

# Gamma Interferon and Interleukin-10 Gene Expression in Synovial Tissues from Patients with Early Stages of *Chlamydia*-Associated Arthritis and Undifferentiated Oligoarthritis and from Healthy Volunteers

SHIGERU KOTAKE,<sup>1†</sup> H. RALPH SCHUMACHER, JR.,<sup>1,2</sup> THURAYYA K. ARAYSSI,<sup>1</sup> HERVE C. GÉRARD,<sup>3</sup> PATRICK J. BRANIGAN,<sup>4</sup> ALAN P. HUDSON,<sup>3</sup> CHERYL H. YARBORO,<sup>1</sup> JOHN H. KLIPPEL,<sup>1</sup> AND RONALD L. WILDER<sup>1\*</sup>

National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, Maryland<sup>1</sup>; Department of Veterans Affairs Medical Center<sup>2</sup> and Department of Microbiology and Immunology, MCP-Hahnemann School of Medicine,<sup>4</sup> Philadelphia, Pennsylvania; and Department of Immunology and Microbiology, Wayne State University Medical School, Detroit, Michigan<sup>3</sup>

Received 28 July 1998/Returned for modification 28 September 1998/Accepted 12 February 1999

**Genetically determined differences in interleukin-10 (IL-10) and gamma interferon (IFN- $\gamma$ ) responses in mice correlate with clearance of *Chlamydia pneumoniae* infection. We measured the synovial expression of IL-10 and IFN- $\gamma$  and additional cytokine genes in patients who had recent-onset *Chlamydia*-associated arthritis (Chl-AA). IL-10 and IFN- $\gamma$  mRNA were relatively abundant in recent-onset Chl-AA.**

*Chlamydiae* are intracellular bacterial pathogens of eukaryotic cells and are responsible for a wide variety of important human and animal infections (21). Reactive arthritis (ReA) is triggered by infection of the urogenital tract with *Chlamydia trachomatis*, the upper respiratory tract with *Chlamydia pneumoniae*, or the gastrointestinal tract with *Yersinia*, *Salmonella*, *Shigella*, or *Campylobacter* (4). In *Chlamydia*-associated arthritis (Chl-AA), chlamydial RNA and/or DNA are present in synovial tissues, and organisms are frequently observed by electron microscopic evaluation or in situ hybridization (2, 15). The organisms are hypothesized to act as a proinflammatory foreign body, initiating the clinical response (20). Moreover, a recent report indicated that *Chlamydia* infection inhibits cell death through apoptosis (6). Cytokines also appear to play important roles in the persistent infection of *C. trachomatis*. Gamma interferon (IFN- $\gamma$ ), the main cytokine produced by Th1 and NK cells, may have a dual role in chlamydial infection, potentially mediating both host resistance and immunopathology within synovial tissue (20). In support of this hypothesis, a recent study showed that genetically determined differences in IFN- $\gamma$  and interleukin-10 (IL-10) responses in mice correlated with clearance of *C. trachomatis* mouse pneumonitis infection (22). Most recently, Perry et al. suggested that the bulk of early chlamydial clearance from the genital mucosa of a murine model is mediated by an IL-12-dependent, IFN- $\gamma$ -independent mechanism and that prevention of disseminated disease requires the action of IFN- $\gamma$  (13). Since very little is known regarding IFN- $\gamma$ , IL-10, or IL-12 expression in synovial tissues from patients with Chl-AA, particularly in the early stages of disease, we addressed this issue in the present study.

From a series of 200 patients with early arthritis, we identi-

fied and analyzed 35 non-rheumatoid arthritis (RA) patients with oligoarthritis of 12 or fewer months of duration who were taking neither prednisone nor second-line antirheumatic drugs. We diagnosed Chl-AA in six patients who were rheumatoid factor negative, had inflammatory mono- or asymmetric oligoarticular arthritis, and exhibited the presence of *C. trachomatis* or *C. pneumoniae* DNA in synovial tissues. Clinical characteristics and laboratory findings of these six patients are shown in Table 1. The other 29 patients with arthritis did not fulfill criteria for any established diagnosis and were classified as undifferentiated oligoarthritis (UO) patients. Chlamydial DNA was not detected in these synovial specimens of UO patients, nor did these patients have any apparent clinically relevant triggering infections prior to the onset of synovitis; i.e., none of the UO patients met criteria for ReA. We also excluded, in addition to patients with RA, patients with psoriatic arthritis, systemic lupus erythematosus, and osteoarthritis. We also analyzed six normal volunteers (NV) who had no evidence of clinical illness. Patients were recruited by physician referral and participated in the biopsy study as part of a protocol (94-AR-0194) approved by an institutional review board from the National Institutes of Health.

Chlamydial DNA encoding 16S rRNA in synovial tissue was detected in synovial specimens of all Chl-AA patients and one of six NV (NV 3) by PCR (3, 7). Primary transcripts from the chlamydial rRNA operons were detected by reverse transcription-PCR (RT-PCR) in three of the six Chl-AA patients (8, 9) (Table 1), suggesting the presence of viable, metabolically active *Chlamydia* in those synovial tissues. In addition, all specimens were also screened for *Borrelia* (OspA) DNA and conserved panbacterial 16S rRNA. All specimens were negative for *Borrelia* DNA, and only the *Chlamydia*-positive specimens were positive with the panbacterial screen.

We quantitated the amounts of 10 cytokines (tumor necrosis factor alpha [TNF- $\alpha$ ], IL-1 $\beta$ , IFN- $\gamma$ , IL-2, IL-12 p40, IL-15, IL-4, IL-6, IL-10, and IL-13) and CD3  $\delta$ -chain mRNAs, using a nested RT-PCR technique as previously reported in detail (10, 11). Briefly, we obtained two to five synovial specimens by

\* Corresponding author. Mailing address: ARB, NIAMS, NIH, 10 Center Dr. MSC 1820, Bldg. 10, Rm. 9N228, Bethesda, MD 20892-1820. Phone: (301) 496-6499. Fax: (301) 402-0012. E-mail: wilderr@arb.niams.nih.gov.

† Present address: Institute of Rheumatology, Tokyo Women's Medical College, Shinjuku-ku, Tokyo 162, Japan.

TABLE 1. Clinical characteristics and laboratory findings of six patients with recent-onset *Chlamydia*-associated arthritis

Patient <sup>a</sup>	Age (yr)/sex <sup>b</sup>	Duration of arthritis (mo)	Chlamydial DNA <sup>c</sup> in synovial tissue	Primary rRNA transcripts <sup>d</sup>	Serum IgG <sup>e</sup> to <i>C. trachomatis</i>	Associated clinical features	HLA-B27
1	22/F	3	+, <i>tr.</i>	–	–	Monoarth.	–
2	52/M	2	+, <i>tr.</i>	+	Low positive	Oligoarth.	+
3	19/M	1	+, <i>pn.</i>	NES	–	Monoarth.	–
4	25/F	1	+, <i>tr.</i>	+	–	Monoarth.	–
5	31/F	5	+, <i>tr.</i> (SF)	+	–	Monoarth.	–
6	30/F	6	+, <i>tr.</i>	NES	Positive	Oligoarth.	+

<sup>a</sup> None had previously received antibiotics.

<sup>b</sup> F, female; M, male.

<sup>c</sup> Chlamydial DNA sequences targeted were the 16s rRNA and MOMP genes (3, 8). *tr.*, *Chlamydia trachomatis*; *pn.*, *Chlamydia pneumoniae*; SF, synovial fluid.

<sup>d</sup> Assays targeting primary transcripts from the chlamydial rRNA operons were performed as described in reference 9. NES, not enough sample.

<sup>e</sup> Specialty Laboratories, San Monica, Calif.

closed-needle biopsy using a Parker Pearson needle from a single tender joint from each patient as previously described (10, 11). Total RNA was prepared from all specimens by the Tri Reagent method. Complementary DNA was synthesized from mRNA by priming total RNA isolated from each whole synovial specimen. We normalized all samples for  $\beta$ -actin cDNA content by competitive PCR and quantitated the amount of each cytokine mRNA in each synovial specimen with a nested PCR technique. The first PCR was conducted with outer sense and outer antisense primers. The nested PCR was conducted with inner sense and inner antisense primers (11). The nested RT-PCR technique is at least 1,000 times more sensitive than a single round of RT-PCR alone and allowed us to analyze each of the small synovial specimens obtained by needle biopsy (11). Polyacrylamide gel electrophoresis of the nested PCR products revealed zero to three bands, depending on the concentration of target cDNA. The relative amounts of each cytokine and CD3 $\delta$  cDNA were graded (4+, 3+, 2+, 1+, or 0) on the basis of the pattern of bands generated by nested PCR. Then, the mean value of the mRNA grades of four synovial specimens from a single joint was calculated. The data were analyzed by using the Spearman's rank correlation coefficient and Mann-Whitney test (StatView; Abacus Concepts Inc., Berkeley, Calif.). A significant difference was defined as  $P < 0.05$ .

Figure 1A shows the relative levels of mRNA for proinflammatory cytokines (TNF- $\alpha$  and IL-1 $\beta$ ), type 1 cytokines (IL-2, IL-12 p40, IL-15, and IFN- $\gamma$ ), and type 2 cytokines (IL-4, IL-6, IL-10, and IL-13) in each synovial specimen for all NV. We detected IL-10 and IL-15 mRNA in at least one specimen from all NV. IFN- $\gamma$  mRNA was detected in only two of six NV. Both IFN- $\gamma$  and IL-10 were detected in NV 2 and NV 3. Interestingly, *C. trachomatis* DNA was also detected in synovial specimens from NV 3, who had no evidence of clinically apparent illness. TNF- $\alpha$  and/or IL-1 $\beta$  mRNAs were detected in specimens from four of the NV. IL-2, IL-4, or IL-13 mRNA was not detected in the NV. Figure 1B and C show cytokine profiles in synovial specimens from all six Chl-AA patients and six representative UO patients, respectively. IFN- $\gamma$  and IL-10 mRNAs were detected in virtually all of the specimens from Chl-AA patients. IL-10 mRNA was detected in all of the specimens from patients with UO, while IFN- $\gamma$  mRNA was not detected in three of the UO patients. TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-15 mRNAs were also frequently detected in Chl-AA patients. IL-2 and IL-12 p40 mRNAs were detected in three of the Chl-AA patients, while IL-4 and IL-13 mRNAs were not detected. Figure 2 shows the relative mRNA levels for IFN- $\gamma$  and IL-10 for all 35 patients and 6 NV studied. The Chl-AA patients clearly had more IFN- $\gamma$  and IL-10 mRNA than did UO patients or NV. The levels of IFN- $\gamma$  and IL-10 mRNA were

significantly higher in Chl-AA patients than in UO patients ( $P = 0.007$  and  $0.014$ , respectively) or than in NV ( $P = 0.011$  and  $0.033$ , respectively). The level of IFN- $\gamma$  mRNA was significantly higher in UO patients than in NV ( $P = 0.027$ ). The levels of other cytokine mRNAs, including IL-12 p40 and IL-4, did not differ between Chl-AA and UO patients. The levels of CD3  $\delta$ -chain mRNA were significantly higher in Chl-AA patients than in UO patients ( $P = 0.001$ ). These results indicate that the number of T cells was higher in Chl-AA patients than in UO patients, because CD3  $\delta$  chain is expressed on the surface of T cells as a part of T-cell receptor. CD3  $\delta$ -chain mRNA was also detected in all NV.

The most notable finding in our study was that mRNA levels of both IFN- $\gamma$  and IL-10 were significantly higher in early Chl-AA patients than early UO patients or NV (Fig. 2). Byrne et al. and Shemer-Avni et al. have reported that IFN- $\gamma$  functions as a cytotoxic cytokine against *Chlamydia*-infected fibroblasts (5, 16). Rank et al. reported that anti-IFN- $\gamma$  antibody treatment resulted in significantly prolonged murine chlamydial genital infection and that passive administration of recombinant IFN- $\gamma$  to chronically infected mice was able to bring about resolution of the infection (14). Thus, their data suggest that IFN- $\gamma$  may play a role in regulating the growth and differentiation of *Chlamydia* in the tissues. The production of IFN- $\gamma$  is, however, probably antagonized by the presence of IL-10. Yang et al. have published data supporting the hypothesis that excessive IL-10 production in BALB/c mice inhibits Th1-like responses, including IFN- $\gamma$  expression and the delayed-type hypersensitivity response following chlamydial infection, and consequently delays resolution of the infection (22). Beatty et al., using an in vitro cell culture system, have demonstrated that the effect of IFN- $\gamma$  on *Chlamydia* is dose dependent (1). Persistent infection is established with low-dose IFN- $\gamma$  and is characterized by the development of noninfectious atypical chlamydial forms, atypical reticulate bodies, that show near-normal levels of the 60-kDa heat shock protein, an immunopathologic antigen, and a paucity of the major outer membrane protein, a protective antigen (1). Moreover, Nanagara et al. reported *C. trachomatis* infection in synovial tissues from patients who had either early or chronic ReA and that most persistent organisms were atypical reticulate bodies with diminished major outer membrane protein (12). In our study, the levels of IL-10 in the Chl-AA group may have been high because of the corresponding high levels of TNF- $\alpha$  and IL-1. In addition, our data suggest that by antagonizing cellular immunity and the production of IFN- $\gamma$ , IL-10 may lead to persistent *C. trachomatis* infection in synovial tissue and that the atypical reticulate bodies of *Chlamydia* are likely to be the predominant form in synovial tissues of Chl-AA.

Simon et al. reported that T cells cloned from the synovial

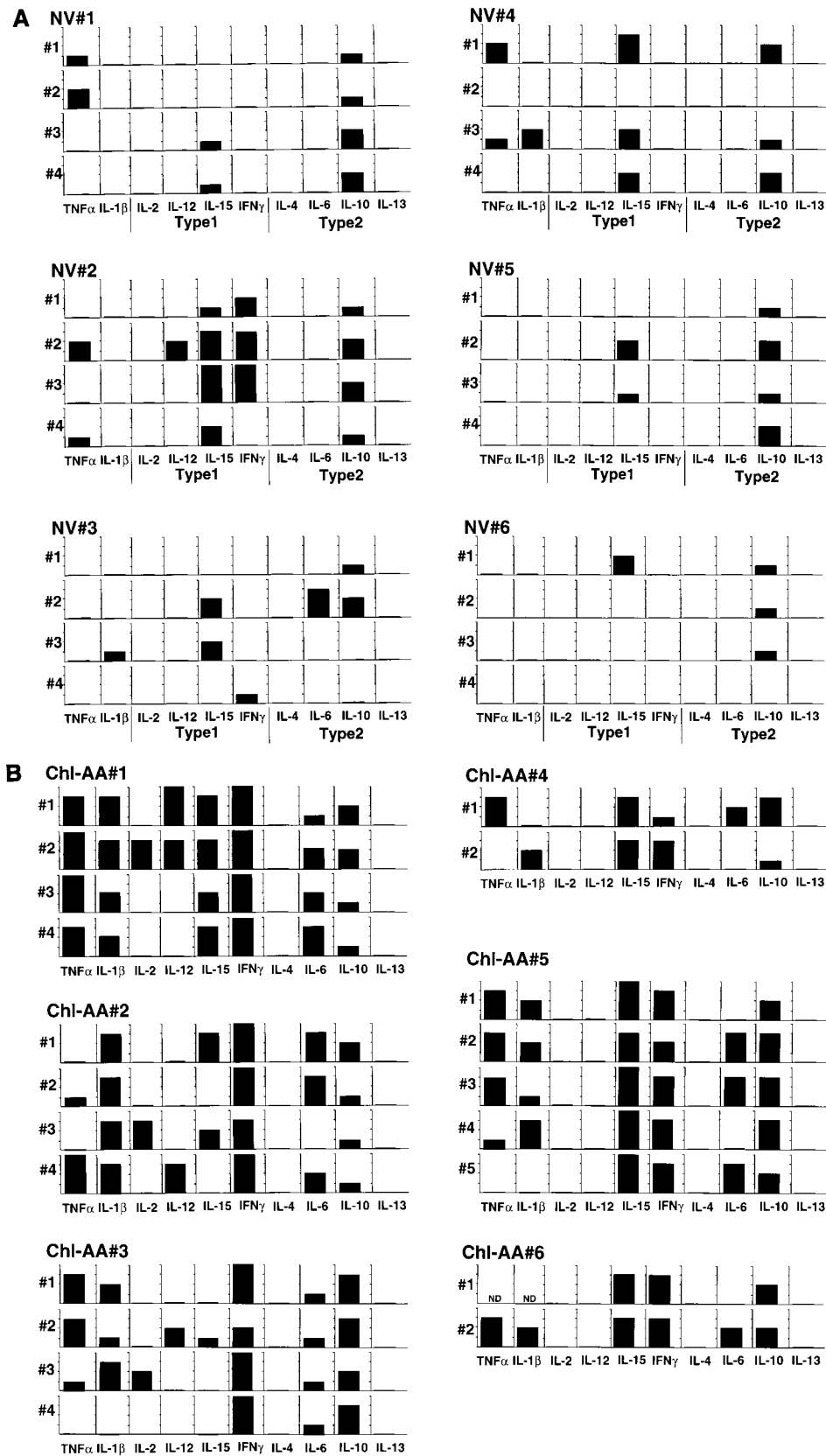


FIG. 1. Relative expression levels of cytokine mRNAs in 6 NV (A), 6 Chl-AA patients (B), and 6 representative UO patients of 29 UO patients (C). The relative amounts of mRNA of CD3  $\delta$  chain and each cytokine in synovial tissues from each patient were quantitated, and the mean value was calculated as described in the text. The relative amounts of the cytokine mRNAs are shown as 0, 1+, 2+, 3+, and 4+ on the y axis. ND, not done. The numbers on the y axis represent the synovial specimen number from each subject.

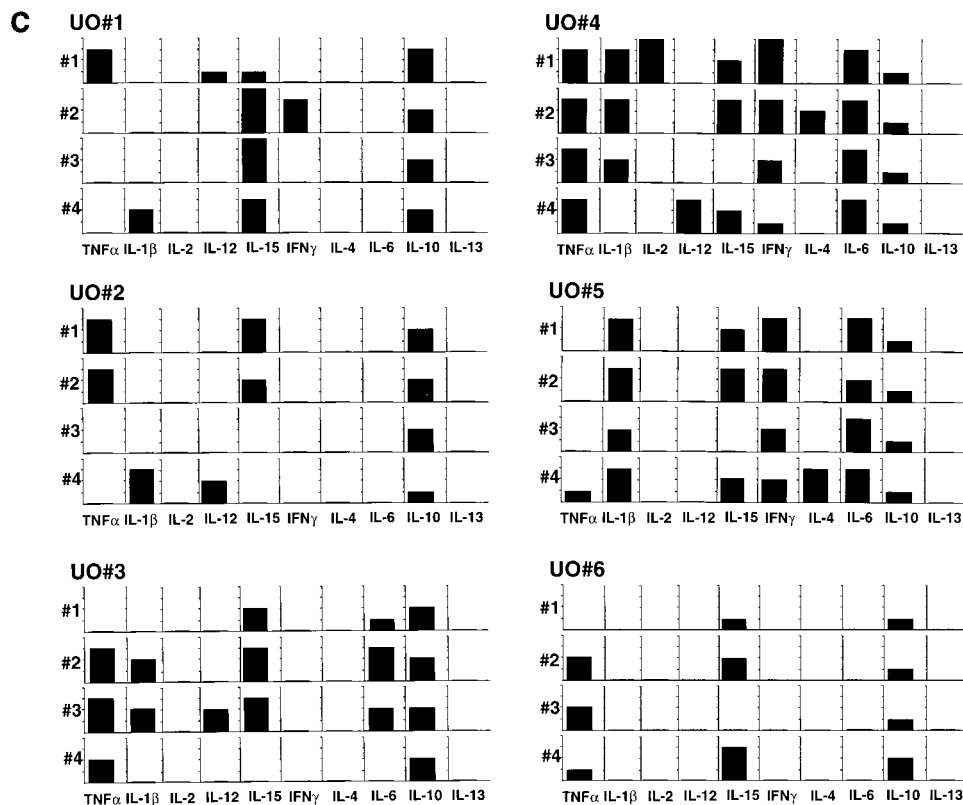


FIG. 1—Continued.

fluids of patients with chlamydial ReA yielded a predominantly Th1 cytokine pattern (19). However, later work suggested that this Th1 predominance might have resulted from bias introduced by the cloning procedure or from insensitive methods of detecting the Th2 cytokine IL-4 (4). Simon et al. also reported that IFN- $\gamma$  mRNA was found in both ReA and RA synovial membranes, while IL-4 mRNA was detected almost exclusively in ReA membranes (4, 18). They also proposed that IL-4 possibly mediates bacterial persistence in the joint by inhibition of IFN- $\gamma$  effects (17). In contrast, we recently reported that we rarely detected IL-4 mRNA in synovial specimens from patients with early ReA or RA (10). In the present study, we also rarely detected IL-4 mRNA in synovial specimens from patients with Chl-AA (Fig. 1B). Our data suggest that IL-10, rather than IL-4, is the major inhibitor of IFN- $\gamma$  production and cellular immunity in early Chl-AA, because we detected IL-10 mRNA but neither IL-4 nor IL-13 mRNA in Chl-AA patients (Fig. 1B, 1C, and 2). Our data may differ from those of Simon et al. (18) for several reasons. First, our analytic techniques and synovial tissue procurement differed, and we analyzed only patients with less than 12 months of disease who were taking neither prednisone nor second-line antirheumatic drugs, with or without chlamydial DNA-positive synovial tissues. Simon et al. examined a group of nine ReA patients that included patients with both early and chronic ReA following infection with not only *Chlamydia* but also *Yersinia*, *Salmonella*, *Shigella*, or *Borrelia*. Medications were not mentioned in their study.

More recently, Yin et al. reported that a Th2 cytokine pattern predominates in the joints of patients with ReA, and they suggested that since Th1 cytokines are necessary for the elimination of ReA-associated bacteria, Th2 cytokines might con-

tribute to bacterial persistence in the joint. In addition, they suggested that the IL-10–IL-12 balance is crucial for regulation of the cytokine pattern in the joints of patients with ReA (23). However, in detection of IL-4, our results also differ from those of Yin et al. We observed that IL-4 gene expression was rare in early disease. They reported a relatively higher number of IL-4-positive cells compared with the number of IFN- $\gamma$ -secreting cells in synovial specimens. Although they studied synovial tissues from the four patients with ReA immunohistologically, three of the patients with Chl-AA had chronic-stage

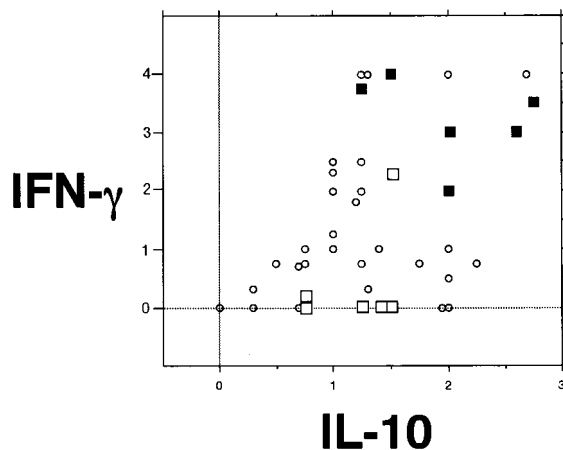


FIG. 2. Relative levels of IL-10 and IFN- $\gamma$  mRNAs in synovial tissue of Chl-AA patient (■), UO patients (○), and NV (□).

disease (durations of 36, 18, and 36 months, respectively). Medications were not mentioned in their study (23).

We also analyzed cytokine mRNA profiles in 24 synovial tissue specimens obtained from 6 NV and compared these data to those for 6 Chl-AA and 29 UO patients taking neither prednisone nor second-line antirheumatic drugs. Cytokine mRNA profiles in NV varied. IL-10 and IL-15 mRNAs were frequently detected, although the amounts of these cytokine mRNAs were smaller than those in synovial tissue from Chl-AA patients (Fig. 1A versus 1B). Thus, our results may indicate that the IL-10 and IL-15 genes are constitutively expressed in the normal synovial tissue. In contrast to the frequent detection of IL-10 and IL-15, IFN- $\gamma$  mRNA was detected only in synovial specimens from NV 2 and NV 3. Moreover, synovial specimens from NV 3 were *C. trachomatis* DNA positive, although the patient did not have clinical symptoms. Thus, the detection of both IL-10 and IFN- $\gamma$  mRNAs in synovial specimens of NV 3 is similar to the cytokine profile of Chl-AA patients as discussed above. The cytokine profile of NV 3 may represent an asymptomatic and extremely early phase of Chl-AA. We are now following up the clinical and laboratory findings of NV 3 to evaluate this possibility.

In summary, our present study indicates that IFN- $\gamma$  and IL-10 are relatively abundant in synovial tissue of early Chl-AA patients. We suggest that high IL-10 levels facilitate persistent chlamydial infection of synovial tissue by antagonizing cellular immunity and the generation of IFN- $\gamma$ -dependent mechanisms required to clear the organism. Our data clearly encourage additional study on the role of host factors in determining the outcome of synovial chlamydial infection.

We express our appreciation to George Poy for oligonucleotide synthesis.

This work was supported in part by NIH grant AR-42541 to A.P.H.

#### REFERENCES

- Beatty, W. L., G. I. Byrne, and R. P. Morrison. 1993. Morphologic and antigenic characterization on interferon  $\gamma$ -mediated persistent *Chlamydia trachomatis* infection *in vitro*. Proc. Natl. Acad. Sci. USA **90**:3998-4002.
- Beutler, A. M., J. A. Whittum-Hudson, R. Nanagara, H. R. Schumacher, Jr., and A. P. Hudson. 1994. Intracellular location of inapparently-infecting *Chlamydia* in synovial tissue from patients with Reiter's syndrome. Immunol. Res. **13**:163-171.
- Branigan, P. J., H. C. Gérard, A. P. Hudson, and H. R. Schumacher, Jr. 1996. Comparison of synovial tissue and synovial fluid as the source of nucleic acids for detection of *Chlamydia trachomatis* by polymerase chain reaction. Arthritis Rheum. **39**:1740-1746.
- Burmester, G. R., A. Daser, T. Kamradt, A. Krause, N. A. Mitchison, J. Sieper, and N. Wolf. 1995. Immunology of reactive arthritis. Annu. Rev. Immunol. **13**:229-250.
- Byrne, G. I., C. S. Schobert, D. M. Williams, and D. A. Kaege. 1989. Characterization of gamma interferon-mediated cytotoxicity to chlamydia-infected fibroblasts. Infect. Immun. **57**:870-874.
- Fan, T., H. Lu, H. Hu, L. Shi, G. A. McClarty, D. M. Nance, A. H. Greenberg, and G. Zhong. 1998. Inhibition of apoptosis in *Chlamydia*-infected cells: blockade of mitochondrial cytochrome c release and caspase activation. J. Exp. Med. **187**:487-496.
- Gérard, H. C., P. J. Branigan, H. R. Schumacher, Jr., and A. P. Hudson. 1995. Screening of synovial tissue from reactive arthritis patients for the presence of *Chlamydia pneumoniae*, abstr. 1444, p. S394. In Abstracts of the 59th National Scientific Meeting of the American College of Rheumatology 1995. American College of Rheumatology, San Francisco, Calif.
- Gérard, H. C., J. A. Whittum-Hudson, and A. P. Hudson. 1997. Genes required for assembly and function of the protein synthetic system in *Chlamydia trachomatis* are expressed early in elementary to reticulate body transformation. Mol. Gen. Genet. **255**:637-642.
- Gérard, H. C., P. J. Branigan, H. R. Schumacher, Jr., and A. P. Hudson. 1998. Synovial *Chlamydia trachomatis* in patients with reactive arthritis/Reiter's syndrome are viable but show aberrant gene expression. J. Rheumatol. **25**:734-742.
- Kotake, S., H. R. Schumacher, Jr., C. H. Yarbboro, T. K. Arayssi, J. A. Pando, K. S. Kanik, M. F. Gourley, J. H. Klippel, and R. L. Wilder. 1997. *In vivo* gene expression of type 1 and type 2 cytokines in synovial tissues from patients in early stages of rheumatoid, reactive and undifferentiated arthritis. Proc. Assoc. Am. Phys. **109**:286-302.
- Kotake, S., H. R. Schumacher, Jr., and R. L. Wilder. 1996. A simple nested RT-PCR method for quantitation of the relative amounts of multiple cytokine mRNAs in small tissue samples. J. Immunol. Methods **199**:193-203.
- Nanagara, P., L. I. Feng, A. M. Beutler, A. P. Hudson, and H. R. Schumacher, Jr. 1995. Alteration of *Chlamydia trachomatis* biologic behavior in synovial membrane. Arthritis Rheum. **38**:1410-1417.
- Perry, L. L., K. Feilzer, and D. Caldwell. 1997. Immunity to *Chlamydia trachomatis* is mediated by T helper 1 cells through IFN- $\gamma$ -dependent and -independent pathways. J. Immunol. **158**:3344-3352.
- Rank, R. G., K. H. Ramsey, E. A. Pack, and D. M. Williams. 1992. Effect of gamma interferon on resolution of murine chlamydial genital infection. Infect. Immun. **60**:4427-4429.
- Schumacher, H. R., Jr., S. Magge, P. V. Cherian, J. Sleckman, S. Rothfuss, G. Clayburne, and M. Sieck. 1988. Light and electron microscopic studies on the synovial membrane in Reiter's syndrome. Immunocytochemical identification of chlamydial antigen in patients with early disease. Arthritis Rheum. **31**:937-946.
- Shemer-Avni, Y., D. Willach, and I. Sarov. 1988. Inhibition of *Chlamydia trachomatis* growth by recombinant tumor necrosis factor. Infect. Immun. **56**:2503-2506.
- Sieper, J., and J. Braun. 1995. Pathogenesis of spondylarthropathies. Persistent bacterial antigen, autoimmunity, or both? Arthritis Rheum. **38**:1547-1554.
- Simon, A. K., E. Seipelt, and J. Sieper. 1994. Divergent T-cell cytokine patterns in inflammatory arthritis. Proc. Natl. Acad. Sci. USA **91**:8562-8566.
- Simon, A. K., E. Seipelt, P. Wu, B. Wenzel, J. Braun, and J. Sieper. 1993. Analysis of cytokine profiles in synovial T cell clones from chlamydial reactive arthritis patients: predominance of the Th1 subset. Clin. Exp. Immunol. **94**:122-126.
- Toivanen, T., and A. Toivanen. 1995. Role of micro-organisms in the pathogenesis of arthritis: lessons from reactive and Lyme arthritis. Scand. J. Rheumatol. **24**(Suppl. 101):191-197.
- Ward, M. E. 1995. The immunobiology and immunopathology of chlamydial infections. APMIS **103**:769-796.
- Yang, X., K. T. HayGlass, and R. C. Brunham. 1996. Genetically determined differences in IL-10 and IFN- $\gamma$  responses correlate with clearance of *Chlamydia trachomatis* mouse pneumonitis infection. J. Immunol. **156**:4338-4344.
- Yin, Z., J. Braun, L. Neure, P. Wu, L. Liu, U. Eggens, and J. Sieper. 1997. Crucial role of interleukin-10/interleukin-12 balance in the regulation of the type 2 T helper cytokine response in reactive arthritis. Arthritis Rheum. **40**:1788-1797.

Editor: R. N. Moore