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Divergent T-cell cytokine patterns in inflammatory arthritis

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ABSTRACT **A major immunoregulatory mechanism in inflammatory infections and allergic diseases is the control of the balance of cytokines secreted by Th1/Th2 subsets of T helper (Th) cells. This might also be true in autoimmune diseases; a Th2 pattern that prevents an effective immune response in infections with intracellular bacteria may favor immunosuppression in autoimmune diseases. The pattern of cytokine expression was compared in the synovial tissue from patients with a typical autoimmune disease, rheumatoid arthritis, and with a disorder with similar synovial pathology but driven by persisting exogenous antigen, reactive arthritis. We screened 12 rheumatoid and 9 reactive arthritis synovial tissues by PCR and *in situ* hybridization for their expression of T-cell cytokines. The cytokine pattern differs significantly between the two diseases; rheumatoid arthritis samples express a Th1-like pattern whereas in reactive arthritis interferon γ expression is accompanied by that of interleukin 4. Studying the expression of cytokines by *in situ* hybridization confirmed the results found by PCR; they also show an extremely low frequency of cytokine-transcribing cells. In a double-staining experiment, it was demonstrated that interleukin 4 is made by CD4 cells. These experiments favor the possibility of therapeutic intervention in inflammatory rheumatic diseases by means of inhibitory cytokines.**

The control of the balance of cytokines secreted by differing T-cell subsets is emerging as a major immunoregulatory mechanism (1, 2). The division of T helper (Th) cells into Th1 [secreting interferon γ (IFN-γ) and interleukin (IL) 2] and Th2 (secreting IL-4 and IL-5) found in the mouse (3) holds good for humans (2), provided that attention is focused on IL-4 (4). Cytokines of the Th1 spectrum are generally elevated in successful responses to a variety of intracellular pathogens (5), and Th2 cytokines are elevated in allergic diseases and in helminth infections (6, 7). The balance appears to be maintained not only by the cytokines considered originally to be of Th1/Th2 type but also by other inhibitory cytokines such as transforming growth factor β and Il-10, which are not confined to one of the original subsets and can additionally be secreted by non-T cells (8, 9). In this context the pattern of T-cell cytokines in reactive and rheumatoid arthritis is of particular interest. Not only is rheumatoid arthritis among the commonest of the candidate autoimmune diseases, it also offers easy access to the site of inflammation. Reactive and rheumatoid arthritis are sister diseases, in the sense that both are characterized by inflammation of joints and the development of similar synovial pathology. Reactive arthritis is triggered by intracellular bacteria that persist in the joint (10, 11), whereas for rheumatoid arthritis, the triggering events are unknown but thought not to involve persistent infection. Comparison of the two diseases should be informative.

The importance of T cells in initiating and maintaining inflammation in the rheumatic diseases is still controversial. Several groups have detected macrophage/monocyte cytokines in the inflamed joint (12), but it has been less easy to detect T-cell cytokines (13). This has led to the suggestion that the pathogenesis of arthritis is mediated solely by macrophages and their effector cytokines (14), although the alternative view is that T-cell cytokines may be important but are expressed at levels too low for detection by conventional methods (13).

To address this issue, the T-cell cytokine pattern of synovial membranes from patients with rheumatoid arthritis and reactive arthritis was examined by PCR and in most cases also by *in situ* hybridization, thus providing cross-validation of the two methods. Such methods offer the most sensitive available means, as exemplified by their success in detecting low level transcription in the thymus (15). The main finding is of significantly differing cytokine patterns: in rheumatoid arthritis Th1 but not Th2 cytokines were often found, whereas in reactive arthritis the Th2 cytokine Il-4 was often detected in addition to Th1 cytokines.

MATERIALS AND METHODS

Patients. Twelve patients had active rheumatoid arthritis fulfilling at least four of the seven American College of Rheumatology (ACR) criteria, and 9 patients had reactive arthritis following infection with *Chlamydia, Yersinia, Salmonella, Shigella*, or *Borrelia*. Control biopsies from the gut of a patient suffering from Crohn disease and from an ulcerative colitis patient were also included. Synovial membrane was obtained by synovectomy (10 patients), diagnostic arthroscopy (4 patients), or needle biopsy of the knee joint (7 patients). Samples were placed in OCT medium (Miles) or normal saline for PCR analysis and snap-frozen in liquid nitrogen. As a control, peripheral blood lymphocytes (PBLs) from a healthy donor were stimulated with Con A (10 μg/ml) for 24 h, then diluted with nonstimulated PBLs, and subjected to RNA extraction or cytospun onto slides.

RNA Extraction and Reverse Transcription. Total cellular RNA was isolated from snap-frozen synovial membranes by using guanidinium thiocyanate buffer for disruption of the tissue followed by centrifugation through cesium chloride gradient (16). Approximately 1 μg of precipitated RNA was reverse-transcribed by following the manufacturer's recommendations (Superscript, GIBCO/BRL). Samples were then heated to 95°C for 10 min and stored at −20°C for further use.

PCR. PCR amplification was performed on a Biometra thermocycler (Göttingen, Germany) using a 35-cycle program consisting of denaturation at 94°C for 1 min, annealing at 65°C (55°C for Il-2 and IFN-γ) for 2 min, and extension at 72°C for 1 min. Each reaction mixture contained PCR buffer (Promega)/3 mM $MgCl_2$/all four dNTPs (each at 0.4 mM)/200 nM of primers (TIB MolBiol, Berlin)/1.5 units of *Taq* polymerase (Promega) to which was added 1 μl of the total 20-μl cDNA reaction mixture. The sequence of oligonucle-

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Abbreviations: Th, T helper; IFN-γ, interferon γ; IL, interleukin; PBL, peripheral blood lymphocyte.

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otide primers specific for β-actin, CD3δ chain, IFN-γ, Il-2, IL-4, and IL-10 have been published (17). PCR results on the same samples were reproducible.

Radioactive Hybridization of PCR Products. To verify PCR results, PCR products were electrophoresed and transferred to Quiabrane nylon membranes (Diagen, Dusseldorf, Germany) by standard methods (16). Blots were hybridized under RNase-free conditions to ^{35}S-labeled RNA probes (prepared as described in the RNA probe section) at 6 ng/ml and washed with Rapid Hybridization buffer by following the supplier's recommendations (Amersham).

RNA Probes. Complementary RNA probes were prepared by subcloning cytokine cDNA fragments into the run-off transcription vector pGEM1 (Promega). All plasmids used were sequenced. After linearization with appropriate restriction enzymes, antisense and sense probes were generated by transcription, with incorporation of ^{35}S-labeled nucleotides (NEN/DuPont) as described (18). The probes for human IFN-γ and IL-4 were kindly provided by Hermann Herbst (Institut für Pathologie, Klinikum Steglitz, Berlin) and for the human IL-10 cDNA were provided by Cornelia Platzer (Institut für Immunologie, Klinikum Steglitz, Berlin).

***In Situ* Hybridization.** Synovial membranes were cryostat-sectioned to 5 μm thickness. Alternatively, stimulated cells were cytospun onto pretreated slides at 500 rpm for 5 min in a Shandon centrifuge (Runcorn, U.K.). Fixation was in 4% (vol/vol) formaldehyde, and hybridization was performed as described (18). The stringent washing conditions generated a high signal/noise ratio but reduced the number of cells scored as positive. There was no signal detected on slides hybridized to the sense probe. Slides were coated with photo emulsion (LM1, Amersham) and developed after 10–30 days.

Combined Immunohistology and *in Situ* Hybridization. Antibodies used were to CD4, CD68, and mouse immunoglobulins (Dakopatts, Glostrup, Denmark). The APAAP (immunoalkaline phosphatase) complex was purchased from Immunotech (Luminy, France). Immobilized antibodies were detected by the APAAP method (19) and slides were immediately subjected to *in situ* hybridization.

RESULTS

Concordance of PCR and *in Situ* Hybridization Results. The concordance between the results of PCR and *in situ* hybridization is shown in Fig. 1. Of the 14 tissues examined in parallel for IFN-γ, 8 gave concordant results, and of the 14 tissues examined in parallel for IL-4, 11 proved concordant.

To determine the sensitivity of the PCR, a dilution of Con A-stimulated PBLs was performed. IL-4 PCR product was still detectable in 1×10^5 PBLs on an ethidium bromide-stained gel, whereas hybridization detected the product down to 1×10^3 PBLs (data not shown). This lower number gave a signal comparable to that of synovial membranes. The lowest detection level, therefore, detected ≈10 IL-4-positive T cells, as *in situ* hybridization of the same PBLs showed that only 1% CD3 lymphocytes were positive for IL-4. Considering the very small number of cytokine-expressing cells, it is likely that PCR as applied here to a whole biopsy would detect mRNA with higher sensitivity; this would explain the biopsies scored as positive by PCR but negative by *in situ* hybridization. In only one sample was IL-4 mRNA detected by *in situ* hybridization but not by PCR. In that case, the two methods were applied to different biopsies taken at the same time. Some of the samples could not be analyzed by PCR for any of the cytokines because β-actin was not detected. The samples omitted from analysis by *in situ* hybridization usually contained no lymphocytic infiltrations.

Cytokine Patterns by PCR Analysis. Synovial membranes of 10 rheumatoid arthritis patients and of 8 reactive arthritis patients were examined by reverse transcriptase PCR for

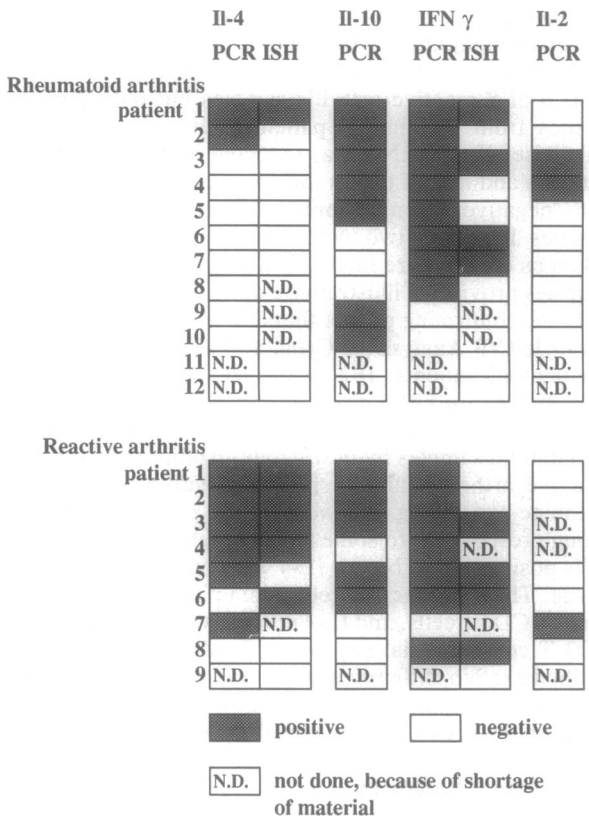

FIG. 1. PCR and *in situ* hybridization (ISH) on synovial membranes.

IL-2 and IFN-γ mRNA (Th1 markers), for IL-4 mRNA (Th2 marker), and for IL-10 mRNA [an inhibitory cytokine, in humans not confined to the Th1 or Th2 subset (20)]. In 8 of 10 rheumatoid arthritis patients and in 7 of 8 reactive arthritis patients, mRNA for IFN-γ was detected by PCR (Fig. 2), and 7 of 10 rheumatoid arthritis and 5 of the 8 examined reactive arthritis patients were positive for IL-10 mRNA. The 35-cycle PCR did not yield a visible mRNA band for IL-4 or IL-2

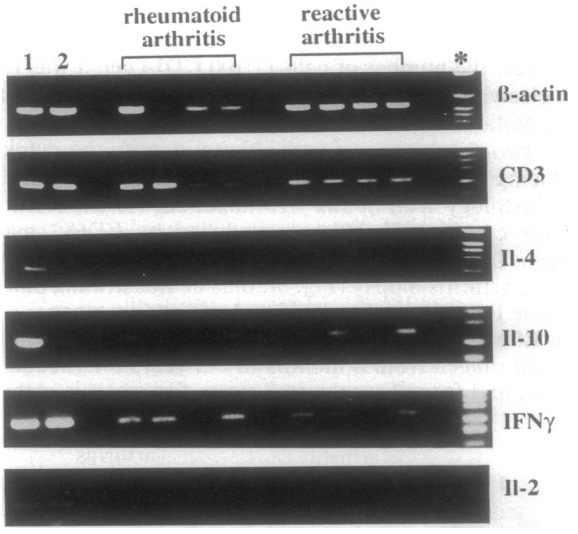

FIG. 2. PCR amplification of cytokine mRNAs with specific primers for β-actin, CD3δ, and various cytokines. Sample 1 is a control, 10^6 PBLs from a healthy donor stimulated for 24 h with Con A; sample 2 is a control, Crohn disease biopsy. Synovial membranes of four rheumatoid arthritis patients and synovial membranes of four reactive arthritis patients are also shown. Lane * contains molecular mass markers.

from either type of synovial membrane. However, the more-sensitive method of hybridizing a radioactive probe to the PCR products did sometimes yield a band (Fig. 3). By this test, 6 of 8 reactive arthritis samples but only 2 of the 10 tissues from rheumatoid patients were positive for IL-4. Likewise, IL-2 mRNA was detected in 1 of the 6 reactive arthritis and in 2 of the 10 rheumatoid samples, which were also negative when examined by normal PCR (Fig. 1). Tissues from the two nonarthritic inflammatory diseases taken as controls are included in Fig. 2. The Crohn disease biopsies provide an illustrative example of unbiased cytokine pattern, confirming results found in other studies (21).

Single-Cell Analysis of Cytokine Patterns. In 4 of the 10 rheumatoid patients, IFN-γ mRNA was detected in single cells by *in situ* hybridization, and 4 of 7 reactive arthritis patients were positive. IL-4 mRNA was detected in 1 of 8 rheumatoid samples by *in situ* hybridization, while 5 of the 8 reactive arthritis samples proved positive (Fig. 1). These cytokine-expressing cells were always present in clusters, as though responding to some local stimulus (Fig. 4). Very few cells positive for IFN-γ or IL-4 were present in the inflamed tissue. The frequency of cells containing IFN-γ mRNA was 1 in 300 CD3 T cells, and IL-4 mRNA was found in >1 in 1000 CD3 T cells in serial sections stained with the appropriate antibodies. No difference was apparent in the number or location of IFN-γ-positive cells between the two diseases or in other features of the synovial histology other than IL-4 expression, which supported the overall similarity of their inflammatory processes.

The difference in IL-4 frequency between the two diseases is statistically significant. Fisher's exact test yields the following probabilities: for number of patients positive by PCR or *in situ* hybridization, rheumatoid arthritis was 2 of 12 and reactive arthritis was 7 of 9, $P < 0.001$; for number of patients positive by both PCR and *in situ* hybridization, rheumatoid arthritis was 1 of 12 and reactive arthritis was 4 of 9, $P < 0.1$; for total number of tests positive, rheumatoid arthritis was 3 of 19 and reactive arthritis was 11 of 16, $P < 0.01$.

In addition to T-cell cytokines, samples were also examined for expression of mRNA for IL-1β as a representative macrophage cytokine in inflamed synovial membranes. As expected, the majority of samples from both diseases proved positive: 5 of 8 synovial membranes from reactive arthritis and 5 of 8 from rheumatoid arthritis (data not shown). However, the number of cells (1:100 CD14 cells) was lower than elsewhere (22), probably because of the stringent washing conditions.

For two cytokines we were able to identify the cytokine-producing cells by their surface markers by combining immunohistology with *in situ* hybridization. As expected, the effector cytokine IL-1β was produced by CD68$^+$ macrophages. IL-4 was produced by CD4$^+$ lymphocytes in a reactive arthritis biopsy (Fig. 5), thus demonstrating that this cytokine is made by T cells in at least one instance.

No Effect of Disease Duration. In this study, the disease duration ranged from 6 months to >2 years for rheumatoid arthritis and from 3 weeks to 3 years for reactive arthritis,

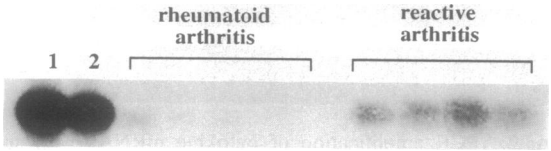

FIG. 3. Hybridization of PCR products with a radiolabeled IL-4 probe. Sample 1 is a control, 10^6 PBLs; sample 2 is a control, 5×10^5 PBLs from a healthy donor stimulated for 24 h with Con A. Synovial membranes of four rheumatoid arthritis patients and synovial membranes of four reactive arthritis patients are also shown.

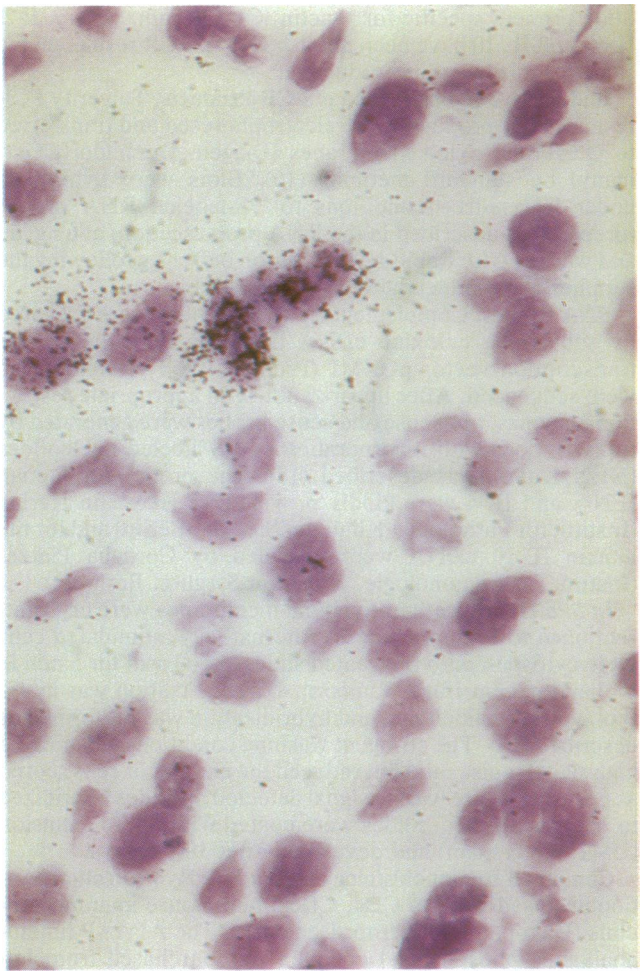

FIG. 4. Rheumatoid arthritis synovial membrane hybridized to IFN-γ RNA probe. (×1000.)

with no trend in cytokine production to be detected by PCR or by single-cell analysis (data not shown).

DISCUSSION

In this study, cytokine patterns were assessed in rheumatoid and reactive arthritis by PCR and *in situ* hybridization. T-cell cytokine mRNA was detected in the majority of samples, and previous failure to find it (12) was probably due to insensitive assays. Both methods gave satisfactorily concordant results. They provide information only of transcriptional activity (i.e., the presence of mRNA) and do not necessarily imply that cytokine protein is synthesized or secreted (23). The detection of these cytokines at the inflammation site strengthens the case for T cells being responsible for inducing and maintaining arthritis (13), although monocytes and synovial lining cells and their products may nevertheless play the principal role in joint inflammation and tissue destruction (14).

The major finding was a marked difference in the frequency of IL-4-positive samples between the two diseases, with a significantly higher number of reactive arthritis samples expressing IL-4 mRNA. This contrasts with the similar overall frequencies of IFN-γ- and IL-10-positive samples in the two diseases. Studies in infectious disease and in allergy have identified IL-4 as the crucial cytokine distinguishing Th1 and Th2 subsets in humans (refs. 24–26; see Introduction). Here, T cells in the rheumatoid arthritis synovial membrane secrete predominantly a Th1 cytokine pattern, whereas in reactive arthritis IL-4 is expressed as well. Contrary to initial indications in the mouse, IL-10 is not regarded as a cytokine

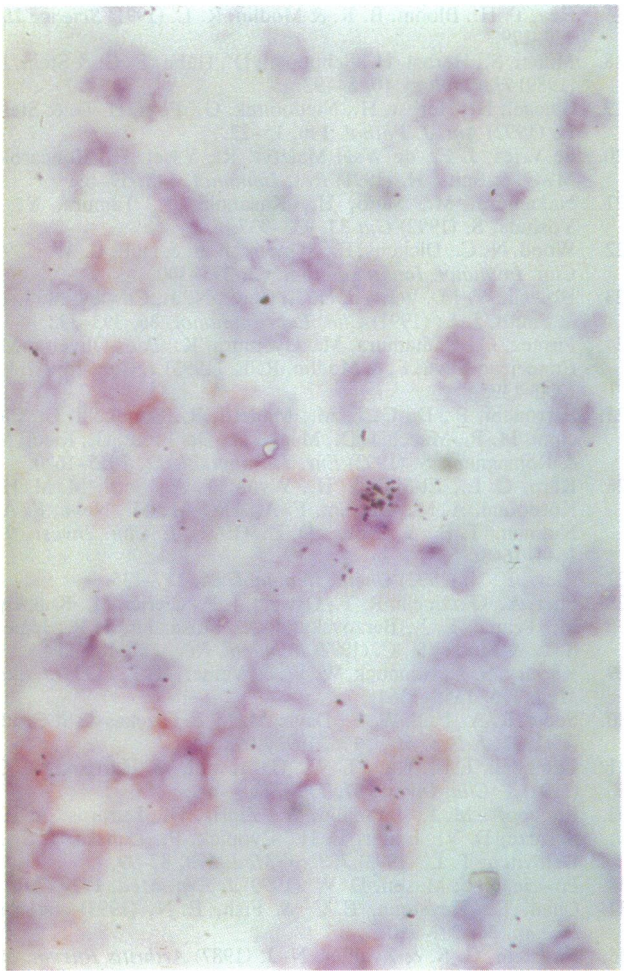

FIG. 5. Reactive arthritis synovial membrane hybridized to IL-4 RNA probe after immunohistological staining of CD4 (red staining). (×1000.)

discriminating between Th1 and Th2 cells (17, 24), and it behaved accordingly in the present study. There was no trend in cytokine pattern related to disease progression, although our data do not exclude that there is one that, early in these diseases, could be of great interest, particularly as possible key players in maintaining the disease states.

This study sets the two inflammatory arthritic diseases within a cytokine landscape hitherto populated mainly by infectious diseases and by atopy. Two leading examples are leishmaniasis (27) and leprosy (17), where Th1 cells exert a protective effect and sometimes also mediate hypersensitivity, subject in both cases to down-regulation by Th2 cells. Like reactive arthritis, these diseases are caused by intracellular pathogens protected against by Th1 cytokines that activate macrophages. The reverse occurs in helminth infections (28).

There is no previous work on cytokine balance in human autoimmune disease with which the present study can be compared. Animal models suggest that preferential activation of Th1 responses is central to the pathogenesis of these diseases. Thus in experimental allergic encephalitis, a leading model of autoimmunity, IFN-γ, IL-2, and tumor necrosis factor α are secreted at the peak of disease and IL-4 and transforming growth factor β are secreted during recovery (29). IL-4 probably participates in suppressing inflammation by down-regulating the macrophage-activating cytokine IFN-γ (30). In nonobese diabetic mice IFN-γ production correlates with disease activity (31), and treatment with anti-IFN-γ antibody or IL-4 prevents onset of the disease (31, 32). Rats can be rendered diabetic by manipulations favoring the Th1 subset, and the otherwise doomed Th1-manipulated rat can be protected by the transfer of Th2 cells (33).

Attempts to detect these antagonistic subsets in inflammatory arthritis *in vivo* have so far been unsuccessful (34). Low levels of IFN-γ (35) and IL-2 (23, 36) mRNAs but not IL-2 protein (23) have been found. There are two reports of Th2 cytokines in synovial fluid or synovial membranes with conflicting results (34, 36). No attempts to detect IL-5 or IL-10 in inflamed joint tissue have been reported. Only in rheumatoid arthritis serum and synovial fluid was an increase in IL-10 levels found (37). *In vitro* T-cell cloning has been reported from rheumatoid arthritis synovial membrane (38) and from synovial fluid of reactive arthritis (39–41); this procedure, with its attendant bias, yields clones mainly of Th1 type, with some production of IL-10 (41).

The amount of cytokine mRNA was small, but so it is even in the infectious diseases where they are believed to play such an important role (2, 26). This study suggests that past difficulties in detecting T-cell cytokines in inflammatory arthritis can be explained by the extremely low frequency of cytokine-expressing cells. At first sight the relevance of these few cells might seem doubtful. However, the production of these cytokines is probably restricted to the minority of T cells that have recently undergone antigenic stimulation, and there is increasing evidence that only a few such cells are needed to maintain an inflammatory lesion. Thus only 2% of T cells from tuberculoid leprosy skin lesions respond to the bacterial antigen (42). In multiple sclerosis, the frequency of T cells responding to myelin-oligodendrocyte glycoprotein in the cerebrospinal fluid is 1:450 (43), and only 1:1000 T cells from synovial fluid in reactive arthritis respond to the bacterial antigen that triggered the disease (44). Additionally, studies on the frequencies of cytokine-expressing cells have shown that only between 0.1 and 1% of all cells present in leprosy lesions express IFN-γ (45). The frequencies observed here are thus in line with those reported previously for other forms of immunopathology.

The combined immunohistology/*in situ* hybridization experiment showed IL-4 secretion by CD4 T cells, but we have no information yet for IFN-γ and IL-10. No doubt a more detailed identification of the cytokine-secreting cells is needed, although this is unlikely to change our view of the importance of these cytokines. After all, evidence is increasing that differentiation into Th1 or Th2 cells is initiated by cytokines secreted by cells of the innate immune system (46). Thus IFN-γ is secreted by natural killer cells and promotes the development of the Th1 subset and so does IL-12 expressed by macrophages (47). The initial source of IL-4 expression, which induces the Th2 subset, is not known yet (46). Therefore, even if the cytokines identified here are not themselves produced by T cells, they would nevertheless be expected to influence the differentiation of these cells.

IL-10 is expressed in both T-cell subsets and also by non-T cells in the inflammatory diseases leprosy and leishmaniasis (24, 26). Regardless of the source of IL-10, like IL-4 (30), it probably contributes to the pathogenesis of arthritis by suppressing the bactericidal effector mechanisms of activated macrophages.

The association of rheumatoid arthritis with the major histocompatibility complex class II gene *DR4* and of ReA with the major histocompatibility complex class I gene *B27* cannot be explained by our data. However, recent evidence suggests that CD8 T cells may also divide functionally into two types, corresponding to Th1 and Th2 CD4 cells (4, 48).

In conclusion, T-cell cytokine transcription was detected in many synovial membranes from both rheumatoid and reactive arthritis patients. The frequency of cytokine-transcribing cells is extremely low, reflecting probably the low frequency of antigen-specific cells in the inflamed joint tissue. In rheumatoid synovial membrane, these few cells display a strong bias toward

the Th1 cytokine pattern, with expression of IFN-γ but not of IL-4. On the other hand, in reactive arthritis the transcription of IFN-γ and IL-2 is usually accompanied by that of IL-4. Under these different circumstances, both of these patterns can be regarded as detrimental, and a case can be made for their playing a causal role in both diseases, rather than merely reflecting disease activity. Could not rheumatoid arthritis resemble the Th1-manipulated rat, with cofactors such as HLA DR4, to account for the particular form of autoimmunity that emerges? As for reactive arthritis, the usual outcome of infection with *Chlamydia* or the other triggering organisms is cure, presumably reflecting the crucial role of Th1 cells in combating intracellular infections (5). A failure to mount this form of protective immune response, brought about by the inhibitory effect of IL-4, could explain why only a minority of patients normally develop the disease in chronic form. If these arguments are accepted, they would strengthen the case for therapeutic intervention by means of inhibitory cytokines or anti-cytokine antibodies. As we come to learn more about the differentiation of Th1 and Th2 cells in early and late stages of these inflammatory rheumatic diseases, it should become possible to design appropriate forms of intervention, including perhaps therapy by implantation of cytokine genes (49). The present study contributes to that enterprise by focusing attention on IL-4 as a candidate agent for intervention in rheumatoid arthritis, and on IFN-γ or perhaps better IL-12 (50) or anti-IL-4 antibodies (27) for reactive arthritis. In a subject as difficult as this, the present data and their implications are unlikely to be accepted at all readily. Therapy will be the judge. Indeed the same may be said of the whole concept of cytokine balance: its importance will be judged by its ability to predict, formulate, and guide various forms of clinical intervention.

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