Streptococcal Cell Wall-Induced Arthritis: Requirements for IL-4, IL-10, IFN-γ, and Monocyte Chemoattractant Protein-1

Ralph C. Schimmer, Denis J. Schrier, Craig M. Flory, Keith D. Laemont, David Tung, Alan L. Metz, Hans P. Friedl, Mary Carol Conroy, Jeffrey S. Warren, Beatrice Beck and Peter A. Ward

Streptococcal Cell Wall-Induced Arthritis: Requirements for IL-4, IL-10, IFN-γ, and Monocyte Chemoattractant Protein-1

Ralph C. Schimmer, Denis J. Schrier, Craig M. Flory, Keith D. Laemont, David Tung, Alan L. Metz, Hans P. Friedl, Mary Carol Conroy, Jeffrey S. Warren, Beatrice Beck, and Peter A. Ward

Intra-articular injection of streptococcal cell wall Ag followed by i.v. challenge ("reactivation") results in a destructive lymphocyte-dependent monoarticular arthritis. To further define the role of immune mechanisms in the model, Abs to Th1 and Th2-related cytokines were evaluated. Treatment of rats with antibodies to IL-4 reduced swelling, while treatment with anti-IL-10 or anti-IFN-γ either had no effect or slightly enhanced the inflammatory response. These results suggest that Th-2 immune mechanisms may be, at least in part, operative in the model. To more precisely define the role of IL-4, the effects of anti-IL-4 on monocyte chemoattractant protein-1 (MCP-1) expression were evaluated. Initial studies demonstrated that mRNA (as determined by in situ hybridization) and protein (as determined by immunofluorescence) for MCP-1 were detectable in inflamed synovial tissue in a time-dependent manner. Anti-IL-4 treatment significantly reduced the expression of mRNA for MCP-1 24 and 72 h after reactivation. In addition, anti-MCP-1 inhibited swelling and reduced influx of 111In-labeled T cells. These data suggest that the reactivation model of streptococcal cell wall Ag-induced arthritis is Th-2 dependent, and that an inter-relationship exists between IL-4 and the expression of MCP-1.


Isolated complexes of peptidoglycan and polysaccharide from the cell walls of Lancefield group A streptococci possess a high inflammatory and antigenic potential. Depending on the size of the cell wall fragments (1) and the route of application, varying inflammatory responses occur. A single systemic (i.p.) injection of a relatively large dose of SCW results in development of a chronic erosive polyarthritis in genetically susceptible, female Lewis rats (2). Other inflammatory reactions caused by SCW in rats and mice include hepatic granulomas (3–5), granulomatous enterocolitis (6), carditis (7), and uveitis (8). While chronic, remittent polyarthritis induced by systemic injection of SCW is a widely used model of arthritis in rats and mice, mechanistic studies are difficult because the relapses occur unpredictably. A variation of this model involves the local injection of SCW directly into a joint followed by systemic challenge with SCW. The initial intra-articular application of SCW causes a local, acute inflammatory reaction, with edema reaching a maximum after 24 h and subsiding thereafter. After 3 wk, only a slight chronic inflammation with diffuse infiltration of the synovial layer by lymphocytes and monocytes remains (9). The i.v. injection of a low dose of SCW into the same rats causes pronounced reactivation of the arthritic response in the joint (10). This model of “reactivation” begins with a comparable low baseline of ankle edema and results in an immunologically mediated inflammatory response that mimics the exacerbation of a chronic arthritic course. The model provides synchronized recurrences, thus allowing analysis of the regulation of these recurrences (11). It has been shown that recurrences of SCW-induced arthritis in the chronic model were completely abolished by treatment with anti-lymphocyte serum (12). Treatment with anti-lymphocyte serum also inhibited the flare-up reaction in the reactivation model (13). The reactivation in SCW-induced arthritis can also be suppressed by cyclosporin A and depletion of T cells (14).

Although results obtained to date strongly suggest that T cells and mononuclear cells are critical participants in the pathophysiology of the reactivation model, little work has been performed to precisely define the nature of the immune response involved. Much evidence from other animal models of arthritis and human disease suggests that Th1 mechanisms are involved. Indeed, in the chronic model of SCW-induced arthritis Allen et al. (15) found that long-term IL-4 treatment decreased the amount of swelling during the chronic phase. In addition, under certain circumstances IFN-γ treatment triggers the onset of collagen-induced arthritis in mice and enhances swelling in adjuvant-induced arthritis (16, 17). However, not all evidence points to a consistent role for Th1 mechanisms in models of arthritis. Alternate IFN-γ treatment protocols in adjuvant arthritis caused transient reduction in ankle swelling followed by a significant exacerbation. In addition, IFN-γ treatment inhibited the development of collagen-induced arthritis and adjuvant arthritis (18, 19). Therefore, the participation of Th1 and Th2 mechanisms in models of arthritis remains controversial.

In rheumatoid arthritis in humans, evidence regarding the nature of the immune response is also inconclusive. Mononuclear cells obtained from synovial fluid and subsequently stimulated in culture demonstrated an increase in IFN-γ and IL-2 biosynthesis (20). Few cells were found that produce IL-4. Similarly, T cell clones...
obtained from synovial tissue were primarily of the Th1 type (21, 22). Another study found high expression of IFN-γ relative to IL-4 in the rheumatoid synovial membrane (23). However, other studies have suggested that Th-2-related processes are operative. Unstimulated CD4+ T cells from synovial fluid produced large quantities of IL-4 and IL-10 compared with low levels of IFN-γ and IL-2 (24). In addition, synovial tissue lymphocytes produced significant quantities of IL-10 (25). The diverse nature of the immune response in rheumatoid arthritis and chronic models may reflect the complex immunoregulatory networks that are evident in human disease. To help define the cellular mechanisms involved in the reactivation paradigm, studies were performed to evaluate the roles of IL-4, IL-10, and IFN-γ in the model. The results suggest that the model is IL-4 (but not IL-10 or IFN-γ) dependent, suggesting that Th-2 mechanisms are involved. In addition, these results suggest a linkage between IL-4 and expression of MCP-1.

Materials and Methods

Reagents and interventions

Except as noted all reagents were purchased from Sigma Chemical Co. (St. Louis, MO). The 100P fraction of the streptococcal preparation (PG-APS) was purchased from Lee Laboratories (Grayson, GA). The material was briefly sonicated. Recombinant murine IL-4 and IL-10 were expressed in Escherichia coli and purified to homogeneity and high sp. act. as previously described (26). Rabbis were immunized with recombinant murine IL-4 and IL-10 in CFA. Production of Ab to rat MCP-1 has been described previously (27). A purified IgG preparation was made from the anti-MCP-1 antiserum using a protein A-Sepharose-4 fast flow column (Pharmacia, Piscataway, NJ). A purified IgG preparation was made from the anti-MCP-1 antiserum using a protein A-Sepharose-4 fast flow column (Pharmacia, Piscataway, NJ). An IgG preparation was made as described above. For interventions involving antisera, 0.5 ml of antiserum or control rabbit serum (Lampire Biologic Laboratories, Pipersville, PA) was injected i.v. just before systemic injection of SCW. On days 1 and 2 after reactivation the animals were given daily i.v. doses of 0.25 ml of antiserum or control serum. A loading dose of 500 μg of anti-MCP-1, anti-IFN-γ, or appropriate control IgG was administered before i.v. challenge with SCW. On days 1 and 2 after reactivation the animals were given daily i.v. doses of 250 μg of Ab or control IgG.

Animals

Female Lewis rats (100–125 g) were purchased from Charles River Laboratories (Portage, MI) and housed in the animal quarters of Parke-Davis Pharmaceutical Research (Ann Arbor, MI). All animal protocols were approved by the Parke-Davis institutional animal care and use committee. Rats were given food and water ad libitum.

Animal model of SCW-induced arthritis

SCW-induced arthritis was induced as previously described (28). A SCW preparation (100P fraction) was suspended in PBS, and 10 μl of the suspension containing 6 μg of PG-APS was injected into the ankle joint using a 25-gauge needle attached to a microliter syringe (Hamilton Co., Reno, NV). Contraateral joints were injected with saline. Swelling was measured by plethysmographic assessment of the ankle volume in mercury, using an edema computer (Buxco Electronics, Inc, Sharon, CT). Reactivation of the arthritis inflammation was induced 21 days after the intra-articular injection by the i.v. injection of 100 μg of PG-APS. This resulted in a marked and prolonged mononuclear arthritis involving the joint originally injected with PG-APS. Blocking Ab treatments were performed by tail vein injection of appropriate IgG. Seven animals per group were used in each study, and two animals in each group were killed at 72 h after flare-up for histologic assessment.

Immunohistochemistry

Whole ankles were harvested immediately after animals had been euthanized. The joints were skinned and flash-frozen in liquid nitrogen. Cryosectioning of whole undecalified joints was accomplished as described previously (29–33). The initial method used in this study was described originally by Rijntjes et al. (31). To overcome the potential risk of shattering the thin sections during the cutting process, a piece of transparent tape was applied to the surface of the cutting block. The section was cut underneath the tape, thus leaving the section attached to the tape strip. The tape was transferred to a slide, and the section was briefly fixed in acetone and further processed for immunostaining.

Whole ankle RNA extraction

Ankles were harvested in the manner described above, skinned, and immediately flash-frozen in liquid nitrogen. The ankles were ground into fine powder using a tissue pulverizer (Biospec Products, Bartlesville, OK) continuously cooled in liquid nitrogen. The resulting tissue powder was then homogenized in Trizol reagent using a tissue homogenizer (Ultra-Turrax T25, Janke & Kunkel, Staufen, Germany) in three 10-s steps with intermittent cooling of the sample in liquid nitrogen. RNA extraction was then performed by phase separation using Trizol reagent (Life Technologies, Gaithersburg, MD) and chloroform, and subsequent RNA precipitation by isopropanol. The resulting pellet was air-dried, washed in 75% ethanol, and dissolved in 100 μl of Tris-EDTA buffer.

Northern blot analysis

Ten micrograms of RNA from each tissue preparation was electrophoretically separated through a denaturing agarose/formaldehyde gel followed by capillary transfer to nylon membranes. The membranes were washed in distilled H2O and subsequently dried in vacuo at 80°C for 2 h. Prehybridization was performed at 65°C for 1 h in 7% SDS, 500 mM NaHPO4, 1 mM EDTA, and 1% (w/v) BSA followed by hybridization to 32P-labeled cDNA probes at 65°C overnight in the above buffer with 100 μg/ml of salmon sperm DNA and 106 cpm/ml probe. Filters were then washed at 65°C for 10 min in 5% SDS, 25 mM NaHPO4, 1 mM EDTA, and 0.5% BSA followed by two washing steps in 1% SDS, 25 mM NaHPO4, and 1 mM EDTA and exposure to XAR5 film (Eastman Kodak, Rochester, NY) at −85°C. Densitometry measurements were made with a densitometer equipped with ImageQuant software (version 3.3, Molecular Dynamics, Sunnyvale, CA).

In situ hybridization of ankle tissue

In situ hybridization studies were performed on cryosections of whole ankle joints. Animals were killed, and the entire joint was harvested, skinned, and immediately placed in 4°C cold decalcification solution containing 10% (w/v) EDTA and 7.5% (w/v) polyvinylpyrrolidone (m.w. = 25,000; pH 6.95) (34, 35). The tissue specimens were placed in Tissue-Tek III cassettes (Polysciences, Warrington, PA) that floated freely in the decalcifying solution. Decalcification was performed over 10 days at 4°C, with the solution exchanged every third day. Thereafter, ankles were embedded in OCT (Polysciences) and flash-frozen in isopentane prechilled on liquid nitrogen.

In situ hybridization studies were performed according to previously described methods using full-length [32P]UTP-labeled sense and antisense riboprobe generated by in vitro transcription of a linear MCP-1 template (36).

In vivo T cell migration assay using isolated and 111In-labeled T cells

Normal donor rats were killed, the spleens were removed immediately, and the tissues were gently teased apart in a petri dish containing cold RPMI with 10% normal rat serum (NRS). The resulting cell suspension was carefully separated from tissue debris, rinsed, and resuspended in 2 ml of RPMI/NRS. To separate B and T cells, the cell suspension was added to a nylon wool column (Polysciences, Inc., Warrington, PA) and incubated at 37°C for 1 h. Thereafter, the nonadherent T cells were eluted with RPMI/NRS and quantitated on a Coulter counter (Coulter Electronics, Luton, U.K.). To label the cells, 5 × 107 cells were suspended in 0.5 ml of RPMI with 7 μCi of 111Inoxyquinoline. Incubation was performed for 20 min, with gentle mixing of the suspension every 5 min. Rats received a tail vein injection of 1 × 107 labeled cells 4 days after i.v. challenge with SCW. The animals were killed 24 h later, the ankles were removed, and the isotopic content of the specimen was determined on a Cobra II Gamma Counting System (Packard Instrument Co., Meriden, CT).

Statistical power

One- or two-way analysis of variance was employed together with Student's t test to evaluate statistical differences among experimental groups.

Results

Requirements for IL-4, but not for IL-10 or IFN-γ

Animals were treated with blocking Abs to IL-4, IL-10, or IFN-γ to determine their effects on the development of joint edema. The
results are shown in Figure 1. In animals treated with anti-IL-4, significantly less ankle edema was found on days 1a, 2a, 3a, 4a, and 5a, with reductions of 38%, 5%, 42%, 46%, and 40%, respectively (Fig. 1A). Thus, the full development of arthritis in this model of arthritis requires MCP-1. We also addressed the question of whether up-regulation of mRNA for MCP-1 might be IL-4 dependent. In the experiments described in Figure 3, treatment with Ab to IL-4 reduced MCP-1 mRNA levels at 24 h to baseline levels. The effects of the Ab were also evident at 72 h. Surprisingly, the inhibitory effect of the Ab was less obvious at 48 h. Ab to IFN-γ had no effect on MCP-1 mRNA at any time point.

Immunohistochemical detection of MCP-1

For these studies, ankles were harvested at the time of maximal swelling (72 h after SCW challenge). MCP-1 was observed mainly on the synovial lining (Fig. 4A) and, to a lesser extent, on mononuclear cells in the subsynovial area (Fig. 4C). Sections of control synovial tissue failed to demonstrate the presence of MCP-1 on the synovial surface (Fig. 4B) or in subsynovial cells (Fig. 4D).

In situ hybridization analysis of MCP-1

The sense and antisense P-33-labeled RNA probes used in these studies were generated by in vitro transcription of full-length MCP-1 cDNA. In the synovial lining of arthritic rats, there was significant expression of mRNA for MCP-1 (Fig. 5A). The specificity of the signal is demonstrated by sections incubated with the sense MCP-1 probe (Fig. 5B).

Requirements for recruitment of splenic T cells into joints

Twenty-four hours following i.v. challenge with SCW, rats were infused with 111In-labeled splenocytes harvested from normal rat spleens. Twenty-four hours later, trafficking of the labeled splenocytes into inflamed joints was assessed. In control animals not challenged with SCW (but receiving normal rabbit IgG), the amount of radioactivity accumulating in joints was minimal (Fig. 6). In animals challenged with SCW (and also treated with normal rabbit IgG), there was a 3.5-fold increase in cell accumulation, as reflected by 111In radioactivity in the joint (Fig. 6). 111In-labeled T cell accumulation was significantly suppressed (by 87%) in the presence of anti-MCP-1 (Fig. 6). Thus, it would appear that naive T cell trafficking into the arthritic joint is MCP-1 dependent.

Discussion

The pleiotropic characteristics of IL-4 are reflected by numerous studies documenting inhibitory and stimulatory effects on inflammation. Many monocyte functions are negatively regulated by
FIGURE 3. Requirements for IL-4 in up-regulation of mRNA for MCP-1 (A) and IFN-γ (B) in synovial tissues undergoing development of arthritic changes after i.v. challenge with SCW. Data represent densitometry tracings of Northern blots. Synovial mRNA was also analyzed in animals treated with blocking Ab to IL-4.

IL-4. IL-4 suppresses in vitro production of IL-1 and TNF-α by human monocytes (37) and enhances the synthesis of IL-1R antagonist (38). IL-4 also suppresses the production of IL-6 (39) and IL-8 (40) by human monocytes. IL-4 directs the development of Th2 cells from naive (Th0) lymphocytes (41) and up-regulates the expression of certain vascular adhesion molecules, in particular vascular cell adhesion molecule-1 (42, 43). While IL-4 alone increases endothelial cell adhesiveness for T cells, but not PMN (44), the combination of TNF-α and IL-4 synergistically enhances T cell adhesiveness (45). Thus, it appears that the combined actions of TNF-α and IL-4 are important in the transition from acute to chronic inflammation, in part due to selective effects on T cell adhesion. MCP-1 expression is also sensitive to IL-4. Increased expression and secretion of MCP-1 are observed in IL-1-stimulated endothelial cells exposed to IL-4 (46). Our studies demonstrate a proinflammatory effect of IL-4 in the reactivation of SCW-induced arthritis. These results are in contrast to the anti-inflammatory effect of this cytokine in the chronic form of this model (15). A recent report, however, has shown a protective effect of IL-4 blockade during the acute inflammatory exacerbation in Ag-induced arthritis (47). While the precise role of IL-4 in this model is unknown, our results suggest that IL-4 may enhance MCP-1 expression. MCP-1 expression in the reactivation of SCW-induced arthritis was up-regulated after 24 h and continued to increase until the end of the observation period. Ab against IL-4 reduced MCP-1 expression at 24 and 72 h. These results suggest that IL-4 has a significant regulatory effect on MCP-1.

The requirements for IL-4, but not IFN-γ, during the reactivation event, as demonstrated by the blocking studies, is indicative of a Th2-type response. Analysis of the relationship between tissue edema and Th1 and Th2 responses has been published recently (48–50). These studies evaluated the role of Th1 and Th2 cells in footpad and ear models of delayed-type hypersensitivity. Th1 cells induce delayed swelling that peaked at 48 h and lasted for 5 days (48), which is consistent with earlier investigations (49). In contrast an early swelling response peaking at 6 h is observed in a system in which Th2 responses predominate (50). The response is primarily neutrophilic at 6 h and changes to a mainly mononuclear cell infiltrate by 48 h. PMN are also involved during the reactivation reaction in SCW-induced arthritis. Footpad edema caused by Th2 cells is highly dependent on IL-4, and the IL-4 dependency of the early phase of SCW-induced arthritis thus might indicate that the ankle edema is predominantly triggered by Th2 cells in conjunction with PMN.

While several studies have defined the requirements for inflammatory mediators in the chronic SCW-induced arthritis model, only a few reports exist regarding the regulation of the reactivation model (51), which may be an attractive model for the acute flares in rheumatoid arthritis. Increased MCP-1 levels are observed in the joint fluid of patients with rheumatoid arthritis (52–54), and synoviocytes obtained from arthritic joints have been found to secrete MCP-1 (3). Thus, it is possible that MCP-1 may contribute to the recruitment of mononuclear cells into the synovium. Our studies demonstrate a major role for this β-chemokine in the reactivation of SCW-induced arthritis. Blocking MCP-1 with polyclonal Abs significantly reduced ankle edema and T cell migration. These results are consistent with the concept that MCP-1 may be a major chemotactic factor for T cells in in vitro and in vivo systems.

In conclusion, the reactivation model of SCW-induced arthritis is useful for the elucidation of cellular inflammatory mechanisms that are immune in nature. These results indicate that this arthritic response is IL-4 dependent, potentially via regulatory effects on MCP-1 expression. The data also suggest that Th2-related mechanisms may contribute to the pathophysiology of the model.

Acknowledgments

We thank Doreen Gasparella and Beverly Schuman for their administrative assistance, Godwin Okonkwo for excellent technical support, and Robin G. Kunkel for graphic support.

References
