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Regulation of Early Complement Components C3 and C4 in the Synovium

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To determine the cytokine inducibility of early complement component (C3 and C4) expression in the synovium, explant tissue was maintained in culture for 7 days. C3 and C4 production was measured by specific enzyme-linked immunosorbent assay, and RNA was evaluated by semiquantitative PCR. The effects of leukemia inhibitory factor (LIF), gamma interferon (IFN-γ), IFN-α, and estrogen on C3 and C4 expression were evaluated. C3 levels were unaffected by 7 days of LIF, IFN-γ, or IFN-α treatment. In contrast, C4 levels were significantly induced in synovial samples treated for 7 days with either IFN-γ or IFN-α. LIF had no effect on C4 levels in this system. Estrogen was found to down-modulate the induction of expression due to IFN-γ. These data provide evidence for cytokine regulation of C4 expression in the synovium and for estrogen modulation of those effects.

The complement system plays an important role in host defense and immunologically mediated inflammation (43). Activation of the complement cascade produces biologically active peptides capable of increasing vascular permeability, stimulating chemotaxis, enhancing phagocytosis, and directly inducing cellular injury. While most serum-derived complement is produced by the liver, many other cells are capable of producing complement components in local inflammatory reactions (15). The synovium is one of the tissues capable of producing multiple complement components (33, 35). The mechanisms by which complement production is regulated in the synovium have not been previously investigated.

In systemic lupus erythematosus (SLE), it is believed that deposition of immune complexes in end organs with subsequent complement activation mediates most of the pathology (27). Synovial pathology in SLE is typically characterized by increases in synovial lining cells and a leukocytic infiltration and is believed to be mediated by complement and polymorphonuclear leukocytes (26). Nevertheless, it is not clear what role complement plays in the pathogenesis of synovitis in SLE. Interestingly, two biologic agents capable of markedly inducing complement gene expression have been implicated in drug-induced SLE. Both alpha interferon (IFN-α) and IFN-γ have been associated with drug-induced SLE (8, 12, 21, 34).

Cytokines that are believed to play a role in inflammatory synovitis include tumor necrosis factor alpha, interleukin-1, interleukin-6, leukemia inhibitory factor (LIF), granulocyte-macrophage colony-stimulating factor, and transforming growth factor β (1, 5, 9, 11, 19, 36, 44). Levels of LIF, in particular, have been demonstrated to be markedly elevated in synovitis, and LIF has been demonstrated to be capable of independently inducing synovial inflammation (6, 32, 41). Locally produced complement factors may be important in inflammation, in which cytokine regulation of complement component expression may increase their levels substantially (17).

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Other immunomodulatory factors, such as sex hormones, may play a role in modulating inflammatory responses. Many rheumatologic disorders associated with synovitis occur with greater frequency in females than in males, and the course of SLE may vary with the menstrual cycle (37). Synovial tissue has recently been demonstrated to contain estrogen binding sites (13) and could be metabolically influenced by that hormone. The effect of estrogen on complement component expression has not previously been investigated.

Local synovial production of complement components accounts for much of the intra-articular complement (35), and the capacity of complement to mediate inflammation suggests that local production may play a role in inflammatory synovitis. In the present study, we examined the regulation of early complement component expression in the synovium by IFN-α, IFN-γ, LIF, and estrogen in an effort to understand the pathways by which complement component expression might be influenced in the synovium.

MATERIALS AND METHODS

Synovial membrane cultures. Synovial samples were obtained from discarded tissue from patients undergoing total knee replacement for osteoarthritis. None of the patients were taking corticosteroids. Synovial villi were dissected from the surrounding adipose tissue and cut into full-thickness squares (2 by 2 mm). These synovial membrane cultures were maintained in minimal essential medium supplemented with 10% fetal bovine serum (Gibco-BRL, Gaithersburg, Md.). The culture media were changed daily and reagents were added as indicated below for the individual experiments. Triplicate or quadruplicate samples from each of eight patients were evaluated. At the end of the test period, the explant tissue was dried and weighed. To examine a pure population of synoviocytes, adherent synoviocytes were grown as monolayers for four passages in minimal essential medium supplemented with 10% fetal bovine serum (Gibco-BRL) by previously described purification techniques (32).

Biologic agents. IFN-γ was a generous gift from Genentech (South San Francisco, Calif.). 17β-estradiol, IFN-α, and LIF were purchased from Sigma (St. Louis, Mo.), Hoffmann-La Roche (Nutley, N.J.), and Genzyme (Cambridge, Mass.), respectively. Time course and dose-response experiments using the synovial membrane cultures were performed with each biologic agent, and the doses and time points in the experiments were selected on the basis of the maximal responses. LIF was used at 10 ng/ml to be representative of levels that can be achieved within the joint space (22, 40).

ELISA. Polyclonal antisera directed against C4 (goat anti-human C4 from Calbiochem, San Diego, Calif., and rabbit anti-human C4 from Behring Diag-

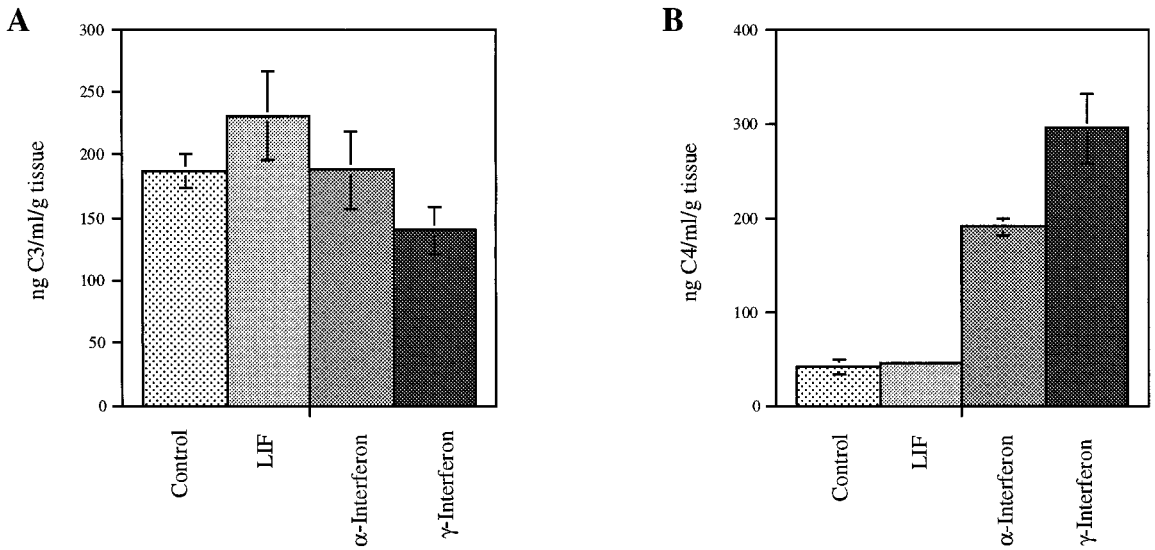

FIG. 1. Cytokine induction of C3 and C4 expression. Triplicate and quadruplicate synovial explant cultures from patients were treated with LIF at 10 ng/ml, IFN-α at 1,000 U/ml, or IFN-γ at 1,000 U/ml for 7 days. Each day the supernatant was removed and replaced with fresh medium and treatment agent. (A) C3 production was not affected. (B) C4 production was significantly increased by IFN-γ ($P < 0.0001$) and IFN-α ($P = 0.0007$).

nostics, Somerville, N.J.) were used in an indirect enzyme-linked immunosorbent assay (ELISA) with horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G from Miles-Yeda (Tel Aviv, Israel). Polyvinyl chloride microtiter plates were coated overnight with a 1:10,000 dilution of goat anti-human C4 in carbonate buffer. Supernatants from synovial cultures were added to the plates at various dilutions in phosphate-buffered saline–Tween with 2% bovine serum albumin. On each plate, dilutions of a standard human control serum containing a known amount of C4 were used for quantification of C4 protein. A 1:1,000 dilution of rabbit anti-human C4 was used as a second antibody, and alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G was used as the final antibody. The plates were developed with *o*-phenylenediamine dihydrochloride (Sigma). The reaction was stopped with a 1 M HCl solution. A modification of the C4 ELISA was used to quantify C3. Goat anti-human C3 at 1:500 (Accurate Antibody, Westbury, N.Y.) was used as the capture antibody. The second antibody was biotinylated goat anti-human C3 at 1:750. A 1:1,000 dilution of avidin-horseradish peroxidase was used in the next step, and the plates were developed as described above.

Reverse transcriptase PCR assay. Total RNA was prepared from synovial samples and monolayer cultures by using guanidinium isothiocyanate (10). Two micrograms of RNA was used as a template for first-strand cDNA synthesis, using oligo(dT) and avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim, Indianapolis, Ind.). The cDNA samples were then used as templates for simultaneous PCR amplifications with actin and C4-specific primers. Actin is an internal control which is not altered by exposure to cytokines. The actin primers (exon 4, 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′; exon 6, 5′-CTAGAAGCATTGCGGTGGACGATGGAGGG-3′) amplified a product of 661 bp, while the C4 primers bracketed exons 38 to 41 and amplified a product of 420 bp (exon 38, 5′-GAAGTGCCCTCGCCAGCGTCG-3′; exon 41, 5′-CCTGGCACCCCTGAGTGCCATAC-3′). Amplification of any potentially contaminating DNA template is not possible with these primer pairs with our standard cycling protocol. Kinetic analysis was performed to ensure that the product was obtained during the linear phase of amplification. Quantitation was performed by using a densitometer and standardization to the actin signal.

Statistical analysis. The results in Fig. 1 are the averaged values for the patients; error bars correspond to the standard errors of the means. Statistical comparisons of the effects of estrogen and IFN-γ on synovial C4 production were performed by the Wilcoxon matched-pairs test, comparing triplicate or quadruplicate control sets with treatment sets for each patient.

RESULTS

To validate the membrane culture system and examine whether cytokine effects on early complement component expression would be detectable in this system, we initially measured C4 production by synovial membrane cultures in the presence or absence of IFN-γ during the first week after explanation. The effect of IFN-γ was maximal at day 7 in culture. The synovial cells remained viable throughout this time period as demonstrated by synoviocyte purification and trypan blue exclusion (5). Therefore, the remainder of the experiments with synovial membrane cultures were performed on day 7.

To examine the effects of three cytokines believed to play a role in different types of inflammatory reactions, we exposed synovial organ cultures to physiologic levels of 10 ng of LIF per ml, 1,000 U of IFN-γ per ml, or 1,000 U of IFN-α per ml. Figure 1 demonstrates the effect of these cytokines on C3 and C4 expression. Examination of C3 expression by ELISA did not reveal any response to LIF, IFN-γ, or IFN-α. In contrast, IFN-γ and IFN-α increased C4 expression sevenfold ($P < 0.0001$) and fivefold ($P = 0.0007$), respectively. LIF did not affect C4 expression in these synovial membrane cultures.

To evaluate whether estrogen could directly affect complement component expression, we treated synovial explants with estrogen, IFN-γ, or both. Estrogen clearly down-modulated the induction of C4 expression by IFN-γ (Fig. 2). This effect was observed in samples from both male and female donors.

To demonstrate that the synoviocytes themselves produced C4 and to confirm that the synovial samples synthesized C4 de novo, semiquantitative PCR studies were performed to demonstrate the presence of C4 message. Figure 3 shows results for simultaneous PCR amplifications of total cDNA isolated from day-7 membrane cultures with primers specific for C4 and with actin as an internal control. The C4-specific bands appear in all of the sample lanes, confirming that the synovium actively transcribes C4 message. To demonstrate that purified synoviocytes produce C4, RNA was isolated from these cultures and assayed by reverse transcriptase PCR. Purified synoviocytes also expressed C4 message, confirming that synoviocytes are capable of C4 production. In both the membrane culture system and the synovial monolayer cultures, C4 message was upregulated two- to threefold in response to IFN-γ. Treatment of the monolayer culture with IFN-α also resulted in increased C4 message levels. These studies confirm that the synovium produces C4 and that synoviocytes participate directly in the production of C4. Expression of C4 in the synovium was shown to be regulated by IFN-α and IFN-γ, and the increases in protein production detected by ELISA were paralleled by in-

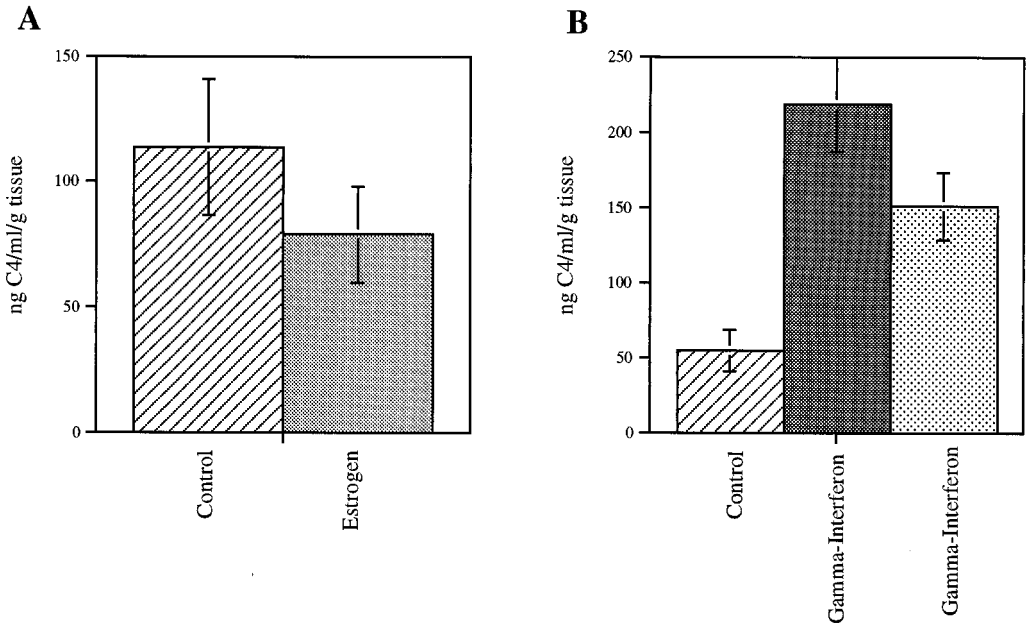

FIG. 2. Estrogen modulation of IFN-γ effects. (A) Triplicate and quadruplicate synovial explant cultures were treated with 10^{-5} M 17β-estradiol for 7 days. The difference between the control cultures and those treated with estrogen approached significance ($P = 0.08$). (B) Cultures were treated with 10^{-5} M 17β-estradiol, 1,000 U of IFN-γ per ml, or both for 7 days. The difference between the control cultures and those treated with IFN-γ is significant ($P = 0.002$), and the difference between the IFN-γ-treated cultures and those which received both IFN-γ and estrogen is significant ($P = 0.01$).

creases in C4 message level. Semiquantitative PCR was not sensitive enough to determine whether the effect of estrogen was also paralleled by changes in C4 transcript levels.

DISCUSSION

It is known that the synovium is capable of producing large amounts of individual complement components and that complement appears to be upregulated in rheumatoid arthritis (19, 35). The mechanisms by which complement production is regulated in the synovium have not been previously investigated.

Inflammatory synovitis has been artificially reproduced in a number of animal model systems. Chronic antigenic challenges with collagen (38), streptococcal cell wall antigen (42), and mycobacterial heat shock protein (39) are all capable of inducing inflammatory synovitis. Certain viral infections are capable of inducing synovitis, and exposure to high levels of inflammatory cytokines may also induce an inflammatory state with arthritis (24). How these different initiators might all be able to induce a common pathologic lesion is not known. The complement system, by virtue of its ability to interface with the humoral immune system, may play a significant role.

Complement components are not coordinately regulated. In addition, the cytokine regulation of complement component expression depends not only on the individual component but also on the tissue type. Cytokine regulation of C3 expression appears to be very tissue specific (3, 4, 7, 14, 16, 18, 28–31). Cytokine regulation of C4 expression has been less extensively studied but has been found to be upregulated in monocytes and scleral fibroblasts in response to IFN-γ (23, 25). In an effort to determine how C3 and C4 expression in the synovium is regulated, we exposed synovial cultures to IFN-γ and IFN-α. C3 expression was unchanged by either cytokine. C4 expression was upregulated in response to both cytokines. Both the explant cultures and the synovial monolayers responded to IFN-γ and IFN-α, and these responses were paralleled by the transcripts as determined by reverse transcriptase PCR. Interestingly, in humans SLE with active synovitis has been induced by exposure to IFN-α and IFN-γ (8, 12, 21, 34).

To examine whether another cytokine which has been demonstrated to play an active role in inflammatory synovitis was capable of inducing C3 or C4 expression, synovial cultures were exposed to LIF (6, 32, 41). There was no evidence of induction of either component by this cytokine. C3 is known to be induced by the cytokine interleukin-6 (17), which shares

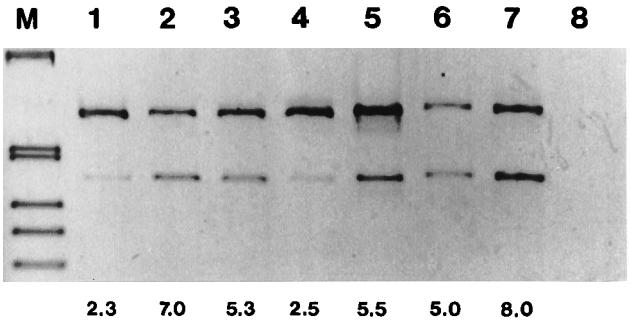

FIG. 3. Reverse transcriptase PCR of C4 transcripts in the synovium. RNA was isolated from synovial explant tissue after treatment for 7 days. Lane 1, no treatment; lane 2, 1,000 U of IFN-γ per ml; lane 3, 1,000 U of IFN-γ per ml with 10^{-5} M 17β-estradiol. RNA was isolated from pure synoviocyte monolayer cultures treated for 3 days. Lane 4, no treatment; lane 5, 1,000 U of IFN-γ per ml; lane 6, 1,000 U of IFN-α per ml; lane 7, 1,000 U of IFN-γ per ml with 10^{-5} M 17β-estradiol. Molecular weight markers (M) are shown to the left. cDNA was produced from the RNA and was amplified by PCR with oligonucleotides specific for actin and C4. The actin bands are 661 bp in length and are the upper band in each lane. The C4-specific bands are 420 bp in length and are the lower band in each lane. A negative control (lane 8) was amplified in parallel without a template. The bands were scanned with a densitometer and standardized to the actin signal for semiquantitative comparisons. The relative intensity of the C4 signal, standardized to that of the actin signal, is shown below each lane.

many characteristics and a subunit of its receptor with LIF (20). Nevertheless, C3 expression was unchanged by LIF at any of the concentrations tested.

Because many rheumatologic disorders associated with synovitis occur with greater frequency in females than in males, it is important to understand the effects of sex steroid hormones on various aspects of the inflammatory response. In the synovial cultures, estrogen markedly down-modulated the induction of C4 expression by IFN-γ and appeared to decrease basal expression as well. This is the first demonstration of an estrogen effect on the production of a complement component. The transcription control element governing estrogen induction of transcription has been identified (2), although the C4 promoter region does not appear to contain this transcription control element. At present, the mechanism of the estrogen effect is not known. Semiquantitative PCR was not able to identify a change in transcript level associated with estrogen treatment of either membrane cultures or synovial monolayers. This may be due to a limited sensitivity of this assay system, or the effect of estrogen on C4 production may occur through posttranslational mechanisms.

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