Dynamics of Early Synovial Cytokine Expression in Rodent Collagen-Induced Arthritis

A Therapeutic Study Using a Macrophage-Deactivating Compound

Karin Palmblad,^{*†} Helena Erlandsson-Harris,^{*} Kevin J. Tracey,[‡] and Ulf Andersson^{*†}

From the Department of Rheumatology, Center for Molecular Medicine, Karolinska Institutet, Stockholm, Sweden; the Department of Rheumatology,† Astrid Lindgren's Children's Hospital, Karolinska Hospital, Stockholm, Sweden; and the Laboratory of Biomedical Science,‡ North Shore University Hospital, Manhasset, New York*

This study was performed to elucidate pathophysiological events before and during the course of collagen-induced arthritis in Dark Agouti rats, a model for rheumatoid arthritis. Kinetic studies of local cytokine responses were determined using immunohistochemical techniques, quantified by computer-assisted image analysis. We recently reported that the macrophage-pacifying agent CNI-1493 successfully ameliorated collagen-induced arthritis. In the present trial, we investigated the potential of CNI-1493 to down-regulate pro-inflammatory cytokines. Synovial cryosections were analyzed at various time points for the presence of interleukin (IL)-1 β , tumor necrosis factor (TNF), and transforming growth factor (TGF)- β . Unexpectedly, an early simultaneous TNF and IL-1 β expression was detected in resident cells in the lining layer, preceding disease onset and inflammatory cell infiltration by >1 week. The predominant cytokine synthesis by synovial (ED1⁺) macrophages coincided with clinical disease. TNF production greatly exceeded that of IL-1 β . CNI-1493 treatment did not affect the early disease-preceding TNF and IL-1 β synthesis in the lining layer. However, after disease onset, CNI-1493 intervention resulted in a pronounced reduced IL-1 β and in particular TNF expression. Furthermore, CNI-1493 significantly up-regulated synthesis of the anti-inflammatory cytokine TGF- β and thereby shifted the balance of pro-inflammatory and anti-inflammatory cytokines in the arthritic joint in a beneficial way. (*Am J Pathol* 2001, 158:491–500)

Rheumatoid arthritis (RA) is a common chronic autoimmune and inflammatory disease. Although the etiology is still unknown there has been recent significant progress in understanding the underlying pathogenetic mechanisms. It is now an established fact that the pro-inflammatory cytokines tumor necrosis factor (TNF) and interleukin 1 (IL-1) are pivotal mediators in the RA process.^{1,2} Controversy persists as to whether TNF-independent IL-1 synthesis occurs in rheumatic synovitis. It has been claimed that TNF induces most of the IL-1 production in the inflamed synovia of RA patients,³ making TNF a prime target for therapy. Clinical trials with patients with active RA revealed that TNF neutralization provides substantial relief of RA symptoms and inflammatory activity.^{4,5} Trials with IL-1 receptor antagonist (IL-1Ra) in RA showed moderate suppression of clinical disease activity and beneficial effects on the progression of joint erosions.⁶

Collagen-induced arthritis (CIA) in susceptible animal strains constitutes a model of autoimmunity that shares a number of pathological, immunological, and genetic features with RA.^{7,8} The Dark Agouti (DA) rat is particularly susceptible and offers a stable, reproducible model with an erosive, chronic polyarthritis developing in 100% of immunized animals when induced with homologous collagen type II in the presence of Freund's incomplete adjuvant.⁸ This allows studies of immunological events preceding disease onset. Expression of CIA can be enhanced by systemic addition of TNF and IL-1, whereas transforming growth factor β (TGF- β) and antibodies to TNF and IL-1 prevented onset of disease.^{9–13} Comparative studies of murine CIA suggested that anti-TNF was effective at the onset of arthritis but less so at later stages, whereas anti-IL-1 administration was also highly effective in established disease, including a reduction of cartilage

Supported by grants from the Swedish Medical Research Council, the Swedish National Cancer Foundation, the Swedish Association against Rheumatism, B. Dahlin's Foundation, B. von Kantzow's Foundation, the Freemason Lodge Barnhuset in Stockholm, N. Svartz' Foundation, af Ugglas' Foundation, and King Gustaf V's Foundation.

Accepted for publication October 17, 2000.

Address reprint requests to Karin Palmblad, M.D., Department of Rheumatology, CMM, L8:04, Karolinska Hospital, S-171 76 Stockholm, Sweden. E-mail: karin.palmblad@cmm.ki.se.

destruction.^{13,14} Uncoupling of joint swelling and ongoing cartilage damage have been suggested as being separate activities of TNF and IL-1, respectively.^{14,15}

Establishing TNF and IL-1 as therapeutic targets has led to a search for other means of blocking their activities. Recently, a tetravalent guanyldiazotization (CNI-1493) was developed as an inhibitor of macrophage activation, which is mediated by a dose-dependent inhibition of the translation of both TNF and IL-1 β mRNAs^{16–18} via interaction with the p38 MAP kinase.¹⁸ In a previous study we could demonstrate that CNI-1493 successfully ameliorated the course of CIA in DA rats after both prophylactic and therapeutic intervention.¹⁹

A question that has not been addressed in RA is the kinetic pattern of pro-inflammatory cytokine expression at early stages of disease. To answer this question, detailed longitudinal studies are required, involving multiple sampling of synovial tissue, which for practical reasons preferably is performed in experimental models. Kinetic studies of synovial cytokine production after onset of clinical disease in murine CIA has been reported.^{20,21} No studies before onset of clinical signs of disease in experimental arthritis have, to our knowledge, previously been published. Neither have studies describing local cytokine expression in rat synovial tissue. The aims of this study were to characterize temporal and spatial changes in synovial cytokine synthesis in DA rats before and after onset of CIA. An additional motive for carrying out this study was to compare the cytokine profile in untreated rats with CIA to that in rats treated prophylactically with the novel cytokine suppressive agent CNI-1493. We have previously developed new immunohistochemical staining methods that enable detection of intracellular cytokine production in rat specimens²² and computerized image analysis for quantification.^{23,24} Here we have analyzed cryopreserved sections from knee joints for the presence of TNF, IL-1 β , and TGF- β at various time points after immunization. Additionally, a phenotypic determination of inflammatory cells in the synovial tissue at the different time points was documented.

Materials and Methods

Animals

Male DA rats, 2 to 3 months old with a mean weight of 220 to 230 g, were kept and bred at the animal unit at the Karolinska Hospital in Stockholm, Sweden. Rats were maintained under climate-controlled conditions with a 12-hour light/dark cycle and were fed standard rodent chow and water *ad libitum*. The health status of the animal colony was monitored according to the guidelines from the Swedish Veterinary Board and reported free from screened pathogens. All procedures in this project were approved by the Ethical Committee of Stockholm North, Sweden.

Induction and Evaluation of CIA

On day 0, DA rats were immunized intradermally at the base of the tail with rat type II collagen emulsified with

Freunds' incomplete adjuvant (Difco, Detroit, MI), as previously described.²⁵ Using this method, chronic polyarthritis is known to develop in 100% of the animals with mean day of onset of clinical disease day 14 after immunization. Inflammatory lesions affecting both forepaws and hindpaws develop with a progressive deterioration of clinical disease until approximately day 21 after immunization. The severity of arthritis thereafter reaches a plateau, with a subsequent transition to a chronic inflammation with subsiding arthritis indices around day 28 after immunization. This chronic phase includes the development of joint ankylosis as well as flares of active arthritis, which affect both previously affected and new joints.

To follow-up the development of the arthritis, the rats were monitored daily for visual inflammatory signs such as erythema and swelling by two observers using a previously described scoring system.¹⁹ Arthritis was graded semiquantitatively with a scale of 0 to 4 for each paw (0, no changes; 1, one type of joint affected; 2, two types of joints affected; 3, three types of joints affected; 4, 3+ maximal erythema and swelling). An arthritis index was calculated for each animal that expresses a cumulative score for all paws with a maximum possible value of 16. A transition from acute to chronic arthritis was established when signs of ankylosis appeared, which was accompanied with a decrease in erythema and swelling and thus in arthritis index.

Although the severity of CIA is generally scored in the paws, profound expression of joint inflammation is concomitantly apparent in the knee joints, which allows a higher degree of standardization of immunohistological sections.

Therapeutic Compound (CNI-1493)

The tetravalent guanyldiazotization CNI-1493 (*N,N'*-bis(3,5-diacetylphenyl) decanediamide tetrakis (amidinohydrazotization) tetrahydrochloride, CAS Reg. No.164301-51-3), was synthesized and purified as previously described.¹⁶ Animals were injected with CNI-1493 intraperitoneally with doses adjusted according to their daily weight. Control animals received equal volumes with the vehicle alone (sterile, deionized water).

Preparation of Samples for Immunohistochemical Analysis

Forty-five animals were included in this kinetic trial and the kinetics of local cytokine expression was determined in untreated and CNI-1493-treated animals, respectively. Three animals that were not immunized were sacrificed at day 0 as normal controls. Thereafter, 21 immunized animals received 5 mg/kg/day of CNI-1493 adjusted to their daily weight and the remaining 21 immunized animals served as controls receiving vehicle alone. At certain time points after immunization six animals were sacrificed, three untreated and three treated with CNI-1493. Three early time points (3, 6, and 10 days after immunization), the time point of expected onset (day 15 after immunization), the time point for expected maximal severity of

arthritis (day 21 after immunization), and the time point for transition to a chronic phase of disease (day 27 after immunization) were selected. The administration of CNI-1493 was withdrawn on day 27 after immunization and three animals from both treatment groups were monitored without treatment until day 38 after immunization, when the trial was terminated.

To examine and compare local immunoreactivity, animals were sacrificed by CO₂ asphyxiation, followed by dissection of the patella with adjacent synovium. The synovial specimens were immediately snap-frozen in isopentane prechilled in dry ice and kept at -70°C until sectioned. Sagittal cryosections of 7- to 8- μ m thickness, encompassing the patella bone with its cartilage and adjacent synovium, were mounted on chromium potassium sulfate and gelatin-coated glass slides (Novakemi, Stockholm, Sweden). Sections were air-dried for 30 minutes and then fixed either for 10 minutes in 2% formaldehyde (Sigma Chemical Co., St Louis, MO) in PBS at room temperature, or in 50% acetone for 30 seconds and 100% acetone for 3 minutes at 4°C. All slides were subsequently stored at -70°C until required for staining. As the arthritic lesions were symmetrical with equal scoring in both hind paws, only one knee specimen per rat was studied.

Immunohistochemical Detection of Intracellular Cytokine Production in Formaldehyde-Fixed Sections

The formaldehyde-fixed cryopreserved sections were stained for intracellular expression of cytokines as previously described.^{19,24} Briefly, permeabilization of the cell membranes was performed by the use of Earle's balanced salt solution (Gibco Ltd., Paisley, UK) supplemented with saponin 0.1% (Riedel de Haen AG, Seelze, Germany) in all subsequent washes and incubation steps. Endogenous peroxidase activity was blocked for 1 hour in the dark with 1% hydrogen peroxide and 2% sodium nitride dissolved in Earle's balanced salt solution-saponin. Sections were thereafter blocked with either 2% normal goat sera or 2% normal human AB-sera for 30 minutes to reduce background staining because of non-specific binding sites. Subsequently, endogenous biotin was blocked with avidin for 30 minutes and biotin for an additional 15 minutes (avidin/biotin blocking kit; Vector Laboratories, Burlingame, CA), both substituted with 0.1% saponin. Thereafter sections were incubated overnight in a humidified chamber with 50 μ l of rat cytokine-specific antibodies: a polyclonal ligand-affinity purified goat anti-rat IL-1 β (AF-501-NA; R&D Systems, Minneapolis, MN), a polyclonal antigen affinity-purified rabbit anti-rat TNF- α (catalog no. CT 061; U-CyTech BV, Utrecht University, The Netherlands) and a monoclonal mouse (IgG₁) anti-rat TGF- β (originally obtained from American Tissue Cultures Collection (ATCC) as a hybridoma and cultured and purified in our laboratory), used at a final concentration of 2 to 5 μ g/ml. Slides were then incubated for 30 minutes with appropriate biotin-labeled antibody (Jackson ImmunoResearch, West Grove, PA): donkey

anti-goat (catalog no. 705-066-147), donkey anti-rabbit (catalog no. 711-066-152), or donkey anti-mouse (catalog no. 715-066-151). They were all Fab₂-fragmented and used at a final concentration of 1:1,000. Thereafter 50 μ l of a solution of Vectastain avidin-biotin-horseradish peroxidase (Vectastain Elite, ABC-kit; Vector Laboratories), prepared according to the directions of the manufacturer, was applied for 30 minutes. After final washes in Earle's balanced salt solution-saponin, the substrate diaminobenzidine (peroxidase substrate kit, Vector) was added. A color reaction was stopped after 5 minutes by washes in deionized water. Sections were counterstained with Mayer's hematoxylin and mounted with buffered glycerol.

Specificity of Cytokine Staining

In each assay, controls for specificity of cytokine stainings were included, based on parallel staining studies omitting the primary antibody or using primary isotype prematched immunoglobulin of irrelevant antigen specificity at the same concentration as the cytokine-detecting antibodies. Additionally, blocking experiments were performed by preabsorption of the cytokine-specific antibodies with their respective target cytokines at a 1:10 molar ratio overnight at 4°C before application to tissues.

Immunohistochemical Detection of Cell Surface Markers on Acetone-Fixed Sections

Phenotypic characterization of cells present in the synovial tissue was performed on separate acetone-fixed slides as previously described.¹⁹ The primary antibodies used for cell surface staining were monoclonal mouse antibodies directed to MHC II (OX-6), α/β -T cell receptor (R73), OX-33 (an isoform of CD45 specific for rat B-cells), and ED1 (a surface membrane antigen expressed on rat macrophages, monocytes and dendritic cells). OX-6 and R73 were originally obtained from ATCC as hybridomas, and cultured and purified in our laboratory. OX-33 and ED1 were purchased from Serotec Ltd (Oxford, UK).

Microscopic Evaluation and Quantification with Computer-Aided Image Analysis

Cytokine and phenotype expression was quantified with a computerized image analysis system, as previously described,^{23,24} by calculating the total positively stained area for a given cytokine or surface marker. Stained tissue sections were evaluated with a Polyvar II microscope (Reichert-Jung, Vienna, Austria) equipped with a three-chip charged couple device color camera (DXC-750P; Sony Corporation, Tokyo, Japan) that digitized the microscope images to be processed in a Quantimet 550S image analyzer (Leica Cambridge, Cambridge, UK) linked to a PC computer. In the present study, analysis of an entire section was defined as all synovial tissue within five microscopic fields (1.2 mm) above and below the patella bone at a magnification of $\times 250$, which typically involved a total of 10 to 42 microscopy fields, depending

on the width of synovial tissue. The stainings for the different cytokines and surface markers were examined in sequential sections to ensure that comparable tissue areas were studied. Using same color detecting thresholds, control quantifications gave reproducible values of the positively stained areas with an intra-assay variation of 2 to 5%. All animals were studied in at least two to three separate staining experiments for each given cytokine or surface marker. Quantifications between stainings for a given antigen on separate sections gave an interassay variation of 5 to 15%.

Statistical Analyses

The differences between CNI-1493-treated and control animals were compared using the Mann Whitney *U* test. *P* values were based on comparisons between the groups at a given time point. A value of *P* < 0.05 was considered as significant.

Results

Evolution of Collagen-Induced Arthritis

The animals had no signs of clinical arthritis at the early time points studied (days 3, 6, and 10 after immunization). At the time point of expected disease onset (day 15 after immunization) there were 12 remaining animals in both groups. Seven of the untreated animals had clinical signs of arthritis, whereas all CNI-1493-treated animals remained healthy. One of the untreated animals remained healthy throughout the trial so that the incidence never reached 100%. At day 21 after immunization eight of nine untreated and one of nine of CNI-1493-treated animals had developed arthritis. Five of six animals had arthritis in both groups day 27 after immunization, with an arthritis index of 8 in the untreated and 4.8 in the CNI-1493-treated animals. Because three untreated animals had developed ankylosis, indicating a transition from acute to a chronic inflammation, treatment was stopped day 27 after immunization. However, as we in an earlier study¹⁹ had experienced that the severity of arthritis promptly increased after CNI-1493 treatment withdrawal, we monitored three rats from each group for an additional 10 days without any therapy to study clinical and immunological consequences (Figure 1).

Phenotypic Characterization and Distribution of Cells in the Synovium

In sections from animals sacrificed before onset of disease (before day 15 after immunization), the synovial tissue appeared nonproliferative, containing only a few cell layers. Some scattered cells stained positive for MHC II and occasionally for the macrophage marker ED1 (Figure 2, D and E). These cells were located mainly as isolated events in the deeper synovial area. An additional MHC II expression of cells in the lining layer was noted from day 6 after immunization on scattered cells and, at

Clinical arthritis

Figure 1. Clinical expression of CIA in CNI-1493-treated animals and controls. Three untreated and three animals treated with CNI-1493 were sacrificed at the time points indicated. Each individual animal is represented with a bar, which shows the arthritis index at time point of sacrifice. CNI-1493 treatment was withdrawn on day 27 after immunization (arrow), and three animals from each group were monitored without therapy until day 38 after immunization, when the trial was terminated.

later time points, on larger proportions of cells. No cells stained positive for α/β -TCR (Figure 2F) or OX-33 at these earlier time points.

Phenotypic characterization of sections after disease onset (day 15 after immunization in untreated animals) revealed a massive cell infiltration, consisting mainly of MHC II⁺ cells (Figure 2D). A large fraction of corresponding areas was ED1⁺ (Figure 2E, also see Figure 4). The predominance of MHC II⁺ and ED1⁺ cells was evident at all stages of disease, after the clinical course reaching a maximal value at day 21 in the untreated control animals. The ED1⁺ cells were observed within the sublining layer and in the pannus area. Only a few cells were ED1⁺ in the thickened synovial-lining layer, but a large fraction was MHC II⁺. A similar distribution of MHC II⁺ and ED1⁺ cells was recorded in sections of CNI-1493-treated animals, but as the degree of inflammation and thus of cell infiltration dominated in untreated animals, a larger number of cells stained positive with significant differences at indicated time points (Figure 2, D and E).

No statistically significant differences were calculated in the distribution of T cells in the two studied animal groups (Figure 2F). Scattered cells expressing α/β -TCR were observed from disease onset (day 15 after immunization), mostly in the deeper layers of the synovia at some distance from cartilage and bone. A stable number of α/β -TCR⁺ cells were noted thereafter throughout the monitoring period. Occasional OX-33⁺ B cells could be detected at later time points in both two groups (data not included).

Location and Morphology of Cytokine Expression before Onset of Arthritis

TNF and IL-1 β expressing cells could be recognized already 3 days after immunization in all six studied animals, which preceded the expected onset of clinical disease by >10 days. These cells were mainly located in

Figure 2. Quantified expression of cytokines and phenotype markers during the course of CIA. Synovial specimens from knee joints were analyzed by immunohistochemistry in animals sacrificed at the time points indicated on the x axis. The expression of the cytokines TNF (A), IL-1 β (B), and TGF- β (C), and the cell surface markers MHC class II (OX-6) (D), surface marker for rat macrophages and monocytes (ED1) (E), and α/β T cell receptor (R73) (F), respectively, were studied and quantified at an original magnification of $\times 250$ using a computerized image analysis system. Each bar represents an individual animal and expresses the total positive area for the given cytokine within 1.2 mm above and below the patella bone. A significant difference ($P < 0.05$) between treatment groups was noted at the time points indicated (asterisk).

the synovial-lining layer, but also to a lesser extent within blood vessel endothelium and occasionally as isolated sublining cells (Figure 3). At this early time point no MHC II or ED1 expression could be detected within the lining layer. A similar distribution of TNF and IL-1 β -producing cells was noted day 6 and day 10 after immunization. In some animals, an additional IL-1 β production was noted

in cells with fibroblast-like morphology within deeper areas of synovial tissue. At these time points before disease onset the synovia was a thin membrane containing only a few cell layers, explaining the low values for total positively stained area. This early disease-preceding synthesis of TNF and IL-1 β in the lining layer was not influenced by CNI-1493 intervention (Figure 2, A and B).

Figure 3. Representative micrographs illustrating brown (diaminobenzidine) immunoperoxidase staining of cryopreserved synovial tissue for expression of TNF (**A**) and IL-1 β (**B**) at an early time point, 3 days after immunization, ie, preceding expected disease onset by 10 days. A thin, nonproliferative synovia is evident at this time point. The TNF- and IL-1 β -producing cells are particularly located in the synovial lining layer but also within the blood vessel endothelium and occasionally as isolated cells. **C:** A sequential section is stained with irrelevant control antibody. Bone is characterized by dark blue staining in all figures. Original magnification, $\times 125$.

Comparison of Cytokine Expression after Day 15 following Immunization

A more evident production of TNF and IL-1 β coincided with onset and progression of clinical disease in untreated animals (Figure 2, A and B; Figure 4, A and B). Accordingly, the maximal production was seen day 21 after immunization, corresponding to the peak of paw swelling and to manifestation of erosive changes in cartilage and bone. TNF production dominated quantitatively with values up to fourfold that of IL-1 β . Particularly in sections with cartilage damage and pannus formation, the local cytokine expressed was mostly TNF although IL-1 β was also detected. A decline in production of these pro-inflammatory cytokines occurred day 27 after immunization, when clinically a transition of the acute inflammation to a chronic phase occurred. The distribution of TNF- and IL-1 β -positive cells was similar with cells detected both within the synovial-lining layer and the deeper synovial tissue. Additionally, scattered cells were distributed in the interstitial tissue, perivascularly, and within vessel endothelium for both cytokines.

Prophylactic therapy with CNI-1493 resulted in a reduction of both pro-inflammatory cytokines, but especially of TNF. At day 21 after immunization the reduction of TNF was 10-fold in CNI-1493-treated animals as compared to untreated animals. Disease onset was delayed in the treatment group. TNF synthesis was down-regulated even at later time points when CNI-1493-treated animals displayed signs of clinical arthritis (Figure 2A and Figure 4E). Intervention with CNI-1493 also resulted in reduction of IL-1 β production (Figure 2B) but the effect was not as evident as for that on TNF.

In addition to cytoplasmatically stained cells, we observed immunoreactivity extending over extracellular areas surrounding cytokine-producing cells. This was particularly evident when staining for TNF, as the detected positive area for TNF at certain time points exceeded the positive area occupied by the presumed producer cells (ED1 $^{+}$) in sequentially stained sections (Figure 4, A and C). The specificities of the extracellular and the intracellular cytokine immunoreactivities were verified by their complete inhibition in blocking experiments with pre-absorption of the cytokine-specific antibody with recombinant target cytokine before staining. No TNF- or IL-1 β -producing cells could be detected in animals that were not immunized.

Distribution and Kinetics of TGF- β -Producing Cells Locally in the Joints

A low TGF- β expression was detected in synovial sections of untreated animals from disease onset and onward. The weak staining observed was localized to the sublining layer on day 15 after immunization and at later time points an additional expression in the lining layer could also be detected. However, in CNI-1493-treated animals a distinct up-regulation of TGF- β was documented that was statistically significant on days 10, 27, and 38 after immunization. At the later time points an

Figure 4. Representative micrographs illustrating brown (diaminobenzidine) immunoperoxidase staining of cryopreserved synovial tissue from arthritic animals. Untreated animal analyzed at the time point of maximal arthritis (day 21 after immunization) with sequential sections stained for expression of TNF (A), IL-1 β (B), ED-1 (C), and an irrelevant isotype-matched control (D). CNI-1493-treated animal with clinical arthritis day 27 after immunization stained for TNF (E) and ED1 (F). Note the marked cell infiltration and area of ED1⁺ cells far exceeding that of TNF-producing cells in sections from the CNI-1493-treated animal. Synovitis, articular cartilage, and bone (dark blue staining) are evident in all figures. Original magnification, $\times 100$.

Figure 5. Representative micrographs illustrating brown (diaminobenzidine) immunoperoxidase staining of cryopreserved synovial tissue day 38 after immunization in a post-CNI-1493-treated animal with CIA (treatment withdrawn day 27 after immunization), stained for TGF- β (**A**) and a sequential section stained with irrelevant control antibody (**B**). Synovitis, articular cartilage, and bone (dark blue staining) are evident in all figures. Original magnification, $\times 100$.

intense immunoreaction for TGF- β was recorded within the synovial lining and sublining layer (Figure 2C and Figure 5A).

Discussion

In the present study we have documented changes in cytokine profiles locally in the inflamed joint before onset and during the course of disease in collagen-induced arthritis in rats. Secondly, we have compared this profile to that in animals treated with the novel cytokine suppressive agent, CNI-1493.

Earlier studies in murine models have suggested a sequential cytokine expression coinciding with clinical disease, with TNF expression preceding IL-1 β .²¹ However, in our rat model TNF and IL-1 β appeared simultaneously, followed by a synchronous peak and decline in expression (Figure 2, A and B). To our knowledge this is the first demonstration of early local cytokine production preceding inflammatory cell infiltration and clinical arthritis. The producer cells of this initial disease-preceding cytokine expression were synoviocytes within the synovial-lining layer, but also cells within blood vessel endothelium (Figure 3). This underlines the potential role of TNF in up-regulating the expression of adhesion molecules on vascular endothelial cells. TNF has also been demonstrated to be chemotactic for monocytes and neutrophils, causing accumulation of these inflammatory cells in the synovium.²⁶ After disease onset macrophages predominated as the major cytokine-producing cells, a finding consistent with earlier studies in murine arthritis models²¹ and RA synovial tissue.^{24,27}

Although the clinical severity of CIA is generally scored in the paws, profound expression of joint inflammation is concomitantly apparent in the knee joints. A great advantage of analysis of knee joints is the higher degree of standardization of immunohistological sections and the

possibility for detailed analysis of cytokine expression in multiple sequential sections. Paw specimens need to be decalcified before cutting, which prevents the usage of our methodology, because the delicate intracellular morphology will be deranged by the chemicals needed for decalcification. In addition, there are major difficulties obtaining a serial of sequential sections with comparable morphology.

After onset of clinical disease, the number of TNF-producing cells clearly exceeded that of IL-1 β , especially in the acute phase of inflammation but also in the chronic phase (Figure 2, A and B). This quantitative TNF dominance compared to IL-1 β in CIA is the opposite result to that we have recorded in human RA using the same methodology.²⁴ One should be cautious with the overall interpretation of data obtained with different antibodies, in view of potential differences in immunostaining capacity. However, we have recently reported that kinetic patterns and quantitative responses of TNF and IL-1 β production were very similar in cultured rat spleen cells²² using the technology of this study to previous results in human blood cell cultures²⁸ and in mouse spleen cultures,²⁹ indicating satisfactory sensitivity of both antibodies. It could also be argued that studies of synovial CIA specimens at much later time points might show a more congruent picture to that in RA with lower TNF expression. The cytokine interplay may be different in acute and advanced arthritis. But in the present trial, we have focused on the time span that is commonly used in evaluating new therapeutic approaches. This further implicates a need for caution when extrapolating findings in animal models to human RA.

Therapeutic cytokine-blocking studies in murine CIA revealed that TNF neutralization gave major suppressive effects when treatment was started shortly before disease onset, but only a marginal effect when given after the arthritis was fully expressed, whereas anti-IL-1 was

also highly effective in established disease.^{13,14} These results could be explained in quantitative terms considering that in experimental arthritis the quantitatively dominating TNF production makes IL-1 a more accessible target for therapeutic down-regulation. The treatment strategy in human RA might be the opposite because the quantitatively higher IL-1 production might require relatively higher doses of IL-1Ra than in CIA for beneficial effects.

We have previously shown that the novel macrophage pacifying compound CNI-1493 can prevent and diminish the severity of CIA.¹⁹ Intervention with CNI-1493 did not have any effect on the early, quantitatively low, disease-preceding synthesis of TNF and IL-1 β in the lining layer. After disease onset, coinciding with macrophage infiltration in the synovium, CNI-1493 clinically resulted in a delayed disease onset and a decrease in arthritis severity with a clear reduction of swelling and a synovitis that was less extensive at all time points when compared to untreated animals. We demonstrate a profound down-regulation of TNF in CNI-1493-treated animals as well as a reduced IL-1 β synthesis although not as evident as for TNF. These findings are in accordance with earlier *in vitro* studies of CNI-1493, when suppression of several other pro-inflammatory cytokines, including IL-1 α and IL-1 β , required 10 to 20 times higher concentrations than needed for TNF inhibition.¹⁷ The profound inhibitory effect of CNI-1493 on TNF synthesis in this TNF-dominating disease implicates that the down-regulatory potential of CNI-1493 in rheumatoid synovitis might even be underestimated based on our present clinical results.

An unexpected finding in this study was that prophylactic intervention with CNI-1493 resulted in an up-regulation of TGF- β (Figure 2C and Figure 5). TGF- β has been mainly implicated as an anti-inflammatory mediator in the synovium with a modulatory role during the progression of arthritis.³⁰ Release of TGF- β by synoviocytes could be an important regulatory mechanism for ameliorating the destruction induced by IL-1 and TNF of cartilage and bone. The balance of proinflammatory cytokines and anti-inflammatory cytokines will dictate the magnitude of the inflammatory response. We hypothesize that CNI-1493-mediated up-regulation of TGF- β production might be one of the mechanisms by which CNI-1493 ameliorates CIA. However, the question remains unanswered whether this TGF- β up-regulation was a direct effect of CNI-1493 on producer cells, or was indirectly caused by the reduced levels of TNF and IL-1 β . The effect of p38 MAP kinase on cytokine expression has been studied most extensively in activated monocytes/macrophages. Stimulators of the phosphorylation of p38 MAP kinase (eg, lipopolysaccharide) cause the release of TNF and IL-1, but do not always activate the release of TGF- β , which is constitutively produced by monocyte cultures. p38 MAP kinase has been implicated in the regulation of the release of TGF- α ,³¹ but evidence for the direct regulation of TGF- β by p38 is lacking. In contrast to previous reports of CIA in rats,³² only a low number of cells stained positive for TGF- β in synovial sections of untreated animals in our study. A possible explanation for this could be that the antibody used in this study recognized TGF- β 1 and

TGF- β 3 but not TGF- β 2. This TGF- β up-regulation apparent in the CNI-1493-treated animals contradicts the notion that the down-regulatory effects on TNF and IL-1 β were caused by nonspecific toxicity of the compound.

In conclusion, this study provides new insights into early cytokine expression by the unequivocal demonstration of local TNF and IL-1 β synthesis in resident cells more than a week before disease onset and inflammatory cell infiltration. We also determined that after disease onset, TNF production clearly exceeded that of IL-1 β , a result in contrast to that in human RA in which the reverse situation is established. Characterizing animal models and elucidating differences are crucial prerequisites when extrapolating findings in experimental systems to the human clinical situation.

Acknowledgment

We thank Assoc. Prof. R. A. Harris for rewarding linguistic advice.

References

1. Brennan M, Maini RN, Feldmann M: TNF- α —a pivotal role in rheumatoid arthritis? *Br J Rheum* 1992; 31:293–298
2. Feldmann M, Brennan FM, Chantry D, Haworth C, Turner M, Abney E, Buchan G, Barrett K, Barkley D, Chu A, Field M, Maini RN: Cytokine production in the rheumatoid joint: implications for treatment. *Ann Rheum Dis* 1990; 49:480–486
3. Brennan FM, Chantry D, Jackson A, Maini R, Feldmann M: Inhibitory effect of TNF alpha antibodies on synovial cell interleukin-1 production in rheumatoid arthritis. *Lancet* 1989; 2:244–247
4. Elliott MJ, Maini RN, Feldmann M, Kalden JR, Antoni C, Smolen JS, Leeb B, Breedveld FC, Macfarlane JD, Bijl H, Woody JN: Randomized double-blind comparison of chimeric monoclonal antibody to tumour necrosis factor α (cA2) versus placebo in rheumatoid arthritis. *Lancet* 1994; 344:1105–1110
5. Moreland LW, Schiff MH, Baumgartner SW, Tindall EA, Fleischmann RM, Buöppitt KJ, Weaver AL, Keystone EC, Furst DE, Mease PJ, Rederman EM, Horwitz DA, Arkfeld DG, Garrison L, Burge DJ, Bloch CM, Lange MLM, McDonnell ND, Weinblatt ME: Etanercept in rheumatoid arthritis. *Ann Intern Med* 1999; 130:478–486
6. Bresnihan B, Alvaro-Gracia JM, Cobby M, Doherty M, Domljan Z, Emery P, Nuki G, Pavelka K, Rau R, Rozman B, Watt I, Williams B, Aitchison R, McCabe D, Musick P: Treatment of rheumatoid arthritis with recombinant human interleukin-1 receptor antagonist. *Arthritis Rheum* 1998; 41:2196–2204
7. Staines N, Wooley P: Collagen arthritis—what can it teach us? *Br J Rheum* 1994; 33:798–807
8. Larsson P, Kleinau S, Holmdahl R, Klareskog L: Homologous type II collagen-induced arthritis in rats. Characterization of the disease and demonstration of clinically distinct forms of arthritis in two strains of rats after immunization with the same collagen preparation. *Arthritis Rheum* 1990; 33:693–701
9. Thorbecke GJ, Shah R, Leu CH, Kuruvilla AP, Hardison AM, Palladino MA: Involvement of endogenous tumour necrosis factor α and transforming growth factor β during induction of collagen type II arthritis in mice. *Proc Natl Acad Sci USA* 1992; 89:7375–7379
10. Hom JT, Bendele AM, Carlson DG: In vivo administration with IL-1 accelerates the development of collagen-induced arthritis in mice. *J Immunol* 1988; 141:834–841
11. Cooper WO, Fava RA, Gates CA, Cremer MA, Townes AS: Acceleration of onset of collagen-induced arthritis by intra-articular injection of tumour necrosis factor or transforming growth factor-beta. *Clin Exp Immunol* 1992; 89:244–250
12. Williams RO, Feldmann M, Maini RN: Anti-tumour necrosis factor ame-

- liorates joint disease in murine collagen-induced arthritis. *Proc Natl Acad Sci USA* 1992, 89:9784–9788
13. Joosten LAB, Helsen MMA, van de Loo FAJ, van den Berg WB: Anticytokine treatment of established type II collagen-induced arthritis in DBA/1 mice. A comparative study using anti-TNF α , anti-IL-1 α/β , and IL-1Ra. *Arthritis Rheum* 1996, 39:797–809
14. Joosten LA, Helsen MM, Saxne T, van De Loo FA, Heinegard D, van Den Berg WB: IL-1 alpha beta blockade prevents cartilage and bone destruction in murine type II collagen-induced arthritis, whereas TNF-alpha blockade only ameliorates joint inflammation. *J Immunol* 1999, 163:5049–5055
15. van den Berg WB, Joosten LAB, Kollias G, van de Loo FAJ: Role of tumor necrosis factor α in experimental arthritis: separate activity of interleukin 1 β in chronicity and cartilage destruction. *Ann Rheum Dis* 1999, 58:140–148
16. Bianchi M, Ulrich P, Bloom O, Meistrell MR, Zimmerman GA, Schmidt-mayerova H, Bukrinsky M, Donnelly T, Bucala R, Sherry B, Manogue KR, Tortolani AJ, Cerami A, Tracey KJ: An inhibitor of macrophage arginine transport and nitric oxide production (CNI-1493) prevents acute inflammation and endotoxin lethality. *Mol Med* 1995, 1:254–266
17. Björk L, Tracey KJ, Ulrich P, Bianchi M, Cohen PS, Åkerlund K, Fehniger T, Andersson U, Andersson J: Targeted suppression of cytokine production in monocytes but not in T lymphocytes by a tetravalent guanlylhydrazone (CNI-1493). *J Infect Dis* 1997, 176:1303–1312
18. Cohen PS, Nakshatri H, Dennis J, Caragine T, Bianchi M, Cerami A, Tracey KJ: CNI-1493 inhibits monocyte/macrophage tumor necrosis factor by suppression of translational efficiency. *Proc Natl Acad Sci USA* 1996, 93:3967–3971
19. Åkerlund K, Erlandsson-Harris H, Tracey KJ, Wang H, Fehniger T, Klareskog L, Andersson J, Andersson U: Anti-inflammatory effects of a new tumour necrosis factor-alpha (TNF- α) inhibitor (CNI-1493) in collagen-induced arthritis (CIA) in rats. *Clin Exp Immunol* 1999, 115:32–41
20. Mussener Å, Litton M, Lindroos E, Klareskog L: Cytokine production in synovial tissue of mice with collagen-induced arthritis (CIA). *Clin Exp Immunol* 1997, 107:485–493
21. Marinova-Mutafchieva L, Williams RO, Mason LJ, Mauri C, Feldmann M, Maini RN: Dynamics of proinflammatory cytokine expression in the joints of mice with collagen-induced arthritis (CIA). *Clin Exp Immunol* 1997, 107:507–512
22. Palmblad K, Andersson U: Identification of rat IL-1 β , IL-2, IFN- γ and TNF- α in activated splenocytes by intracellular immunostaining. *Bio-tech Histochem* 2000, 75:101–109
23. Cunnane GC, Björk L, Ulfgrén A-K, Lindblad S, FitzGerald O, Bresnahan B, Andersson U: Quantitative analysis of synovial membrane inflammation: a comparison between automated and conventional microscopic measurements. *Ann Rheum Dis* 1999, 58:493–499
24. Ulfgrén A-K, Gröndal L, Lindblad S, Khademi M, Johnell O, Klareskog L, Andersson U: Interindividual and intra-articular variation of proinflammatory cytokines in patients with rheumatoid arthritis: potential implications for treatment. *Ann Rheum Dis* 2000, 59:439–447
25. Andersson M, Holmdahl R: Analysis of type II collagen-reactive T cells in the mouse. I. Different regulation of autoreactive vs. non-autoreactive anti-type II collagen T cells in the DBA/1 mouse. *Eur J Immunol* 1990, 20:1061–1066
26. Tak PP, Taylor PC, Breedveld FC, Smeets TJ, Daha MR, Kluin PM, Meinders AE, Maini RN: Decrease in cellularity and expression of adhesion molecules by anti-tumor necrosis factor alpha monoclonal antibody treatment in patients with rheumatoid arthritis [see comments]. *Arthritis Rheum* 1996, 39:1077–1081
27. Chu CQ, Field M, Feldmann M, Maini RN: Localization of tumor necrosis factor α in synovial tissues and at the cartilage-pannus junction in patients with rheumatoid arthritis. *Arthritis Rheum* 1991, 34:1125–1132
28. Sander B, Andersson J, Andersson U: Assessment of cytokines by immunofluorescence and the paraformaldehyde-saponin procedure. *Immunol Rev* 1991, 119:65–93
29. Sander B, Höiden I, Andersson U, Möller E, Abrams JS: Similar frequencies and kinetics of cytokine producing cells in murine peripheral blood and spleen: cytokine detection by immunoassay and intracellular immunostaining. *J Immunol Methods* 1993, 166:201–214
30. Palladino MA, Morris RE, Starnes HF, Levinson AD: The transforming growth factor-betas. A new family of immunoregulatory molecules. *Ann NY Acad Sci* 1990, 593:181–187
31. Fan H, Derynck R: Ectodomain shedding of TGF- α and other transmembrane proteins is induced by receptor tyrosine kinase activation and MAP kinase signaling cascades. *EMBO J* 1999, 18:6962–6972
32. Mussener Å, Funa K, Kleinau S, Klareskog L: Dynamic expression of transforming growth factor-betas (TGF- β) and their type I and type II receptors in the synovial tissue of arthritic rats. *Clin Exp Immunol* 1997, 107:112–119