ± SEM. The percentages of macrophage staining in a control mouse without arthritis were: WT (for $Bf^{-/-}$ exp.) 1.82 and $Bf^{-/-}$ 1.13; and WT (for $C4^{-/-}$ exp.) 2.33 vs. $C4^{-/-}$ 4.35.
[b] $p = 0.03$ for neutrophil staining in the synovium of $Bf^{-/-}$ vs. WT mice.

incubated with primary Ab for 30 min at room temperature. The sections were then serially incubated with biotinylated rabbit anti-rat Ig (DakoCytomation), followed by Envision-plus rabbit (DakoCytomation). Staining was developed with liquid DAB-plus (DakoCytomation) and counterstained with hematoxylin. In a similar fashion, another slide was stained for murine neutrophils using a rat anti-mouse neutrophil mAb (anti-Gr1; Serotec), which recognizes a polymorphic 40-kDa Ag expressed by polymorphonuclear neutrophils but is absent on resident tissue macrophages. The secondary Ab and the full developing procedure were as presented above. All slides were read and scored blinded by an experienced observer. To calculate the percentages of macrophages or polymorphonuclear neutrophils within the synovium, slides were viewed under a Zeiss Axioskop 2-plus microscope, and digital images were taken at ×25 with a Zeiss AxioCam HR digital camera using Axiovision version 4.4 software. The area of the synovium was traced in each image, and the percentage of positive cells within the synovium was calculated using an Excel spreadsheet. The formula used to calculate the percentages of positive macrophages or neutrophils in the synovium was as follows: area of positive F4/80-positive cells or of Gr1-positive cells per total measured area of the synovium × 100.

Immunohistochemistry for C3 or factor H deposition

At sacrifice on day 10, the left ankle and paw were excised and fixed in formalin as described previously (12). The presence of C3 in WT, $Bf^{-/-}$, and $C4^{-/-}$ mice with and without disease was immunohistochemically localized with polyclonal goat anti-mouse C3 antisera (ICN Pharmaceuticals). Histology sections were incubated with anti-murine C3 Abs overnight at 4°C. The tissue sections were sequentially incubated with biotinylated rabbit anti-goat Ig (Vector Laboratories) and then additionally treated with Dako LSAB2 Streptavidin-HRP (DakoCytomation). Staining was developed with Liquid DAB$^+$ (DakoCytomation) and counterstained with hematoxylin.

Scoring for C3 staining was performed on the synovium and surrounding tissues combined and separately on the cartilage. The synovium and surrounding tissue were scored using a 3-point scoring system in which 0 represented no staining and 1, 2, and 3 represented low, moderate, and high staining, respectively. The criteria for cartilage staining was as follows: 0, no staining present; 0.5, one area of minimal staining of chondrocytes in one joint; 1, one area of moderate staining of chondrocytes in one joint; 2, multiple areas of moderate staining of chondrocytes with multiple joints affected; and 3, multiple areas of intense staining of chondryocytes and/or diffuse multifocal staining of articular cartilage lesions. For each animal, the synovium and cartilage scores were determined separately for each of the five joints. The sum of synovium and cartilage scores for a single joint could be a maximum of 6; an animal score for 5 joints (mean ± SEM) and a sum total animal score (all 5 joints, with a maximum score of 30) were determined. In addition, the mean values for each of the individual synovium or cartilage scores were calculated separately for 5 joints per animal (maximum of 3 for synovium or cartilage), as well as the sums of all 5 joints in each animal (maximum of 15 for synovium or cartilage).

Similar experiments were conducted to identify factor H in the joints. Slides with these formalin-fixed tissues were first treated with proteinase K (DakoCytomation), then the tissue sections were incubated with a polyclonal goat anti-human factor H Ab (Quidel) for 30 min at room temperature. Staining with the secondary Ab and counterstaining with hematoxylin were conducted as described above. Factor H was only found on the cartilage surface, and the scoring system for its deposition was the same as described above. Joints from a factor H-deficient gene-targeted mouse (provided by Dr. M. Braun, University of Texas, Houston, TX) served as an additional negative control.

mRNA levels for cytokines and complement-related molecules in knee joints assessed by quantitative RT-PCR

Cytokine induction in the knee joints at day 10 was quantified by measuring levels of steady-state mRNA for various cytokines from all WT, $Bf^{-/-}$, and $C4^{-/-}$ mice with and without disease. This approach was also used to measure the joint levels of steady-state mRNA for complement components, receptors, and regulatory molecules. Quantitative RT-PCR was performed by U. Pugazhenthi of the University of Colorado Cancer Center core facility (Denver, CO). Sequences of the mouse oligonucleotides used for quantitative RT-PCR are listed in Table I. Samples omitting reverse transcriptase were included in all analyses as negative controls and to exclude the possible presence of contaminating genomic DNA. Total RNA was extracted by pulverizing the frozen individual knee joints (synovium, bones, and some adjacent muscles) using Tri-reagent (Sigma-Aldrich), following the manufacturer's instructions. The quality of the RNA was assured by agarose gel electrophoresis and use of an internal standard mRNA. Joint mRNA levels were measured for each cytokine/chemokine/complement factor by real-time RT-PCR using appropriate primers and probes. Primers and probes were designed using Primer Express Software, version 1.0 (Applied Biosystems). Reactions and analysis of mRNA was performed on an ABI prism 7700 Sequence Detector System located at the University of Colorado Cancer Center QRT-PCR Core (Applied Biosystems). The mouse macrophage cell line RAW (provided by Dr. S. H. Kim,

Table IV. *Cytokine and TLR mRNA levels in knee joints of mice with arthritis*

	WT and $Bf^{-/-}$ Mice ($n = 6$ for each)			WT and $C4^{-/-}$ Mice ($n = 3$ for each)		
Cytokine[a]	WT	$Bf^{-/-}$	p value	WT	$C4^{-/-}$	p value
TNF-α	8.68 ± 0.97	5.74 ± 1.10	0.08	19.3 ± 7.6	26.8 ± 12.4	0.6
IL-1β	137.8 ± 18.6	64.0 ± 9.9[b]	<0.01	163.5 ± 83.7	258.1 ± 79.1	0.5
IL-1α	28.9 ± 4.6	21.0 ± 2.6	0.2	36.8 ± 7.3	56.8 ± 0.3	0.3
IFN-γ	1,159.5 ± 307.2	1,802.3 ± 442.5	0.2	8,909.6 ± 4,531.1	10,842.9 ± 3,583.3	0.8
MIP-1α	0.29 ± 0.15	0.10 ± 0.04[b]	<0.01	0.18 ± 0.10	0.27 ± 0.06	0.3
IL-1Ra	384.8 ± 29.5	294.6 ± 36.5	0.08	292.8 ± 93.0	463.8 ± 115.5	0.3
IL-4	40.1 ± 9.9	43.1 ± 10.5	0.8	25.0 ± 18.0	7.15 ± 0.74	0.4
IL-10	1,356.7 ± 224.1	1,441.5 ± 478.2[b]	0.9	13,972.9 ± 9,048.2	17,443.5 ± 6,631.7	0.8
TLR4	5,527.2 ± 791.3	9,208.5 ± 1,409.0	<0.05	16,271.3 ± 5,959.2	25,535.6 ± 9,442.4	0.4

[a] mRNA levels are expressed in pg cytokine or TLR4 RNA/ng 18S ribosomal RNA; mean ± SEM.
[b] The values for IL-1β, MIP-1α, and IL-10 were lower in $Bf^{-/-}$ mice without arthritis in comparison to WT (data not shown).

Table V. *Cytokine protein levels in knee joints of mice with arthritis (n = 6 for each group)*[a]

	WT	$Bf^{-/-}$	p Value
TNF-α	277.5 ± 62.5	411.0 ± 75.9	0.2
IL-1β	11.5 ± 0.8	8.59 ± 2.13	0.2
IFN-γ	70.7 ± 8.3	95.6 ± 13.8	0.1
MIP-1α	14.2 ± 0.7	15.2 ± 6.8	0.7

[a] Protein levels are expressed as pg protein/ng total protein extracted; mean ± SEM.

University of Colorado Health Sciences Center, Denver, CO) was stimulated with LPS (10 μg/ml) for 3 h, and cell lysates were used to make a standard curve for TNF-α, IL-1β, IL-1α, IFN-γ, TLR-4, MIP-1α, C3, C4, CR1, CR3, C3aR, C5aR, CD59, MCP, and factor B. This cell line was also stimulated with LPS (10 μg/ml) for 24 h, and cell lysates were used to make a standard curve for IL-1Ra. The mouse T cell line EL4 (provided by Dr. R. Tucker, University of Colorado Health Sciences Center) was stimulated with PHA (10 μg/ml) for 3 h, and cell lysates were used to make a standard curve for IL-4. A leukemia cell line K562 transfected with murine CR2 (provided by Dr. S. Boackle, University of Colorado Health Sciences Center) was used to make a standard curve for complement receptor CR2. RNA extracted from mouse liver was used to make a standard curve for complement component C5, factor H, C4BP, DAF1, and Crry. Age-matched, disease-free WT, $Bf^{-/-}$, and $C4^{-/-}$ mice that had not been treated were also sacrificed to measure baseline knee joint mRNA levels of cytokines, as well as of complement components and receptors. 18S ribosomal RNA was also measured as an additional internal control for analysis of equal amounts of each sample. Quantitative RT-PCR was performed in duplicate for each cytokine from every joint RNA sample. The results were expressed as mRNA of specific cytokine/chemokine (pg)/18S ribosomal RNA (ng).

Cytokine protein levels in knee joints

The levels of cytokines, as measured by ELISA, were also determined in extracts of proteins from the other knee joint not used for RNA extraction. At day 10, knee joints (including some skin and muscle, patella, cartilage, bone, and synovium) from $Bf^{-/-}$ and WT mice were homogenized in 2 ml of RPMI 1640 medium free of serum or antibiotics. After centrifugation to remove tissue fragments, supernatants from these homogenates were removed and stored at −20°C. The pelleted tissue fragments and residual intact cells were lysed for 10 min in 1 ml of radioimmunoprecipitation assay buffer, followed by centrifugation. The suspended material from these lysed cells was removed and stored at −20°C. Cytokines in the homogenate supernatants were measured using commercial ELISA kits: IL-1β, TNF-α, and IFN-γ (BD Biosciences) and MIP-1α (R&D Systems). The total protein levels in the suspended lysates were determined using the Bio-Rad protein assay (Bio-Rad). The results were expressed as pg cytokine/ng total joint protein.

Statistical analyses

All mice were included in the final analysis of arthritis susceptibility, disease severity, histology, immunohistochemistry, and quantitative RT-PCR of cytokine mRNA. Performance of the W statistic indicated that these results were all normally distributed so an unpaired two sample t test was used to analyze these data. Pearson (parametric) correlation coefficient was used to examine the linear relationship between clinical disease activity score and time (days) after the induction of disease. All data were expressed as the mean ± SEM with $p < 0.05$ considered significant.

Results

Passive transfer arthritis in wild type, $Bf^{-/-}$ and $C4^{-/-}$ mice

Passive anti-collagen Ab-induced arthritis was examined in WT, $Bf^{-/-}$, and $C4^{-/-}$ mice over 10 days. The clinical disease activity score was substantially lower in $Bf^{-/-}$ mice in comparison to control WT mice with significant differences ($p < 0.001$) at days 7, 8, 9, and 10 (Fig. 1*A*). The date of disease onset was delayed, and the incidence was also lower in $Bf^{-/-}$ mice in comparison to control mice, although 50% of the $Bf^{-/-}$ mice eventually developed very mild clinical disease (Fig. 1*B*). In contrast, different results were obtained with $C4^{-/-}$ mice where the clinical disease activity and incidence of disease were no different in comparison to WT animals studied in the same experiment (Fig. 2). These results are identical to those reported when transferring anti-GPI serum into $Bf^{-/-}$ and $C4^{-/-}$ mice (26).

Histological analyses on the synovium of joints obtained at day 10 showed the same overall results. The scores for inflammation, pannus, cartilage damage, bone damage, and total animal were all

Table VI. *mRNA levels of complement components and receptors in knee joints of mice with arthritis*[a]

	WT and $Bf^{-/-}$ (n = 6 for each)[b]			WT and $C4^{-/-}$ (n = 3 for each)		
	WT	$Bf^{-/-}$	p value	WT	$C4^{-/-}$	p value
C3	10,080.5 ± 1,739.22	4,928.7 ± 1,074.7	<0.02	6,004.6 ± 987.0	14,343.4 ± 2,949.5	0.1
C4	8,042.8 ± 609.8	5,986.1 ± 502.3	<0.03	8,228.5 ± 2,507.0	4284.5 ± 2,464.2	0.3
C5	0.61 ± 0.10	0.37 ± 0.05	0.1	0.62 ± 0.17	1.14 ± 0.21	0.1
CR1	555.9 ± 28.4	491.4 ± 27.4	0.1	704.7 ± 185.0	1,138.7 ± 103.9	0.1
CR2	1.21 ± 0.11	0.66 ± 0.13	<0.01	0.64 ± 0.18	0.11 ± 0.10	0.1
CR3	188.4 ± 31.4	76.2 ± 16.7	<0.01	103.1 ± 37.2	287.6 ± 84.7	0.1
C3aR	335.0 ± 43.6	172.6 ± 23.9	<0.01	294.8 ± 81.5	415.6 ± 70.2	0.3
C5aR	232.4 ± 33.3	128.8 ± 19.6	<0.02	207.8 ± 88.9	318.7 ± 106.2	0.5
Factor B	10,279.6 ± 1,132.3	2,677.8 ± 416.5	<0.0001	7,591.0 ± 1,426.2	10,834.0 ± 1,509.8	0.1

[a] Complement component and receptor mRNA levels are expressed in mRNA (pg)/ rRNA (ng); mean ± SEM.
[b] In control $Bf^{-/-}$ mice without arthritis, the value for C5 was significantly higher compared with WT mice.

Table VII. *Localization and intensity of C3 immunohistochemical staining*[a]

	WT and $Bf^{-/-}$ Mice (n = 6 for each)[b]			WT and $C4^{-/-}$ Mice (n = 3 for each)		
Tissue	WT	$Bf^{-/-}$	p value	WT	$C4^{-/-}$	p value
Synovium	4.75 ± 1.34	1.33 ± 0.56	<0.04	2.68 ± 0.88	10.7 ± 3.84[c]	<0.05
Cartilage	3.58 ± 0.90	1.25 ± 0.04	<0.04	3.00 ± 2.00	5.33 ± 2.67	0.5
Total score	8.33 ± 2.20	2.58 ± 0.83	<0.03	5.67 ± 2.73	16.0 ± 6.5	0.2

[a] C3 deposition data from front paw 1, front paw 2, rear paw, rear ankle, and knee (synovium and cartilage). The data are expressed as mean ± SEM.
[b] Some nonspecific staining was present equally both in muscle, connective tissue, and skin of WT and $Bf^{-/-}$ mice.
[c] In $C4^{-/-}$ mice without induced arthritis, high baseline levels of C3 deposition were observed in the synovium compared with cartilage (data not shown).

significantly lower in $Bf^{-/-}$ *mice* in comparison to WT mice (Table II). In contrast, joint damage was modestly increased in the $C4^{-/-}$ in comparison to the WT mice, although the values were not statistically different.

The percentages of macrophages and polymorphonuclear neutrophils also were assessed in the synovia of mice with arthritis. Neutrophils were 90% reduced ($p = 0.03$) in the joints of $Bf^{-/-}$ mice with arthritis, whereas macrophages were only modestly reduced ($p > 0.05$) (Table III). In contrast, increases in the numbers of macrophages and neutrophils were observed in $C4^{-/-}$ mice in comparison to WT controls, although the differences were not statistically significant.

Synovial cytokines

Since cytokines are important mediators of inflammatory arthritis, mRNA for a panel of cytokines was analyzed in the total knee joints of WT, $Bf^{-/-}$, and $C4^{-/-}$ mice. Significant decreases in the steady-state levels of mRNA for IL-1β and MIP-1α (both $p < 0.01$) were observed in $Bf^{-/-}$ in comparison to WT mice, all with arthritis (Table IV). However, cytokine protein levels determined by ELISA on joint extracts showed no significant differences for IL-1β or MIP-1α in $Bf^{-/-}$ in comparison to WT mice with arthritis (Table V). Cytokine mRNA levels were not significantly different between $C4^{-/-}$ mice and their WT controls (Table IV). Cytokine protein studies in knee joints were not performed on these mice. These results suggest that the differences in disease between WT, $Bf^{-/-}$, and $C4^{-/-}$ mice are not due to substantial variations in cytokine levels.

mRNA for complement proteins and receptors

The levels of various complement proteins and their receptors also may influence the severity of inflammatory arthritis. Steady-state levels of mRNA for relevant complement proteins or complement receptors were measured in the knee joints of WT, $Bf^{-/-}$, and $C4^{-/-}$ mice using quantitative RT-PCR. The knee joints of $Bf^{-/-}$ mice with arthritis demonstrated significantly lower levels of mRNA for C3, C4, CR2, CR3, C3aR, C5aR, and factor B in comparison to WT control mice (Table VI). In control $Bf^{-/-}$ mice without arthritis, the level of C5 was significantly higher than seen with WT mice, in contrast to all other complement components and receptors which showed no differences (data not shown). Similar studies on WT and $C4^{-/-}$ mice with arthritis demonstrated no significant differences in steady-state levels of mRNA for the complement components and receptors, following induction of arthritis (Table VI). It should be noted that the small amounts of mRNA for factor B or C4 present in the respective knockout mice are not functional as Western blots failed to reveal any detectable protein (data not shown).

C3 immunohistochemical staining

To examine for the tissue expression and localization of C3 protein, staining for C3 was performed on formalin-fixed joints of WT, $Bf^{-/-}$, and $C4^{-/-}$ mice. The $Bf^{-/-}$ mice exhibited significantly decreased staining for C3 both in the synovium and on the articular cartilage in comparison to WT mice (Table VII). In WT mice, C3 deposition was present in the synovium, surrounding connective tissue and at the edges of the damaged articular cartilage, while little C3 deposition was observed in these locations in the $Bf^{-/-}$ mice (Fig. 3). Minimal background staining for C3 was present in $Bf^{-/-}$ and WT mice without arthritis. In the $C4^{-/-}$ mice with arthritis, significantly high levels of C3 staining were observed in the synovium in comparison to WT mice; no significant differences between deficient and WT mice were seen in the strong intensity of C3 staining in the cartilage (Fig. 3 and Table VII).

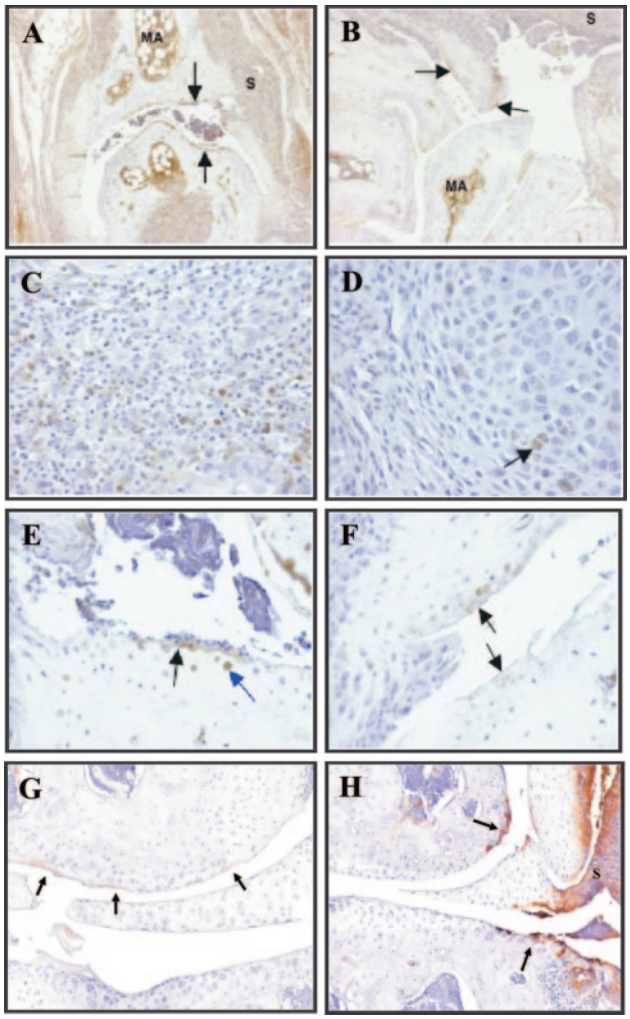

FIGURE 3. Increased C3 immunohistochemical staining in the forepaws of a WT mouse compared with a $Bf^{-/-}$ mouse with arthritis. *A*, WT mouse digit joint has marked synovitis and severe cartilage damage. Bone marrow (MA) is intensely stained and less intense, and diffuse staining is evident in the synovium (S) and surrounding connective tissue. Damaged articular cartilage edges have intense staining for C3 (black arrows). *B*, $Bf^{-/-}$ mouse wrist has markedly reduced inflammation with similar staining of bone marrow, cartilage edges/chondrocytes (black arrows), and minimal C3 staining of mildly inflamed synovium. *C*, WT mouse synovium shows mixed infiltrates with many neutrophils. C3 staining is evident in cytoplasm, nuclei, and within the extracellular material associated with exudate. *D*, $Bf^{-/-}$ mouse synovium shows little C3 staining of periarticular connective tissue (black arrow) and inflammatory cells. *E*, WT mouse with severely damaged cartilage shows C3 staining on edge of collagen and exudate (black arrow) and chondrocytes (blue arrow). *F*, $Bf^{-/-}$ mouse cartilage shows minimal C3 deposition in matrix and chondrocytes (black arrows) in the absence of exudate. *G*, WT mouse with arthritis showing staining of C3 on the articular cartilage and mild cartilage damage (black arrows). *H*, $C4^{-/-}$ mouse with arthritis showing intense C3 staining of the articular cartilage in association with cartilage damage (black arrow) and in the synovium (S) associated with severe inflammatory synovitis. The joint sections are from WT, $Bf^{-/-}$, and $C4^{-/-}$ mice, which demonstrated maximum clinical disease activity scores. Original magnification: *A* and *B*, $\times$100; *D–F*, $\times$400; and *G* and *H*, $\times$200.

mRNA for complement regulatory proteins

Some of the differences between WT, $Bf^{-/-}$, and $C4^{-/-}$ mice in the development of clinical and histological arthritis could have been due to variations in the expression of complement regulatory proteins in the joints. To examine for this possibility, steady-state

Table VIII. *Complement regulatory protein mRNA levels in knee joints of mice with arthritis*[a]

	WT for $Bf^{-/-}$	$Bf^{-/-}$	WT for $C4^{-/-}$	$C4^{-/-}$
C4BP	35.3 ± 11.8	59.6 ± 31.0	31.9 ± 21.9	125.5 ± 39.8
Crry	863.5 ± 76.1	861.2 ± 75.5	1,246.6 ± 224.1	2,488.3 ± 210.1[b]
CD59	3,870.5 ± 603.3	5,190.7 ± 579.2	2,369.8 ± 397.7	5,535.3 ± 1718.8
DAF1	2,939.0 ± 202.3	3,080.0 ± 289.2	3,263.2 ± 717.7	5,803.7 ± 1674.7
MCP	5,781.7 ± 999.0	6,277.0 ± 1726.0	2,625.8 ± 439.8	10,311.2 ± 5086.5
fH	318.8 ± 12.5	311.4 ± 45.6	408.7 ± 143.8	629.5 ± 73.7

[a] Complement component and receptor mRNA levels are expressed in mRNA (pg)/rRNA (ng). The data represent the mean ± SEM for $n = 6$ in the WT and $Bf^{-/-}$ mice and $n = 3$ for the WT and $C4^{-/-}$ mice.
[b] $p < 0.02$ comparing $C4^{-/-}$ with WT.

levels of mRNA for complement regulatory proteins were determined in the joint knees of mice with arthritis using quantitative RT-PCR. The levels of C4BP, Crry, CD59, DAF1, MCP, and factor H were all comparable between the different groups of mice except for Crry, which was significantly increased in the joints of $C4^{-/-}$ mice in comparison to WT mice ($p < 0.02$) (Table VIII). All of the other regulatory proteins showed nonsignificant increases in the $C4^{-/-}$ mice compared with the WT. Importantly, no significant differences in mRNA levels of complement regulatory proteins were observed between the $Bf^{-/-}$ mice and their WT controls.

Factor H immunohistochemical staining

The possibility existed that the differences in severity of arthritis observed in the $Bf^{-/-}$ and $C4^{-/-}$ mice could have been due in large part to altered levels of factor H on the cartilage surface where immune complexes are known to form in anti-CII mAb-passive transfer arthritis. Therefore, immunohistological staining for factor H on the surface of articular cartilage was conducted in WT, $Bf^{-/-}$, and $C4^{-/-}$ mice. Significantly decreased cartilage staining for factor H was observed with $Bf^{-/-}$ mice in comparison to WT mice ($p < 0.01$), whereas factor H staining was not different between $C4^{-/-}$ mice and their WT controls (Fig. 4 and Table IX).

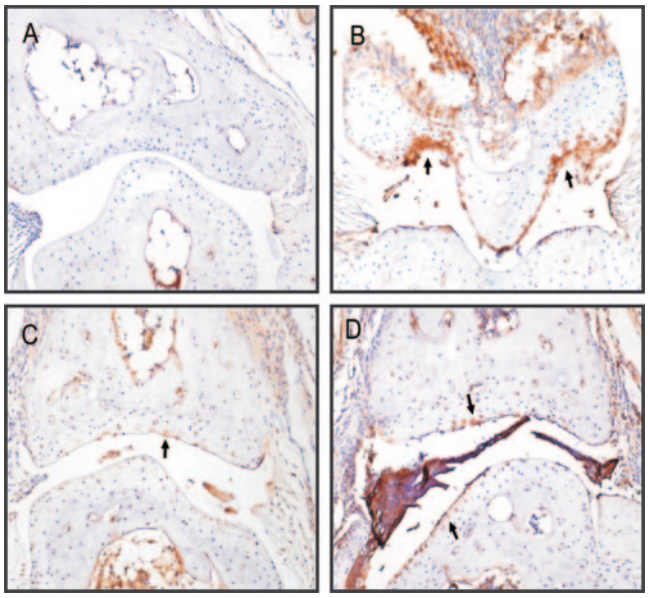

FIGURE 4. Representative immunohistochemical staining for factor H in informative strains of mice with and without arthritis. *A*, WT mouse with no arthritis showing no staining for factor H in the articular cartilage. *B*, WT mouse with arthritis demonstrating increased factor H staining localized to the chondrocytes in the articular cartilage (arrows). *C*, $Bf^{-/-}$ mouse with arthritis demonstrating decreased staining of factor H as compared with the WT control mouse. *D*, $C4^{-/-}$ mouse demonstrating intense staining for factor H in both the chondrocytes in the articular cartilage (arrows) and inflammation in the joint space. The joint sections are from WT, $Bf^{-/-}$, and $C4^{-/-}$ mice, which demonstrated maximum clinical disease activity scores. Staining for factor H in a control factor H-deficient mouse appeared essentially identical to WT mouse without arthritis (data not shown). Original magnification: *A–D*, ×20.

Discussion

Herein we have demonstrated that factor B and the alternative complement pathway play an essential role in the development of joint inflammation and damage in the anti-CII mAb-passive transfer arthritis model. These results are essentially identical to those found in the anti-GPI Ab serum transfer model where, using the same gene-targeted mouse $Bf^{-/-}$ and $C4^{-/-}$ strains, the alternative pathway but not the classical pathway was found to be essential (26). Thus, rather than a phenotype that is unique to the special features of the anti-GPI model, a requirement for alternative pathway activation to develop disease is the common thread between both informative models of immune complex-induced inflammatory arthritis. In both models, substantially less C3 is deposited on the cartilage and in the synovium in the absence of factor B, demonstrating the expected effect of complement inhibition upstream of this major component.

These results are in apparent contradiction to those of Hietala et al. (18), who concluded that both the classical and alternative pathways are involved in the anti-CII mAb-passive transfer model. However, it is important to point out that the relative role of the classical pathway was not evaluated directly in that series of experiments by using mice deficient in C4, but rather was inferred by comparing disease in C3- vs factor B-deficient mice. An identical result was obtained by this group using the active CII immunization model of CIA (15). However, because C3 is activated by both the classical and alternative pathways, decreased disease in C3-deficient mice does not prove involvement of one pathway over the other. Furthermore, in both studies the authors were unable to exclude a confounding role for *H-2* genes. This is because the $Bf^{-/-}$ and $C3^{-/-}$ strains used differed; the gene-targeted *Bf* allele (in the class III region of H-2) came from the H-2^b allotype, whereas the DBA/1j strain and backcrossed $C3^{-/-}$ DBA/1j mice exhibited the H-2^q allotype. The possibility exists that a gene in H-2^b may modify the inflammatory response in a manner unrelated to complement. An additional reason for this difference in results may be that the studies of Hietala et al. (18) were performed on a DBA1/j background while ours were on a C57BL/6 background.

Increased steady-state mRNA levels of complement activation components and receptors accompanied inflammation in WT in comparison to $Bf^{-/-}$ mice (Table VI). It is not known whether

Table IX. *Intensity of immunohistochemical staining for factor H on the surface of the articular cartilage in WT,* $Bf^{-/-}$, *and* $C4^{-/-}$ *mice with arthritis*

WT and $Bf^{-/-}$ Mice[a]			WT and $C4^{-/-}$ Mice[b]		
WT	$Bf^{-/-}$	*p* value	$C4^{-/-}$	WT	*p* value
1.68 ± 0.43	0.21 ± 0.16	<0.01	0.73 ± 0.30	1.68 ± 0.65	0.2

[a] The data represent the mean ± SEM of staining in five joints each in six separate animals. There was no staining observed in control WT or $Bf^{-/-}$ mice without arthritis.

[b] The data represent the mean ± SEM of staining in five joints each in three separate animals. There was no staining in a WT control mouse without arthritis, but a $C4^{-/-}$ mouse without arthritis demonstrated factor H staining on the cartilage at an average level of 0.80.

these increases are due to synthesis by synoviocytes or by infiltrating inflammatory cells, but the lack of factor B appears to interrupt a cascade of increased complement synthesis in the joint. We have not attempted to measure local complement component production because it would of greater interest to determine what cells in the inflamed joint were responsible. However, the absence of alternative complement pathway activation appears not to affect cytokine protein levels in the joint. Decreased steady-state levels of cytokine mRNAs for IL-1β and MIP-1α were observed in the knee joints of $Bf^{-/-}$ mice, but cytokine protein levels were found to be equivalent in anti-CII mAb-treated $Bf^{+/+}$ and $Bf^{-/-}$ mice. These results reinforce that cytokine mRNA levels may not be reflected in comparable levels of proteins due to posttranscriptional effects. Based on these results, we conclude that the lack of active arthritis secondary to alternative pathway inhibition is independent of major effects on the levels of proinflammatory and anti-inflammatory cytokines in the joint.

The $C4^{-/-}$ mice demonstrated greater histological changes and a higher content of neutrophils and macrophages in the synovium than did the WT mice, although these differences were not statistically different. The clinical disease activity score was not different between the $C4^{-/-}$ and WT mice. The apparently increased synovial inflammatory changes in the $C4^{-/-}$ mice may have been secondary to increased levels of inflammatory mediators, as the serum C3 levels were higher in these mice in comparison to WT (data not shown). The mechanisms whereby $C4^{-/-}$ mice may have more active disease will be explored in future studies. However, we conclude that the presence of robust disease in the $C4^{-/-}$ mice certainly indicates that activation of the classical complement pathway is not necessary to induce disease in the passive Ab model of CIA.

We have approached the question of how the alternative complement pathway is activated in the absence of engagement through the classical pathway-initiated amplification loop, i.e., in the absence of C4. Steady-state levels of mRNA for complement regulatory proteins were not different in the knee joints of WT, $Bf^{-/-}$, or $C4^{-/-}$ mice. Factor H is an important regulatory protein of the alternative pathway of complement that inhibits C3 convertase activities in the solution phase and on cell surfaces to which it can bind through either charge interactions or to covalently attached C3b/C3d (34). Factor H levels were found at the expected high levels on the cartilage and in the synovium in WT mice with arthritis. However, factor H was present at lower levels in $Bf^{-/-}$ mice, presumably because the lack of complement activation in these mice provides less fixed C3 to which factor H would bind (35). These results are consistent with the known role for factor H in binding to C3-targeted sites and do not support the possibilities that increased factor H deposited on the cartilage of $Bf^{-/-}$ mice could have contributed to the inhibition of arthritis or that excluded and/or decreased factor H binding to the cartilage of WT and $C4^{-/-}$ mice contributed to the development of active arthritis. Other complement inhibitors (Crry, DAF, MCP, and CD59) were also found at readily detectable levels in the joints and that were equivalent in all strains tested, which is consistent with previous reports (9).

The mechanism of activation of the alternative pathway in this model, like the anti-GPI Ab serum transfer model, remains unexplained to date. Possibilities to study in the future include: 1) changes in pH, as the alternative pathway is activated at low pH (36); 2) a relative imbalance of activators as compared with inhibitors; 3) the potential for specific features of cartilage as a tissue that might allow alternative pathway activation in the presence of immune complexes; and 4) the possibility that in mice the lectin pathway does not require C4 and might directly trigger C3 following the binding of mannose-binding lectin to carbohydrates on anti-GPI or anti-CII Abs (37, 38).

Whatever the mechanism of activation of the alternative pathway, it is likely that many murine models of inflammatory arthritis are mediated by C3a-C5a and do not necessarily involve the terminal complement components. Although the prevention or amelioration of CIA in C5-deficient mice or after treatment with anti-C5 mAbs does not clarify this issue, the absence of arthritis in the K/B × N disease model in mice deficient in C5aR suggests an important role for C5a (26).

Finally, these data further support the hypothesis that inhibition of complement activation might be a relevant therapeutic approach in human diseases (20). Although inhibition of complement serum proteins found at substantial levels is challenging with regard to pharmacokinetics and pharmacodynamics of inhibitors, approaches that block specific complement receptors such as the C5aR (17) or that target complement inhibitors to sites of complement activation (39, 40) continue to hold substantial promise.

Disclosures

The authors have no financial conflict of interest.

References

1. Arend, W. P. 2001. The innate immune system in rheumatoid arthritis. *Arthritis Rheum.* 44: 2224–2234.
2. Firestein, G. S. 2003. Evolving concepts of rheumatoid arthritis. *Nature* 423: 356–361.
3. Plows, D., G. Kontogeorgos, and G. Kollias. 1999. Mice lacking mature T and B lymphocytes develop arthritic lesions after immunization with type II collagen. *J. Immunol.* 162: 1018–1023.
4. Rantapaa-Dahlqvist, S. B. A. de Jong, E. Berglin, G. Hallmans, G. Wadell, H. Stenlund, U. Sundin, and W. J. van Venrooij. 2003. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. *Arthritis Rheum.* 48: 2741–2749.
5. van Venrooij, W. J., and G. J. M. Pruijn. 2000. Citrullination: a small change for a protein with great consequences for rheumatoid arthritis. *Arthritis Res.* 2: 249–251.
6. van Gaalen, F. A., A. Ioan-Facsinay, T. W. Huizenga, and R. E. M. Toes. 2005. The devil in the details: the emerging role of anticitrulline autoimmunity in rheumatoid arthritis. *J. Immunol.* 175: 5575–5580.
7. Fearon, D. T., and R. M. Locksley. 1996. The instructive role of innate immunity in the acquired immune response. *Science* 272: 50–53.
8. Holers, V. M. 2001. Complement. In *Principles and Practices of Clinical Immunology.* R. Rich, ed. Mosby, St. Louis, pp. 21.1–21.8.

9. Linton, S. M., and B. P. Morgan. 1999. Complement activation and inhibition in experimental models of arthritis. *Mol. Immunol.* 36: 905–914.
10. Neumann, E., S. R. Barnum, I. H. Tarner, J. Echols, M. Fleck, M. Judex, F. Kullmann, J. D. Mountz, J. Scholmerich, S. Gay, and U. Muller-Ladner. 2002. Local production of complement proteins in rheumatoid arthritis synovium. *Arthritis Rheum.* 46: 934–945.
11. Banda, N. K., D. Kraus, A. Vondracek, L. H. Huynh, A. Bendele, V. M. Holers, and W. P. Arend. 2002. Mechanisms of effects of complement inhibition in murine collagen-induced arthritis. *Arthritis Rheum.* 46: 3065–3075.
12. Banda, N. K., D. M. Kraus, M. Muggli, A. Bendele, V. M. Holers, and W. P. Arend. 2003. Prevention of collagen-induced arthritis in mice transgenic for the complement inhibitor complement receptor 1-related gene/protein y. *J. Immunol.* 171: 2109–2115.
13. Dreja, H., Annenkov.A., and Y. Chernajovsky. 2000. Soluble complement receptor 1 (CD35) delivered by retrovirally infected syngeneic cells or by naked DNA injection prevents the progression of collagen-induced arthritis. *Arthritis Rheum.* 43: 1698–1709.
14. Goodfellow, R. M., A. S. Williams, J. L. Levin, and B. P. Morgan. 2000. Soluble complement receptor one (sCR1) inhibits the development and progression of rat collagen-induced arthritis. *Clin. Exp. Immunol.* 119: 210–216.
15. Hietala, M. A., I.-M. Jonsson, A. Tarkowski, S. Kleinau, and M. Pekna. 2002. Complement deficiency ameliorates collagen-induced arthritis in mice. *J. Immunol.* 169: 454–459.
16. Wang, Y., S. A. Rollins, J. A. Madri, and L. A. Matis. 1995. Anti-C5 monoclonal antibody therapy prevents collagen-induced arthritis and ameliorates established disease. *Proc. Natl. Acad. Aci. USA* 92: 8955–8959.
17. Woodruff, T. M., A. J. Strachan, N. Dryburgh, I. A. Shield, R. C. Reid, D. P. Fairlie, and S. M. Taylor. 2002. Antiarthritic activity of an orally active C5a receptor antagonist against antigen-induced monarticular arthritis in the rat. *Arthritis Rheum.* 46: 2476–2485.
18. Hietala, M. A., K. S. Nandakumar, L. Persson, S. Fahlen, R. Holmdahl, and M. Pekna. 2004. Complement activation by both classical and alternative pathways is critical for the effector phase of arthritis. *Eur. J. Immunol.* 34: 1208–1216.
19. Sunyer, J. O., and J. D. Lambris. 1998. Evolution and diversity of the complement system of poikilothermic vertebrates. *Immunol. Rev.* 166: 39–57.
20. Holers, V. M., and J. M. Thurman. 2004. The alternative pathway of complement in disease: opportunities for therapeutic targeting. *Mol. Immunol.* 41: 147–152.
21. Thurman, J. M., and V. M. Holers. 2006. The central role of the alternative complement pathway in human disease. *J. Immunol.* 176: 524–530.
22. Thurman, J. M., D. M. Kraus, G. Girardi, D. E. Hourcade, H.-J. Kang, P. A. Royer, L. M. Mitchell, P. C. Giclas, J. E. Salmon, G. S. Gilkerson, and V. M. Holers. 2004. A novel inhibitor of the alternative complement pathway prevents antiphospholipid antibody-induced pregnancy loss in mice. *Clin. Immunol.* 42: 87–97.
23. Watanabe, H., G. Garnier, A. Circolo, R. A. Wetsel, P. Ruiz, V. M. Holers, S. A. Boackle, H. R. Colten, and G. S. Gilkeson. 2000. Modulation of renal disease in MRL/*lpr* mice genetically deficient in the alternative complement pathway factor B. *J. Immunol.* 164: 786–794.
24. Elliott, M. K., T. Jarmi, P. Ruiz, Y. Xu, V. M. Holers, and G. S. Gilkeson. 2004. Effects of complement factor D deficiency on the renal disease of MRL/*lpr* mice. *Kidney Int.* 65: 129–138.
25. Stahl, G. L., Y. Xu, L. Hao, M. Miller, J. A. Buras, M. Fung, and H. Zhao. 2003. Role for the alternative complement pathway in ischemia/reperfusion injury. *Am. J. Pathol.* 162: 449–455.
26. Ji, H., J. Ohmura, U. Mahmood, D. M. Lee, F. M. Hofhuis, S. A. Boackle, K. Takahashi, V. M. Holers, M. Walport, C. Gerard, et al. 2002. Arthritis critically dependent on innate immune system players. *Immunity* 16: 157–168.
27. Thurman, J. M., C. Ljubanovic, C. L. Edelstein, G. S. Gilkeson, and V. M. Holers. 2003. Lack of a functional alternative pathway ameliorates ischemic acute renal failure in mice. *J. Immunol.* 170: 1517–1523.
28. Wipke, B. T., and P. M. Allen. 2001. Essential role of neutrophils in the initiation and progression of a murine model of rheumatoid arthritis. *J. Immunol.* 167: 1601–1608.
29. Lee, D. M., D. S. Friend, M. F. Gurish, C. Benoist, D. Mathis, and M. B. Brenner. 2002. Mast cells: a cellular link between autoantibodies and inflammatory arthritis. *Science* 197: 1689–1692.
30. Wipke, B. T., Z. Wang, W. Nagengast, D. E. Reichart, and P. M. Allen. 2004. Staging the initiation of autoantibody-induced arthritis: a critical role for immune complexes. *J. Immunol.* 172: 7694–7702.
31. Ratnoff, W. D., D. T. Fearon, and K. F. Austen. 1983. The role of antibody in the activation of the alternative complement pathway. *Springer Sem. Immunopath.* 6: 361–371.
32. Matsumoto, M., Fukuda, A. Circolo, Goellner, J. Strauss-Schoenberger, X. Wang, S. Fujita, T. Hidvegi, D. D. Chaplin, and H. R. Colten. 1997. Abrogation of the alternative complement pathway by targeted deletion of murine factor B. *Proc. Natl. Acad. Sci. USA* 94: 8720–8725.
33. Wessels, M. R., P. Butko, M. Ma, H. Warren, A. Lage, and M. C. Carroll. 1995. Studies of group B streptococcal infection in mice deficient in complement C3 or C4 demonstrate an essential role for complement in both innate and acquired immunity. *Proc. Natl. Acad. Sci. USA* 92: 11490–11494.
34. Friese, M. A., T. Manuelian, S. Junnikkala, J. Hellwage, S. Meri, H. H. Peter, D. L. Gordon, H. Eibel, and P. F. Zipfel. 2003. Release of endogenous anti-inflammatory complement regulators FHL-1 and factor H protects synovial fibroblasts during rheumatoid arthritis. *Clin. Exp. Immunol.* 132: 485–495.
35. Hellwage, J., T. S. Jokiranta, M. A. Friese, T. U. Wolk, E. Kampen, and S. Meri. 2002. Complement C3b/C3d and cell surface polyanions are recognized by overlapping binding sites. *J. Immunol.* 169: 6935–6944.
36. Lardner, A. 2004. The effects of extracellular pH on immune function. *J. Leukocyte Biol.* 69: 522–530.
37. Presanis, J. S., M. Kojima, and R. B. Sim. 2003. Biochemistry and genetics of mannan-binding lectin (MBL). *Biochem. Soc. Trans.* 31: 748–752.
38. Malhotra, R., M. R. Wormald, P. M. Rudd, P. B. Fischer, R. A. Dwek, and R. B. Sim. 1995. Glycosylation changes of IgG associated with rheumatoid arthritis can activate complement via the mannose-binding protein. *Nat. Med.* 1: 237–243.
39. Song, H., C. He, C. Knaak, J. M. Guthridge, V. M. Holers, and S. Tomlinson. 2003. Complement receptor 2-mediated targeting of complement inhibitors to sites of complement activation. *J. Clin. Invest.* 111: 1875–1885.
40. Atkinson, C., H. Song, B. Lu, F. Qiao, T. A. Burns, V. M. Holers, G. C. Tsokos, and S. Tomlinson. 2005. Targeted complement inhibition by C3d recognition ameliorates tissue injury without apparent increase in susceptibility to infection. *J. Clin. Invest.* 115: 2444–2453.