Role of chemokines and cytokines in a reactivation model of arthritis in rats induced by injection with streptococcal cell walls

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Abstract: Intraarticular injection of streptococcal cell wall (SCW) antigen followed by intravenous challenge results in a T cell-mediated monoarticular arthritis in female Lewis rats. Initial studies showed that this reactivation response to intravenous SCW antigen is dependent on the presence of interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α) and that the early phase of swelling is neutrophil-dependent. Neutrophil depletion or passive immunization with antibodies to P-selectin or macrophage inflammatory protein-2 reduced the intensity of ankle edema and the influx of neutrophils. After the first few days, however, the arthritic response is mediated primarily by mononuclear cells. Joint tissues showed up-regulation of mRNA for monocyte chemotactic protein-1 (MCP-1), which could be inhibited in part by anti-IL-4; treatment of rats with antibodies to IL-4 or MCP-1 significantly suppressed development of ankle edema and histopathological evidence of inflammation. Antibodies to interferon-γ or IL-10 had no effect. Treatment with anti-MCP-1 also suppressed influx of ^{111}In-labeled T cells into the ankle joint. These data suggest that the late, mononuclear-dependent phase of SCW-induced arthritis in female Lewis rats requires cytokines that up-regulate MCP-1, which in turn may facilitate recruitment and extravasation of mononuclear cells into the joint. *J. Leukoc. Biol.* 63: 359–363; 1998.

Key Words: *interleukin-1 · tumor necrosis factor α · mononuclear cells*

INTRODUCTION

Rheumatoid arthritis is a prevalent chronic inflammatory disease in which large numbers of mononuclear cells infiltrate the synovial tissue. In addition, large numbers of neutrophils accumulate in the synovial fluid and neutrophil-related mediators are elevated in the joint. Although many cell types may be involved in the pathophysiology of the disease, the interrelationship of various mediators and cells is not well defined. It is known that migration of inflammatory cells into tissues is a pivotal aspect of arthritis and other inflammatory diseases and is regulated by a complex process involving a host of adhesion molecules and cellular mediators [1–4]. The first step in this cellular cascade involves a tentative interaction between the leukocyte and the endothelial wall of the vasculature. Depending on the cell type, specific adhesion molecules and cytokines are elicited which allow tethering and then more firm leukocyte attachment in preparation for eventual emigration into the extravascular space. Other cytokines and adhesion molecules help create an opportunity for the cells to enter an inflammatory site and display a range of effector functions.

One complexity in the study of arthritic processes is the lack of synchronized chronic animal models. In most models the arthritic response develops gradually and unpredictably over a period of days or weeks. Understanding the temporal interrelationships of various mediator systems is more practical when the various phases of the inflammatory response occur simultaneously. Therefore, a model of arthritis was developed that is induced by intraarticular (i.a.) injection into an ankle joint of a small dose of purified peptidoglycan-polysaccharide polymers isolated from cell walls of group A streptococci (PG-APS), producing a synovitis with maximal swelling at 24 h that subsides over the next few days. The response is reactivated by an intravenous injection of a small subarthropathic dose of PG-APS, which produces a more prolonged, severe inflammation confined to the joint previously injured with PG-APS. The flare response develops synchronously, allowing precise definition of regulatory mechanisms [5–9]. Although T cell dependency has been demonstrated in cellular studies, a precise role for T cells has not been elucidated [10].

In this report we describe the results of studies done to evaluate the role and interrelationship of cytokines, adhesion molecules, and chemokines in the arthritic response induced by reactivation with PG-APS. The results indicate that the model is neutrophil dependent and that interleukin-1 (IL-1)

Abbreviations: SCW, streptococcal cell wall; IL, interleukin; TNF-α, tumor necrosis factor α; MIP-2, macrophage inflammatory protein-2; MCP-1, monocyte chemotactic protein-1; PG-APS, peptidoglycan-polysaccharide polymers isolated from cell walls of Group A streptococci; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetate; CIA, collagen arthritis.

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and tumor necrosis factor (TNF) are essential for full expression of the arthritic response. Moreover, antibodies to IL-4, but not interferon-γ, block swelling when administered during the reactivation phase. The results also show that antibodies to IL-4 reduce mRNA for MCP-1, perhaps helping to explain the IL-4 dependence of the model. Finally, antibodies to MCP-1 block swelling and T cell influx into the joint, suggesting an important role for this chemokine in the reactivation response induced by streptococcal cell walls.

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 in a Branson Sonifier, Model 200. Antibodies against rat TNF-α, macrophage inflammatory protein-1α (MIP-1α), and IL-1 were generously provided by Dr. S. Kunkel, University of Michigan. Recombinant murine IL-4 and IL-10 were expressed in *Escherichia coli* and purified to homogeneity and high specific activity as previously described [11]. Rabbits were immunized with recombinant murine IL-4 and IL-10 in complete Freund's adjuvant. Production of antibody to rat MCP-1 has been described elsewhere [12]. A purified IgG preparation was made from the anti MCP-1 antiserum with the use of a Protein A Sepharose-4 fast flow column (Pharmacia, Uppsala, Sweden). Anti-interferon-γ was produced in goats after immunization with rat interferon-γ. An IgG preparation was made as described above. Antibody to MIP-2 similarly was raised in rabbits by immunization with recombinant rat MIP-2 protein [13]. For interventions involving antisera, 0.5 mL of antiserum or control rabbit serum (Lampire Biological Laboratories; Pipersville, PA) was injected intravenously 20 min before systemic injection of streptococcal cell wall (SCW). On days 1 and 2 after reactivation the animals were given daily intravenous doses of 0.25 mL of antiserum or control serum. A loading dose of 500 μg of anti-MCP-1, anti-interferon-γ, or appropriate control IgG was administered before intravenous challenge with SCW. On days 1 and 2 after reactivation the animals were given daily intravenous doses of 250 μg of antibody 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 [7]. A SCW preparation (100 p fraction) was suspended in phosphate-buffered saline (PBS) and 10 μL of the suspension containing 6 μg PG-APS was injected into one ankle joint; the contralateral joint was injected with PBS. Swelling was measured by mercury plethysmography. Reactivation of the arthritic inflammation was induced 21 days after the intraarticular injection by the intravenous injection of 100 μg PG-APS. This resulted in a monoarticular arthritis involving the joint originally injected with PG-APS. Blocking antibody treatments were performed by tail vein injection of appropriate Ig's. Seven animals per group were used in each study and two rats from each group were killed for histological assessment at 72 h after flare-up.

Histological techniques

When rats were killed, the ankles were injected intraarticularly with 20 μL of 10% neutral buffered formalin, removed, skinned, and fixed for another 24 h in buffered formalin. Before paraffin embedding, the joints were decalcified in 10% ethylenediaminetetraacetate (EDTA) for 7 days. Paraffin sections were stained in Safranin-O for cartilage proteoglycan and counter-stained with fast green and hematoxylin.

Whole ankle RNA extraction

Ankles were harvested, 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 homogenized in Trizol reagent using a tissue homogenizer (Ultra-Turrax T25, Janke & Kunkel) 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 (GIBCO-BRL, Bethesda, 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 TE buffer.

Northern blot analysis

Ten micrograms RNA from each tissue preparation were electrophoretically separated through a denaturing agarose/formaldehyde gel followed by capillary transfer to nylon membranes. The membranes were washed in distilled H_2O and subsequently dried in vacuo at 80°C for 2 h. Prehybridization was performed at 65°C for 1 h in 7% sodium dodecyl sulfate (SDS), 500 mM $NaHPO_3$, 1 mM EDTA, and 1% (w/v) bovine serum albumin (BSA) followed by hybridization to ^{32}P-labeled cDNA probes at 65°C overnight in the above buffer with 100 mg/mL of salmon sperm DNA and 10^6 cpm/mL probe. Filters were then washed at 65°C for 10 min in 5% SDS, 25 mM NaHPO, 1 mM EDTA, and 0.5% BSA followed by two washing steps in 1% SDS, 25 mM NaHPO, 1 mM EDTA, and exposure to XAR5 film (Kodak) at −85°C. Densitometry measurements were made with a densitometer equipped with ImageQuant software (V. 3.3) by Molecular Dynamics (Sunnyvale, CA).

Statistics

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

RESULTS AND DISCUSSION

Role of neutrophils, TNF-α, and IL-1 in arthritis induced by PG-APS in the reactivation model

To examine the role of neutrophils in the model, anti-neutrophil or appropriate control antibodies were administered to animals 1 day before reactivation of the arthritic response, and for two subsequent days after reactivation. The rats had been immunized in one hind paw with the cell wall preparation 21 days earlier. Circulating neutrophil counts and extravasation of neutrophils into the synovial fluid were almost completely suppressed during the reactivation period. Neutrophil depletion also inhibited swelling for 3 days after reactivation (**Fig. 1**). Inhibition was maximal on day 2 (84% inhibition, $P < 0.05$). Consistent with these observations, antibodies to P-selectin also significantly inhibited swelling (data not shown).

To evaluate the role of TNF-α and IL-1 in the reactivation model, antibodies were administered as described above. Appropriate control antibodies were used in each instance. Results shown in Figure 1 indicate that antibodies to either TNF-α or IL-1 almost completely suppressed the inflammatory response in the hind paws 72 h after reactivation. Inhibition was sustained throughout the observation period. Histopathological analysis indicated a significant reduction in the pansynovial infiltration of mononuclear cells.

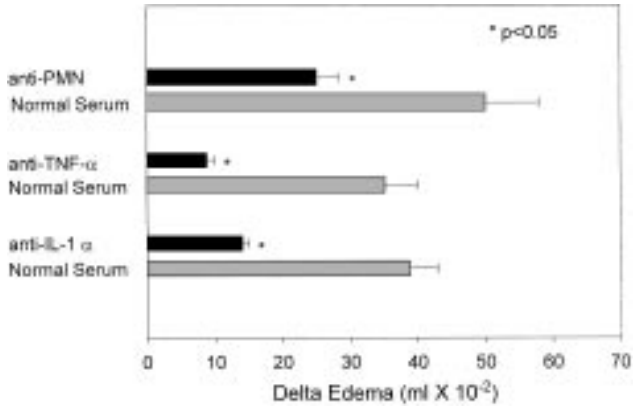

Fig. 1. Changes in ankle volume 72 h after the secondary intravenous injection with PG-APS. Animals were depleted of neutrophils or treated with an antisera to TNF-α or IL-1α. Control animals received an equivalent amount of normal serum. Results are shown as the mean ± SEM (n = 7/experimental group).

These observations parallel those from collagen arthritis (CIA) and the chronic SCW paradigms. In CIA, the IL-1 receptor antagonist decreased the severity of arthritis during the acute phase [7, 14], as did antibodies against IL-1β [15, 16]. In contrast, IL-1 does not play a role in antigen-induced arthritis in mice and rabbits [14, 17]. TNF-α is a proinflammatory cytokine that has been proven to be involved in many animal models of arthritis. The dependence of TNF-α in CIA was shown by Wooley et al. [18] and Williams et al. [19], who found a synergistic relationship between IL-1 and TNF-α. A positive correlation between TNF-α and phagocyte recruitment has also been demonstrated [20]. Our results suggest that both IL-1 and TNF-α are essential for the reactivation response induced by systemic challenge with PG-APS.

The role of IL-4, IL-10, and interferon-γ in the reactivation response induced by PG-APS

To evaluate the involvement of Th1- and Th2-related mechanisms in the development of the reactivation model, antibodies to IL-4, interferon-γ, or IL-10 were administered as described above during the reactivation phase of the arthritic response. Appropriate control antibodies were administered in each instance. The results shown in **Figure 2** indicate that treatment with antibodies to IL-4 significantly reduced the reactivation response to PG-APS. Moreover, results from histological analysis indicates that synovial infiltration and damage to the articular surface were reduced in treated rats (**Fig. 3**). In contrast, antibodies to IL-10 or interferon-γ had no significant effect on the inflammatory response. Similar results were also observed when the antibodies were administered within 7 days of the initial intraarticular injection (data not shown). These results suggest that Th2 mechanisms may be operative in this model and are in contrast with results obtained from other models and in human arthritis, which indicate that Th1 mechanisms are predominant. For instance, Allen et al. [21] found that long-term IL-4 treatment in the chronic SCW model decreased the amount of swelling during the chronic phase. In addition, interferon-γ treatment, depending on the dosing protocol, triggers the onset of collagen arthritis in mice and enhances swelling in adjuvant-induced arthritis [22, 23]. However, not all evidence points to a consistent role for Th1 mechanisms in models of arthritis. Alternate treatment protocols in adjuvant arthritis causes transient reduction in ankle swelling followed by a significant exacerbation. In addition, interferon-γ treatment inhibits the development of CIA and adjuvant arthritis [24, 25]. Therefore, the participation of Th1 and Th2 mechanisms in models of arthritis remains controversial.

In rheumatoid arthritis, evidence regarding the nature of the immune response is also inconclusive. Some studies suggest that Th1-related mechanisms predominate. For instance, mononuclear cells obtained from synovial fluid and subsequently stimulated in culture demonstrated an increase in interferon-γ and IL-2 biosynthesis [26]. Few cells were found that produce IL-4. Similarly, T cell clones obtained from synovial tissue were primarily of the Th1 type [27, 28]. Another study found high expression of interferon-γ relative to IL-4 in the rheumatoid synovial membrane [29]. However, other studies also indicate that Th2-related processes are operative. Unstimulated $CD4^+$ T cells from synovial fluid produce large quantities of IL-4 and IL-10 and deficient levels of interferon-γ and IL-2 [30]. In addition, synovial tissue lymphocytes produce significant quantities of IL-10 [31]. The diverse nature of the immune response in rheumatoid arthritis may reflect the complex immunoregulatory networks that are evident in human disease. The SCW reactivation model may allow a more precise definition of Th2 mechanisms in arthritis.

Chemokine involvement in the reactivation response

To explore the role of chemokines in the inflammatory response induced by reactivation with PG-APS, antibodies to two CC chemokines, MCP-1 and MIP-1α, or one CXC chemokine, MIP-2, were administered during the reactivation phase of the arthritic response. Appropriate control antibodies were administered in each instance. Results shown in **Figure 4** indicate that all of the antibodies have a significant effect on swelling. The effects of antibodies to MCP-1 were most pronounced and

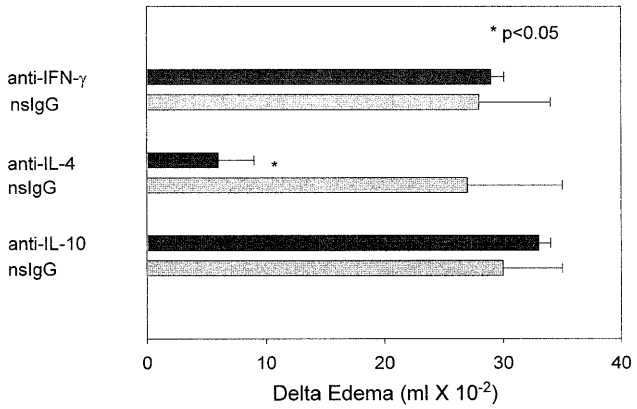

Fig. 2. Changes in ankle volume 72 h after the secondary intravenous injection with PG-APS. Animals were treated with antibodies to interferon-γ (IFN-γ), IL-4, or IL-10. Control animals received an equivalent amount of normal serum or normal serum IgG. Results are shown as the mean ± SEM (n = 7/experimental group).

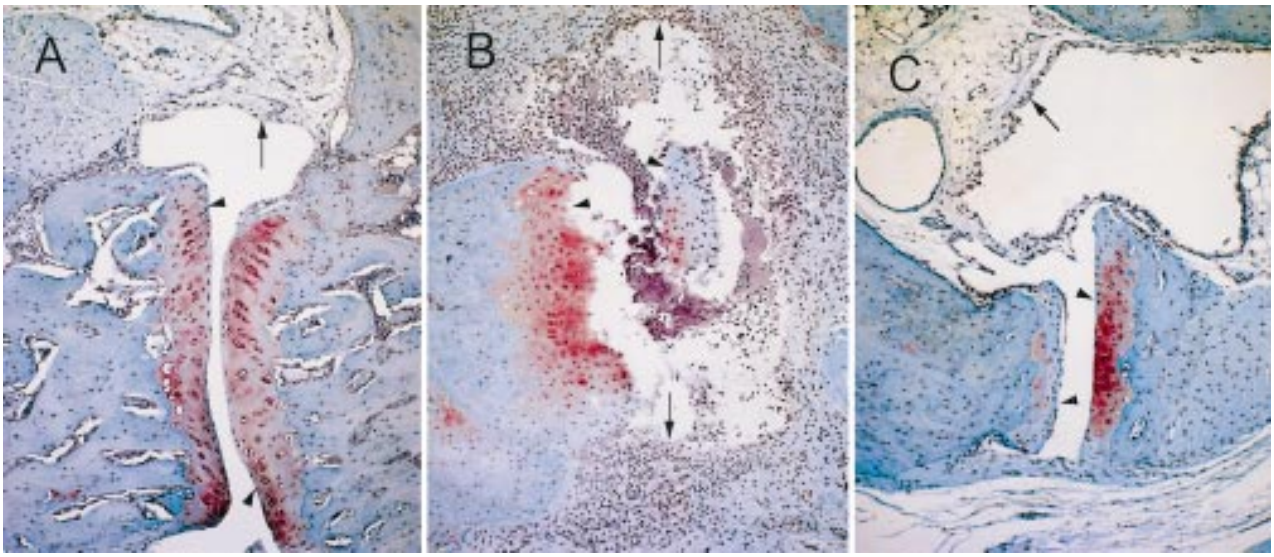

Fig. 3. Effect of anti-IL-4 antibody treatment on joint histopathology. (A) Control joint; (B) positive control joint 72 h post-reactivation; (C) 72-h joint treated with anti-IL-4 antibody. Note the marked reduction in synovial inflammation (arrows) and preservation of joint integrity (arrowheads) in anti-IL-4-treated rats compared with 72-h positive controls.

were studied further by evaluating the effects of the antibody on T cell migration. Antibody treatment significantly reduced T cell migration into the inflamed joint (results not shown). The results demonstrate the important role of MCP-1 in the cellular component of the immune response to PG-APS.

The interrelationship between IL-4 and MCP-1 in the reactivation model

Because previous reports have suggested a connection between the expression of IL-4 and MCP-1 expression in other models [32–35], the effect of an antibody to IL-4 on MCP-1 message expression was evaluated. The antibody was administered during the reactivation phase as described above. Animals were killed at various time points and total RNA from the ankle joints was analyzed by Northern analysis for MCP-1 message. An appropriate control antibody was used as a control. As the results in **Figure 5** suggest, antibody to IL-4 blocks the expression of mRNA for MCP-1 at 24 and 72 h after reactivation. These results indicate a close relationship between IL-4 and the expression of MCP-1, and perhaps help to explain the anti-inflammatory effect of anti-IL-4 antibodies.

In conclusion, the results from these studies suggest that reactivation arthritis induced by PG-APS is a Th2-related immunological response that is tightly regulated by an array of chemokines, cytokines, and adhesion molecules. In addition to the involvement of IL-1 and TNF-α, the model is highly dependent on neutrophils, and swelling is inhibited by antibodies to MIP-2, a chemokine that is chemotactic for neutrophils. Consistent with Th2-related processes and the neutrophil dependence of the model, antibodies to IL-4, but not interferon-γ, attenuated swelling in the model. This effect appears to be mediated in part by the inhibitory effects of antibodies to IL-4 on MCP-1 expression because MCP-1 is critical for both swelling and T cell infiltration.

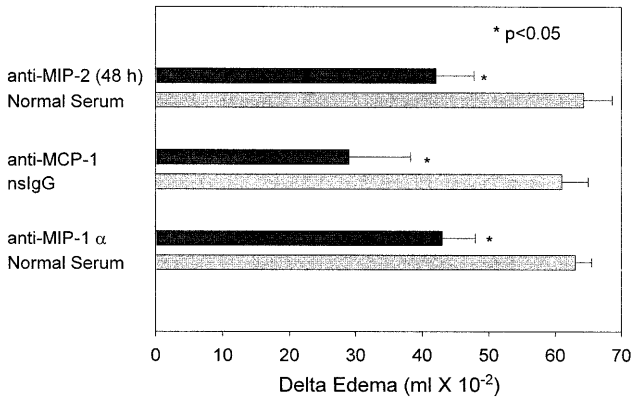

Fig. 4. Changes in ankle volume 72 h (48 h for MIP-2) after the secondary intravenous injection with PG-APS. Animals were treated with antibodies to MIP-2, MCP-1, or MIP-1α. Control animals received an equivalent amount of normal serum or normal serum IgG. Results are shown as the mean ± SEM (n = 7/experimental group).

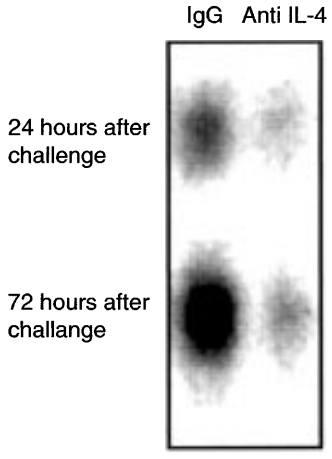

Fig. 5. Changes in expression of MCP-1 mRNA levels 24 and 72 h after the secondary intravenous injection of PG-APS. Animals were treated with antibodies to IL-4. Control animals received an equivalent amount of normal serum IgG. A representative experiment is shown.

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