A Distinct Multicytokine Profile Is Associated with Anti-Cyclical Citrullinated Peptide Antibodies in Patients with Early Untreated Inflammatory Arthritis

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ABSTRACT. ***Objective.*** Early inflammatory arthritis is clinically heterogenous and biologically-based indicators are needed to distinguish severe from self-limited disease. Anti-cyclical citrullinated peptides (CCP) have been identified as potential prognostic markers in early arthritis cohorts. Since cytokine networks are known to play a critical role in the pathogenesis of rheumatoid arthritis (RA) and other forms of inflammatory arthritis, a panel of pro- and antiinflammatory cytokines was measured to identify biologically-based subsets of early arthritis, relating cytokine profiles to clinical measures and to the presence of RA-associated autoantibodies.

Methods. Plasma concentrations of cytokines [interleukin 1ß (IL-1ß), IL-2, IL-4, IL-5, IL-6, IL-7, CXCL8 (IL-8), IL-10, IL-12p70, IL-13, IL-17, granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), interferon-γ (IFN-γ), CCL2 (monocyte chemoattractant protein-1, MCP-1), CCL4 (MIP-1ß), and tumor necrosis factor-α (TNF-α)] were measured in patients with early, untreated inflammatory arthritis [symptom duration ≤ 12 months; ≥ 1 swollen joint; RA, n = 41; undifferentiated arthritis (UA), n = 23]. Cytokine expression patterns were determined using cluster analysis.

Results. Both pro- and antiinflammatory cytokines were elevated in patients over controls (n = 21). RA clustered into subgroups based solely on cytokine profiles. The "mild" RA subgroup (n = 23) had higher CCL4 (MIP-1ß), CXCL8 (IL-8), IL-2, IL-12, IL-17, IL-5, and IL-10 levels, lower IL-6, IFN-γ, GM-CSF, and IL-4 levels, less CCP positivity (52% vs 82%; $p < 0.05$), and lower CCP titers [71 (78) vs 153 (94); $p < 0.01$], but similar erythrocyte sedimentation rate, C-reactive protein, and joint counts compared to the "severe" RA groups. CCL4 (MIP-1ß), IL-13, IL-12, TNF-α, and IL-4 best distinguished the groups. Combining UA with RA samples preserved cytokine subgroups and strengthened the autoantibody associations. Fewer UA patients in the "mild" cluster (n = 16) were RF-positive (24% vs 100%; $p < 0.002$) or CCP-positive (24% vs 66%; $p < 0.08$) compared to the "severe" group.

Conclusion. Early untreated inflammatory arthritis can be categorized into distinct subgroups based on cytokine profiles. These subgroups are associated with CCP and RF autoantibodies. Integration of cytokine profiles with autoantibody status may assist prognostication and treatment decisions in these patients. (J Rheumatol 2004;31:2336–46)

Key Indexing Terms:

CYTOKINES	CHEMOKINES	RHEUMATOID ARTHRITIS
ARTHRITIS	CITRULLINE	AUTOANTIBODIES

The clinical presentation of different inflammatory arthropathies is similar early in the course of disease. As a result, it is often difficult to distinguish patients who are at risk of developing the severe and persistent synovitis that leads to erosive joint damage from those whose arthritis is more self-limited. Such distinction is critical in order to target therapy appropriately, treating aggressively those with erosive disease and avoiding unnecessary toxicity in patients

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with more self-limited disease. Current clinical criteria for diagnosing erosive arthropathies such as rheumatoid arthritis (RA) are less effective in early disease, and traditional markers of disease activity such as joint counts and acute phase response do not adequately identify patients likely to have poor outcomes[1]. Measures of the pathologic events occurring in the synovium are most likely to be of significant prognostic value.

Recent efforts to identify predictors of poor outcome in early inflammatory arthritis have identified the presence of RA-specific autoantibodies, in particular antibodies towards citrullinated peptides, to be associated with erosive and persistent disease in cohorts of patients with early inflammatory arthritis. On the basis of this, a cyclical citrullinated peptide (CCP) has been developed to assist in identification of anti-CCP antibodies in patient sera. Using this approach, the presence of anti-CCP antibodies has been shown to be highly specific and sensitive for RA, can distinguish RA from other arthropathies, and can potentially predict persistent, erosive synovitis before these outcomes become clinically manifest[2]. Importantly, anti-CCP antibodies are often detectable in sera many years prior to clinical symptoms, suggesting that they may be reflective of subclinical immune events[3,4].

Cytokine networks are critical for the initiation and perpetuation of both systemic and local inflammatory responses seen in chronic inflammatory arthritis. These cytokine networks are a key component of the immunopathology of inflammatory arthritis and likely influence whether the synovitis is destined to be self-limited or sustained. Proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin 1 (IL-1), and IL-6 are derived primarily from macrophages and fibroblasts and dominate RA synovitis. The critical importance of these cytokines in mediating and perpetuating inflammatory arthritis is evident in the dramatic clinical responses seen in patients treated with anticytokine-directed therapies[5-10]. However, not all patients respond to these directed therapies and it is clear that T cell-derived cytokines are also important in driving and perpetuating synovitis[11].

T cells have been broadly categorized as Th1 or Th2 based on their cytokine profile. Th1-type cytokines are important for cell-mediated immunity and delayed-type hypersensitivity leading to the production of proinflammatory cytokines. In contrast, Th2-type cytokines are important for humoral immunity and allergic responses and lead to the production of antiinflammatory cytokines. IL-2 and interferon-γ (IFN-γ) are characteristic Th1 cytokines, while IL-4, IL-13, IL-10, and IL-5 are characteristic Th2 cytokines[12]. This Th1/Th2 paradigm was developed from animal models of infection and autoimmunity and has been used to classify human autoimmune diseases[12,13]. RA has been considered a Th1 disease on the basis of the cytokine profile that is typical of the synovitis, while some evidence suggests that other inflammatory arthritides such as the spondyloarthropathies are Th2 diseases[12,13]. Previous studies of patients with early inflammatory arthritis based on limited cytokine expression patterns in synovial tissue, synovial fluid, and cultured peripheral blood mononuclear cells were somewhat inconsistent; however, taken together their findings suggested the cytokine profile in early RA may be skewed toward a Th1 pattern, whereas that of early reactive arthritis is skewed towards a Th2 pattern[14]. The complexity of both cytokine and chemokine networks[13] clearly masks the role a single cytokine may play in the pathogenesis of the disease.

We hypothesized that the early immunopathogenetic processes are different in subsets of recent onset inflammatory arthritis that are destined to have different clinical outcomes, and that measuring a panel of inflammatory cytokines, chemokines, and growth factors that are reflective of these events could distinguish mechanistically based subsets of disease. We show that plasma concentrations of a wide spectrum of both Th1 and Th2 cytokines are elevated in early disease. In addition, the production of RA autoantibodies that are known to be predictive of aggressive disease, in particular CCP, is associated with a characteristic cytokine profile that does not correlate with a strictly Th2 or Th1 cytokine pattern.

MATERIALS AND METHODS

Subjects. Patients with inflammatory arthritis, defined as at least one swollen joint of less than 12 months' duration, were enrolled as part of a prospective cohort at the University of Manitoba. A diagnosis of RA was assigned if American College of Rheumatology (ACR) criteria[15] were met and a diagnosis of undifferentiated arthritis (UA) assigned if ACR criteria for RA or European Spondylarthropathy Study Group criteria for spondyloarthropathies[16] were not met. An additional 21 healthy controls were also studied. Patients with connective tissue diseases or other arthropathies were excluded. Clinical and demographic information was recorded for all patients including swollen joint counts, measurement of acute phase response [erythrocyte sedimentation rate (ESR), C-reactive protein (CRP)], global assessments of disease activity (visual analog scale), and functional status (modified Health Assessment Questionnaire). Rheumatoid factor (RF) titers were measured using nephelometry (> 20 IU/ml considered positive). Anti-CCP was measured by ELISA (InterMedico, Markham, ON, Canada) and interpreted using the manufacturer's instructions. Blood samples obtained at the initial clinic visit were centrifuged at 3300 RPM, and plasma was collected and stored at –20°C for batch analysis of CCP and cytokine measurements. The study was approved by the University of Manitoba biomedical ethics board (protocol B2001-070) and all patients provided informed consent to participate.

Multiplex cytokine assay. Plasma levels of 17 cytokines and chemokines were measured: IL-1ß, IL-2, IL-4, IL-5, IL-6, IL-7, CXCL8 (IL-8), IL-10, IL-12p70, IL-13, IL-17, G-CSF, GM-CSF, IFN-γ, CCL2 (MCP-1), CCL4 (MIP-1ß), and TNF-α. Plasma samples were thawed and run in duplicate using the Bio-Plex protein array system (Bio-Rad, Hercules, CA, USA). This sandwich immunoassay contains dyed microspheres conjugated with a monoclonal antibody specific for a target protein. Antibody-coupled beads were first incubated with the plasma sample, then incubated with the biotinylated detection antibody, and finally incubated with streptavidin-phycoerythrin. The captured immunoassay was read by the Bio-Plex array reader, a flow-based dual laser detector and real-time digital signal pro-

cessing instrument (Bio-Rad) that can analyze up to 100 different families of color-coded polystyrene beads and allows multiple measurements of the sample, ensuring effective quantitation of cytokines. Cytokine standards ranging from 1.95 to 32,000 pg/ml were tested to allow quantitation of cytokine levels in patient plasma samples, and a standard curve was generated from which sample concentrations were predicted.

Statistical analysis. Univariate comparisons of individual cytokines between groups were performed using nonparametric Mann-Whitney U tests. There was a wide range of measurements for a given cytokine and among different cytokines. To express the variability among cytokines and patients in a scaled manner that was not distorted by the original measurement scales of the raw data, the raw data were normalized by calculating a Z score using the formula: (mean value – observed value)/standard deviation. The normalized values thus represent the number of standard deviations above or below the mean cytokine level for each individual cytokine tested. Normalization was done across cytokine and patient variables.

Microarray cluster analysis using the normalized data was performed to identify patterns of cytokine expression. Cluster analysis computes the similarity of the cases among a set of attributes in a matrix of similarities and then sorts the cases so that similar cases are grouped together. The data are depicted in a branching dendrogram in which each section contains closely related cases. Microarray analysis, a variation of cluster analysis in which both the cytokines and cases are clustered simultaneously, reveals how the cases cluster together based on their cytokines, how the cytokines cluster together and how these 2 groupings relate to each other. Microarray cluster analysis was performed using Genesis 1.0 software (used under license from the author, Alexander Sturn, Graz University of Technology, Graz, Austria). This program uses an uncentered Pearson correlation as the measure of similarity and the group average (average linkage) or UPGMA (unweighted pair group method using arithmetic averages) sorting strategy[17]. The adequacy of the classification determined by the variance accounted for was used to determine the appropriate number of clusters.

Discriminant function analysis (DFA) using standardized cytokine levels and SPSS® software (SPSS Inc., Chicago, IL, USA) was performed to identify the cytokines contributing to the cluster formation. Unclustered cases were assigned to the cluster predicted by the DFA, provided the case and assigned cluster cytokine profiles appeared similar. Assigned cluster groupings were used for comparisons of clinical features. Similar results were found when unassigned cases were omitted from comparisons of clinical features.

Additional univariate analysis was performed using chi-squared, Mann-Whitney U, or Kruskal-Wallis comparisons (SPSS software) and corrected for multiple comparisons where indicated. P values < 0.05 were considered significant. Results are shown as mean (SD).

RESULTS

Description of cohort studied. The clinical characteristics of the patients are shown in Table 1. Patients had disease duration of less than 12 months from symptom onset. Untreated patients had not been exposed to prednisone or disease modifying antirheumatic drugs (DMARD). Compared to patients with untreated UA, patients with untreated early RA were more likely to have RA-specific autoantibodies (RF and CCP) with higher autoantibody titers, had a greater acute phase response with higher ESR and CRP values, and were more likely to have polyarthritis (≥ 4 swollen joints) (72% vs 35%; p = 0.02). Functional status and physician global assessments of disease activity were similar.

Cytokine and chemokine levels differed between patients with untreated early arthritis and controls. Cytokines and chemokines were measured in the plasma of patients with untreated (DMARD and prednisone-naive) early RA and early UA and compared to healthy controls (Figure 1). Plasma levels of all tested cytokines, with the exception of IL-2, IL-12, CCL2 (MCP-1), and CCL4 (MIP-1ß), were higher in patients than in controls. Thus absolute levels of both Th1 and Th2 cytokines were elevated in early arthritis. Compared to untreated early UA, patients with early RA had higher levels of all cytokines with the exception of GM-CSF, G-CSF, IL-4, IL-7, IL-10, IL-5, IL-17, and CCL2 (MCP-1), which were statistically similar between the 2 diagnoses.

DMARD and prednisone-naive early RA clusters into subgroups based on cytokine profile. The patients with early RA who had received no DMARD or prednisone therapy (n

Table 1. Clinical features at the initial clinic visit for patients with early untreated RA activity and early untreated undifferentiated arthritis (< 12 months of disease activity). Values represent mean (SD).

	Early RA, n = 41	UA, n = 23	p
Clinical feature			
Swollen joint count*	9 (8)	4 (5)	0.01
mHAQ	0.58 (0.56)	0.49 (0.63)	NS
MD global VAS, mm	22 (23)	14 (19)	NS
Inflammatory markers			
ESR, mm/h; normal < 20*	34 (31)	20 (28)	0.01
CRP, mg/l; normal < 8*	18.9 (25.6)	11.5 (18.3)	0.05
Rheumatoid factor			
Percentage positive**	78%	43%	0.005
Titer, IU/ml; normal < 20*	224 (208)	108 (145)	0.03
CCP			
Percentage positive**	63%	34%	0.03
Titer, units; normal < 20*	103 (94)	61 (85)	0.04

* Mann-Whitney U test, ** chi-square test. CCP: anti-cyclical citrullinated peptide, mHAQ: modified Health Assessment Questionnaire, VAS: visual analog scale assessment of disease activity.

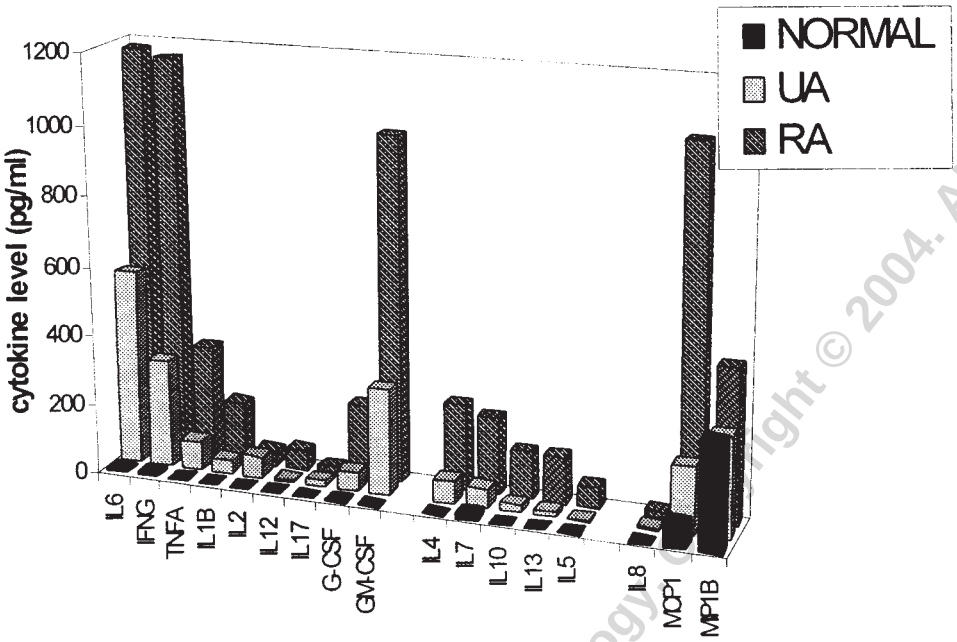

Figure 1. Cytokine, chemokine, and growth factor levels in sera of patients with early untreated inflammatory arthritis, compared to healthy controls. Bars represent mean cytokine level (pg/ml) determined using the Bio-Plex protein array system. RA: rheumatoid arthritis, UA: undifferentiated arthritis.

= 41) were analyzed separately and clustered based on their cytokine and chemokine profiles (Figure 2). The ranges of concentrations observed among cytokines tested were highly variable. A Z score was therefore calculated from the cytokine concentrations prior to clustering to provide a means of more equally weighting each cytokine. The transformed values used to form the clusters represent the number of standard deviations above or below the mean cytokine level for each individual cytokine tested. The majority of cases formed a single cluster (n = 22), shown in red. Sixteen of the remaining patients formed 3 separate subgroups (Figure 2, subgroups 1, 2, and 3; n = 4, n = 4, and n = 8, respectively) shown in green. Three cases did not readily fall into any cluster. For clinical comparisons of cluster groupings, 2 of these cases were assigned to the cluster group predicted by DFA of the cytokines as described below. The third case (Figure 2, number 38, RF-negative RA) was not grouped, as its cytokine profile did not appear to be similar to cases in its predicted group (subgroup 3).

Distinct patterns emerged in the cytokine profiles. The largest patient group (n = 22, shown in red) generally had CCL4 (MIP-1ß), CXCL8 (IL-8), IL-2, IL-12, IL-17, IL-5, and IL-10 levels above the mean values observed, and IL-6, IFN-γ, GM-CSF, and IL-4 levels below the mean values observed. In contrast, the remaining cases, shown in green, had IL-6, IFN-γ, GM-CSF, and IL-4 levels above the mean (Table 2). Importantly, this group had significantly higher CCP and RF titers than the first group (see below). As these indicators are associated with aggressive disease and poor outcome, the cytokine profiles of the second group (shown in green) predict a more "severe" phenotype, and the first group (shown in red) a "mild" phenotype. These "severe" cases formed 3 separate subgroups. One subgroup (subgroup 1) had lower IL-7, IL-13, IL-5, IL-1ß, and G-CSF, but higher TNF-α and MCP levels compared to either of the other 2. A second subgroup (subgroup 2) had lower levels of IL-1ß, IL-2, IL-12, IL-17, and CXCL8 (IL-8), but higher IL-7 and IL-13 compared to subgroup 3. Thus, patients with early RA tested in this analysis are characterized by a complex immunologic profile with increases in cellular and humoral modulating cytokines.

The absolute cytokine levels for each of the RA cluster groups are shown in Figure 4. Significant differences ($p < 0.001$) in cytokine levels were seen between the "mild" red cluster and the "severe" green cluster for all cytokines except IL-17 ($p = 0.008$), CCL2 (MCP-1) ($p = 0.19$), CXCL8 (IL-8) ($p = 0.18$), and CCL4 (MIP-1ß) ($p = 0.77$).

Individual cytokines also clustered into 3 distinct subgroups, and these groups were not based on classical Th1/Th2 distinctions (Figure 2). Classical Th1 cytokines were highly associated with Th2 cytokines. For example, IL-6, IFN-γ, and TNF-α were clustered with IL-4. IL-7 and IL-13 clustered with IL-1ß. IL-10 and IL-5 were clustered with IL-2, IL-12, and IL-17, suggesting that disease activity is complex in these patients, with simultaneous activation of both humoral and cellular immunity.

CCL4 (MIP-1ß) contributed the most to distinguishing between the cases with untreated early RA. DFA was applied

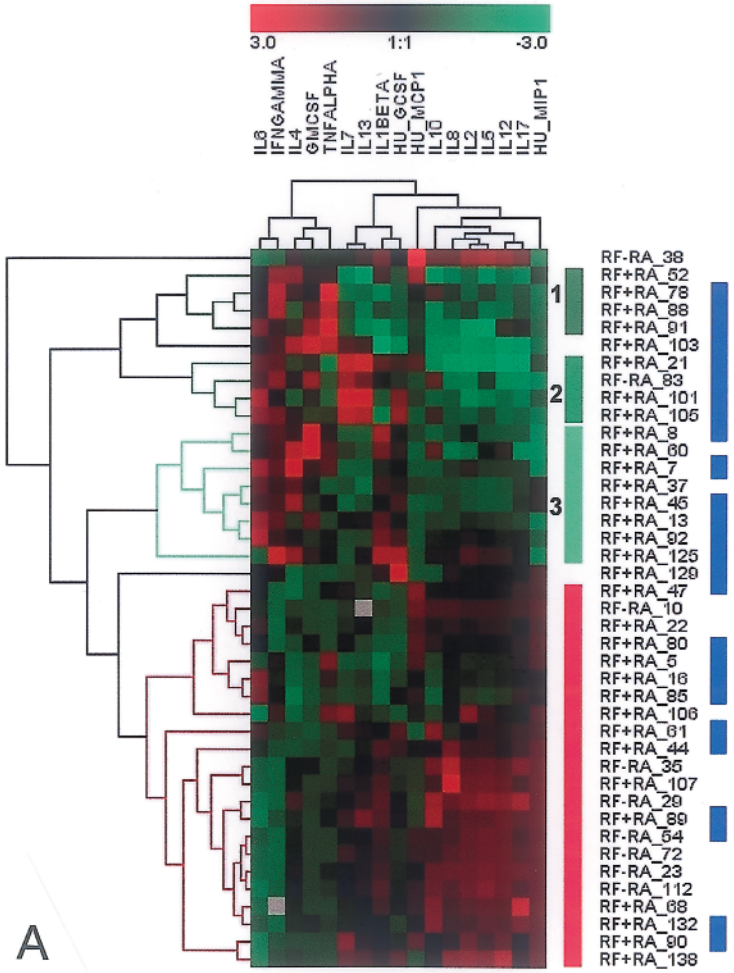

Figure 2. Early untreated inflammatory arthritis clusters into distinct subgroups based entirely on cytokine profiles, and these subgroups differed in CCP positivity. Cytokine levels were normalized and clustered as described in Materials and Methods. CCP levels were determined by ELISA. The "mild" group is shown in red, the "severe" groups in green. Patients with early untreated RA clustered into 2 distinct groups, one with 3 subgroups (denoted 1, 2, 3). Cytokines also clustered into 3 groups. Blue bars represent CCP-positive RA patients.

to determine which cytokines were the most important in discriminating between the cluster groups. A combination of 5 cytokines and chemokines was able to differentiate the 4 clusters obtained in the above analysis, and correctly classified 38 of the 41 RA cases, accounting for 99.3% of the variance among cytokines. CCL4 (MIP-1ß) contributed to 81% of the variance among clusters, with IL-13, IL-12, TNF-α, and IL-4 sequentially adding additional discrimination among the clusters.

Cluster groups were associated with CCP autoantibodies. The clinical features of each untreated early RA subgroup are shown in Table 3. The largest subgroup (n = 23; 22 original cases plus one assigned by DFA) was less likely to be CCP positive than the remainder, and when RF or CCP antibodies were detected they had lower RF titers [168.8 (170) vs 308.3 (231); $p < 0.06$] and lower CCP titers [70.6 (78.1) vs 152.7 (94.1); $p < 0.01$]. This suggests that this group has "mild" disease. No significant differences in joint counts, inflammatory markers, or functional status were seen. Seventeen of the remaining patients formed 3 separate subgroups (n = 4, n = 5, and n = 8); these 3 subgroups were similar in autoantibody status, but differed slightly in CRP and the physician global disease assessment (Table 3). Thus the cytokine profiles of the 2 major groups were associated primarily with autoantibody status, in particular CCP, but not with traditional measures of disease activity.

The cluster groupings are preserved with the addition of untreated early undifferentiated arthritis. To determine if inflammatory arthritis, regardless of whether patients met criteria for RA, could be differentiated on the basis of cytokine profiles early in the course of disease, a cluster analysis was performed on patients with UA (n = 23) and with RA. The groupings observed when only RA patients were clustered were relatively preserved in this analysis, with 2 groups forming, one that had 3 subgroups (Figure 3). All but 2 RA patients remained in their original cluster

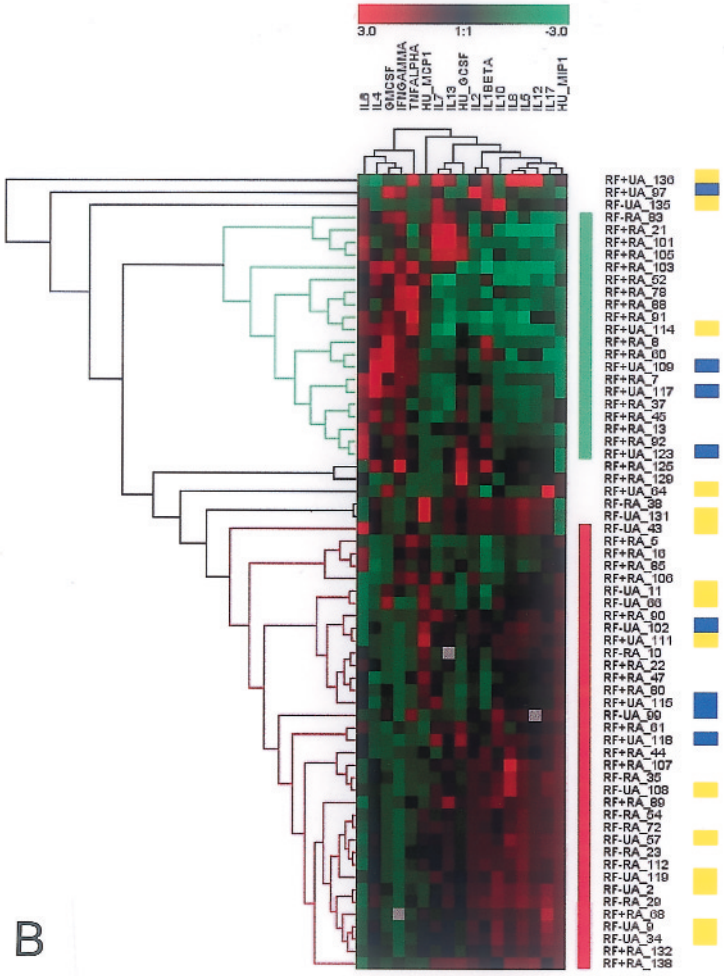

Figure 2. B. Patients with early untreated RA and early untreated undifferentiated arthritis (UA) formed similar clusters. Blue bars represent CCP-positive UA patients. Yellow bars represent CCP-negative patients with UA.

groupings. The majority of patients with UA (n = 16) fell into the larger "mild" red cluster. Four were distributed among the other 3 "severe" green clusters, and 3 did not fall into a specific group. Using discriminant function analysis, 2 of these were classified with the "severe" green groups and one with the "mild" red group. All UA patients falling into one of the 3 "severe" green groups were RF-positive, compared to only 24% of UA patients falling into the larger "mild" red group (Fisher's exact test, p = 0.002). Four of the 6 UA patients falling into the "severe" green cluster were CCP-positive compared to 4 of the 17 patients falling into the "mild" red cluster (one-sided Fisher's exact test, p = 0.08). The clinical characteristics of the 2 clusters of early arthritis are shown in Table 4. The clusters differed in RF and CCP positivity and titers, but not in any other clinical variable that was measured.

DFA of RA and UA clustering indicated that IL-12, CCL4 (MIP-1ß), IL-4, IL-5, CCL2 (MCP-1), and IL-17 were able to correctly classify 98.3% of the 63 RA and UA cases into one of 2 clusters, and accounted for 84.6% of the variance among cytokine variables. Thus CCL4 (MIP-1), IL-12, and IL-4 are able to discriminate groupings of RA and RA/UA patients, and likely are of considerable importance in differentiating cytokine profiles in early arthritis.

Patient outcome at one year. Outcome data were available for 15 patients with early inflammatory arthritis (RA or UA; mean followup 17.4 months, median 17, range 3–31). At the initial clinic visit, the cytokine profile of 5 patients fell into the "severe" green cluster and 10 into the "mild" red cluster. Clinical interventions were not controlled and were all done at the discretion of the patient's attending physician, who was blinded to the patient's autoantibody (CCP) and cytokine data. All 5 green cluster patients were receiving DMARD at followup and 3 were undergoing combination therapy. Seven "mild" red cluster patients were taking DMARD and 2 were on combination therapy. Radiographic erosions were present in 2 of the 5 "severe" green cluster patients, but in only one of the 10 "mild" red cluster

Table 2. Standardized cytokine values of each grouping of patients with untreated early RA. The normalized values represent the number of standard deviations above or below the mean cytokine level for each individual cytokine tested. Normalization was done across cytokine and patient variables.

Cytokine	"Mild" Red Cluster	"Severe" Green Cluster	Mann-Whitney U, corrected p ‡
IL-6†	–0.51 (0.78)	0.76 (0.76)	< 0.002‡
IFN-γ†	–0.68 (0.49)	0.99 (0.67)	< 0.002‡
TNF-α	–0.27 (0.52)	0.38 (1.36)	
IL-1ß	–0.02 (0.70)	-0.03 (1.33)	
IL–2†	0.55 (0.58)	-0.84 (0.84)	< 0.002‡
IL–12†	0.58 (0.57)	-0.86 (0.81)	< 0.002‡
IL-17†	0.63 (0.56)	–0.92 (0.73)	< 0.002‡
G-CSF	–0.05 (1.06)	0.07 (0.98)	
GM-CSF†	–0.53 (0.20)	0.74 (1.21)	< 0.002‡
IL-4*	–0.49 (0.49)	0.64 (1.18)	< 0.02‡
IL-7	0.02 (0.59)	–0.01 (1.42)	
IL-10**	0.40 (0.82)	–0.60 (0.95)	
IL-13	0.00 (0.43)	–0.14 (1.49)	
IL-5†	0.58 (0.58)	–0.88 (0.76)	< 0.002‡
CCL4 (MIP-1ß)†	0.80 (0.26)	–1.00 (0.58)	< 0.002‡
CCL2 (MCP-1)**	0.11 (0.64)	–0.42 (0.68)	
CXCL8 (IL-8)†	0.60 (0.74)	–0.87 (0.60)	< 0.002‡

† Mann-Whitney U red vs green cluster, $p < 0.0001$; * Mann-Whitney U red vs green, $p < 0.001$; ** Mann-Whitney U red vs green, $p < 0.01$; ‡ Mann-Whitney U red vs green cluster corrected p for multiple comparisons.

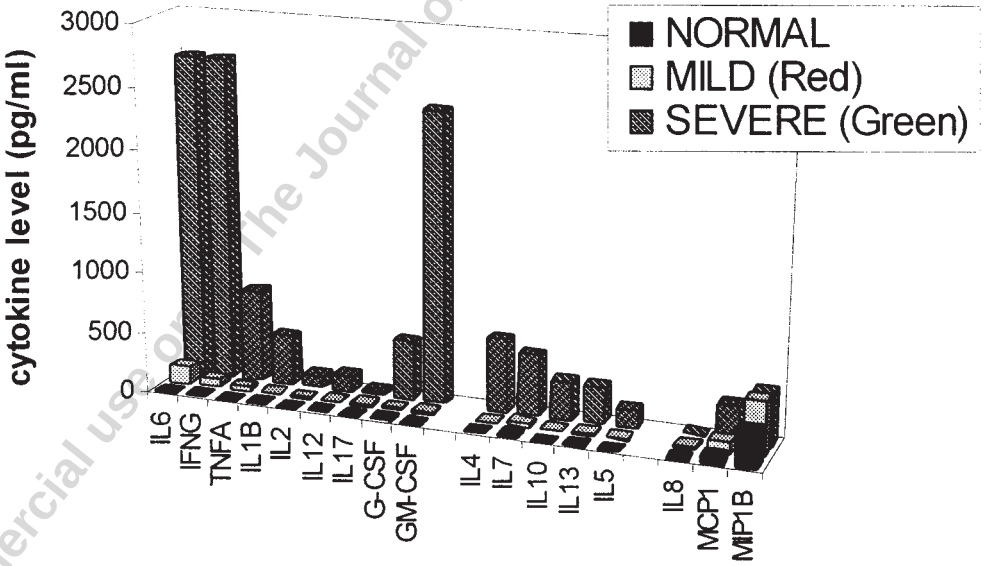

Figure 3. Comparison of cytokine, chemokine, and growth factor levels between the RA cluster groups and healthy controls. Bars represent mean cytokine level (pg/ml) determined using the Bio-Plex protein array system.

patients. One "mild" red cluster patient had periarticular osteopenia without erosions.

DISCUSSION

The clinical similarity of different inflammatory arthritides at presentation complicates the assessment of patients at a time when it is most crucial to intervene with therapy in order to obtain good clinical outcomes. Characterization of patients based on pathogenetic mechanisms is likely to be of value at this stage of disease. We have shown that both pro-

Table 3. Clinical features of untreated patients (no prednisone or DMARD) with early RA at initial presentation. Values expressed as percentage of cluster membership or mean (SD) (3 cases assigned to grouping based on cytokine profile predicted by discriminant function analysis of cytokine profile. One case assigned to each of cluster 1, green 2). One case (number 38) remained ungrouped and was not included.

	Mild Cluster (Red), n = 23	Severe Cluster (Green), n = 17	Green 1 Subcluster, n = 4	Green 2 Subcluster, n = 5	Green 3 Subcluster, n = 8
Clinical feature					
Swollen joint count††	7.6 (6.9)	8.9 (7.8)	4.5 (1.7)	16.8 (7.9)	7 (7.2)
mHAQ	0.52 (0.43)	0.67 (0.72)	0.40 (0.43)	1.25 (1.00)	0.48 (0.56)
MD global VAS, mm†	17.9 (22.6)	28 (22.5)	7 (10.9)	49.3 (4.8)	27.9 (22.7)
Inflammatory markers					
ESR, mm/h	28.3 (22.4)	41.8 (38.5)	23.5 (12.1)	68.6 (56.4)	34.3 (27.2)
CRP, mg/l†††‡	18.2 (24.8)	20.7 (27.7)	3.3 (4.3)	15.3 (10.6)	32.2 (35.4)
RF					
Percentage positive, > 20 IU/ml	70	94	100	80	100
Titer, IU/ml #	168.8 (170)	308.3 (231.3)	305.8 (197.9)	400 (346.4)	275.3 (226.1)
CCP					
Percentage positive**	52.2	82.4	75	100	75
Titer*	70.6 (78.1)	152.7 (94.1)	99.5 (110.4)	221.1 (23.6)	136.6 (97.1)

** Chi-square "mild" cluster vs "severe" cluster, $p < 0.05$; * Mann-Whitney U "mild" cluster vs "severe" cluster, $p < 0.01$; # Mann-Whitney U "mild" cluster vs "severe" cluster, $p = 0.06$; †† Mann-Whitney U green 1 vs green 2, $p = 0.06$; † Mann-Whitney U green 1 vs green 2, $p = 0.03$; ‡ Mann-Whitney U green 2 vs green 3, $p = 0.03$. mHAQ: modified Health Assessment Questionnaire, RF: rheumatoid factor, VAS: visual analog scale assessment of disease activity, CCP: anti-cyclical citrullinated peptide.

Table 4. Clinical features of patients with untreated early RA and undifferentiated arthritis at initial clinic presentation (4 unclassified cases assigned to cluster predicted by discriminant functional analysis: one into the "mild" cluster, 3 into "severe" cluster). Values expressed as percentage of cluster membership or mean (SD).

	Mild Cluster (red), n = 42	Severe Cluster (green), n = 22	p (mild vs severe cluster)
Percentage with early RA	73	60	NS
Clinical feature			
Swollen joint count*	7 (7.3)	7 (7.2)	NS
mHAQ*	0.65 (0.80)	0.49 (0.42)	NS
MD global VAS, mm*	21.2 (22.6)	18.3 (21)	NS
Inflammatory markers			
ESR, mm/h*	35.1 (36.7)	25.5 (25.7)	NS
CRP, mg/l*	16.5 (17.7)	16.6 (26.4)	NS
RF			
Percentage positive, > 20 IU/ml**	95	50	0.001
Titer, IU/ml*	326 (210)	112 (144)	< 0.0001
CCP			
Percentage positive**	77	42	< 0.005
Titer*	144.8 (96.7)	58.4 (74.9)	< 0.0001

* Mann-Whitney U test, ** 2-sided Pearson chi-square. mHAQ: modified Health Assessment Questionnaire, RF: rheumatoid factor, VAS: visual analog scale assessment of disease activity, CCP: anti-cyclical citrullinated peptide.

and antiinflammatory cytokines are elevated in patients with early, untreated inflammatory arthritis, and that early in the disease the classic Th1 predominance associated with RA has not been established. Importantly, we have shown that patients with early inflammatory arthritis meeting ACR criteria for RA can be categorized into distinct subgroups based entirely on their cytokine profile, and that these profiles are associated with CCP and RF autoantibody status but not with other traditional markers of inflammatory disease activity such as joint counts and acute phase response. As CCP and RF titers are prognostic of poor outcome, comprehensive cytokine profiling may help to define the

immunologic foundation from which severe disease will evolve, thus providing a powerful tool for future drug development.

The important cytokines differentiating the cluster groups in RA included the chemotactic cytokine CCL4 (MIP-1ß), Th2 cytokines IL-13 and IL-4, the Th1 cytokine IL-12, and TNF-α. Inclusion of undifferentiated arthritis expanded this list of discriminatory cytokines to include IL-17, IL-5, and CCL2 (MCP-1). Both chemokines shown to be of importance, CCL4 (MIP-1ß) and CCL2 (MCP-1), can be produced by fibroblasts. CCL4 (MIP-1ß) is chemoattractant for cells expressing CCR5, including monocytes and Th1 T cells that are enhanced in RA synovium[18,19], and expression of CCL4 (MIP-1ß) and its ligand CCR5 coincided with peak inflammation in a rat model of arthritis[20]. CCL4 (MIP-1ß) is often present as a heterodimer with MIP-1ß (CCL3), which is a chemoattractant for cells expressing CCR5 or CCR1. CCL2 (MCP-1) induces chemotaxis of cells expressing CCR2 and CCR10 receptors, including monocytes, T cells, natural killer cells, and basophils. Surprisingly, levels of both chemokines were lowest in the subgroup with higher TNF-α levels and more autoantibodies; however, it is not known if this also indicates an absence of infiltrating cells. In the case of CCL2 (MCP-1), lower levels may be the result of downregulation by hypoxia caused by microvascular damage or inadequate neovascularization[21]. It is also known that a subset of RA lymphocytes with reduced phosphorylation of G protein-coupled receptors have an increased responsiveness to CCL3, CCL4, and CCL5[22], suggesting that even low levels of these chemokines can have significant influence in inflammatory processes.

Th2 cytokines are generally considered to have antiinflammatory properties in RA including the inhibition of proinflammatory cytokines and proteinases. The Th2 cytokines IL-4 and IL-13 were important in discriminating between cytokine cluster groups; however, IL-4 was elevated in the group with higher TNF levels and increased CCP positivity, whereas IL-13 was particularly increased in a subset of this group. This may reflect a counter-regulatory response to inflammatory cytokines. However, both IL-4 and IL-13 can have immunostimulatory effects on T cells and macrophages, and importantly on B cells. The association of IL-4 and IL-13 with the CCP-positive cluster may indicate the potential of these cytokines to enhance B cell activity and possibly antibody production. The recent reports of B cell depletion ameliorating RA symptoms indicate that B cells play a fundamental role in disease pathology[23]. Increased levels of IL-4 and IL-13 in early RA may therefore be of consequence in the evolution of B cell dysregulation, and thereby contribute directly to the severe outcomes likely to occur in these patients. Rather than being antiinflammatory in this context, these cytokines may be provocative targets for future biologic treatment of early RA.

The association of CCP autoantibodies with particular cytokine subgroups of early RA suggests that the cytokine profile may predict disease severity, and that both the cytokine profile and the presence of autoantibodies may reflect events leading to autoimmunity. The importance of citrullination in the pathogenesis of RA is increasingly recognized. Citrulline is an amino acid formed by posttranslational modification of arginine by the constitutive enzyme peptidylarginine deiminase (PAD), and citrullination plays a key role in the recognition of self-peptides by the shared epitope HLA-DR0401. The affinity of HLA-DR0401 toward vimentin and other peptides is dramatically increased when the peptide has citrulline substituted for arginine at the site binding the HLA peptide-anchoring pocket P4. Citrulline at this site is required for subsequent T cell responses including T cell proliferation and IFN-γ production[24]. Consistent with this, in our study the group with the highest proportion of CCP positivity had increased expression of IFN-γ. In addition, GM-CSF, which is known to induce macrophage HLA-DR expression[25,26] and is produced by synovial macrophages and stimulated synoviocytes, was enhanced in this group. This suggests that one of the pathologic mechanisms characteristic of this RA subgroup may relate to enhanced antigen presentation.

Distinct histological patterns with associated tissue and serum cytokine profiles have been described in patients with established RA. Diffuse infiltrates of T and B cells had low tissue levels of IFN-γ, IL-1ß, TNF-α, IL-10, and IL-4. Patients with follicular synovitis had high tissue levels of IFN-γ, IL-1ß, and TNF-α, IL-10 but no IL-4[27]. In a separate cohort of patients with established RA, diffuse synovitis was associated with reduced serum TNF-α; however, follicular synovitis was associated with reduced serum IFN-γ[28]. It has been suggested that these histological subtypes do not coexist and are preserved within a given individual. The cytokine profiles found in patients with early disease do not easily fit the pattern found in established disease. We recently observed that in early inflammatory arthritis, diffuse synovitis of lymphocytes or plasma cells is relatively common, while the presence of follicular structures is uncommon and when present, is highly associated[29] with anti-CCP autoantibodies and predicts a worse outcome. In addition, high endothelial venules, microvascular damage, stromal fibrin deposition, and mesenchymal transformation are independent predictors[29] of clinical disease remission at one year of followup. Importantly, these histologic predictors are also highly associated with CCP and RF and add additional predictive value to clinical variables in models predicting clinical remission[29]. It is interesting to speculate that the histological features in early disease may also have a particular cytokine profile. Patients clustering in the green subgroup 3 (Figure 2) had elevated absolute levels of IFN-γ, TNF-α, and IL-1ß compared to the other subgroups and were also likely to be CCP positive, suggesting that this subgroup may

have a greater tendency for lymphoid organization. Patients clustering in the red group generally had lower IFN-γ and TNF-α, and a group also had high expression of MCP and CCL4 (MIP-1ß), suggesting that these patients may have diffuse cellular infiltrates that are less likely to be associated with CCP and potentially predict a better prognosis. Further confirmation of the histologic and cytokine correlates in early disease is needed.

This study looked only at cytokine concentrations at presentation and did not address issues regarding the dynamic changes in cytokine levels or profiles with disease evolution and treatment. Effective therapies will likely change the cytokine profile; however, the pretreatment profile may predict responsiveness to a specific therapy[30]. In our study, only patients who had not received prednisone or DMARD were analyzed, eliminating the potential confounding effects of therapy on cytokine levels. Nonsteroidal antiinflammatory drugs (NSAID) were not restricted, as it is difficult to control for nonprescription usage and the majority of patients had been prescribed NSAID prior to enrollment. It is possible that NSAID use may have affected cytokine levels. Other factors potentially influencing acute changes in plasma cytokine levels, including concomitant infections, vigorous exercise, or other stressors, were potential confounders to the study, although samples were not drawn during times of serious infection or exacerbations of nonrheumatic illnesses, or after vigorous exercise.

All patients had disease of less than 12 months' duration based on symptom onset; however, the duration of symptoms was not controlled for in this study. Thus it is possible that the subgroups we found are reflective of different stages of disease evolution at the time of sampling, and do not represent distinct categories of arthritis. It is noteworthy that most of the patients with UA, who generally have less severe disease in longterm followup studies[31], fell into a single category, suggesting that this category, characterized by lower prevalence of autoantibodies, does represent a "milder" form or stage of arthritis. Of particular interest are the outcomes of the patients with undifferentiated arthritis falling into the green subgroups, which, although small in numbers, had distinct cytokine profiles. Further longitudinal data are required to determine whether a specific cytokine profile is truly reflective of the potential for more severe disease, as suggested by the autoantibody associations.

Interpretation of plasma cytokine levels in the context of understanding mechanisms of disease pathogenesis assumes that plasma cytokine levels reflect synovial cytokine expression. The extent to which plasma cytokine levels reflect actual pathologic events occurring in the synovium is still incompletely understood; however, studies have shown that plasma levels of many cytokines and chemokines are generally reflective of synovial fluid levels and synovial tissue expression, although levels in plasma are often lower than in synovial fluid, as expected with primary synovial tissue sources[32-36]. In addition, selected serum cytokine levels often correlate with measures of disease activity including joint damage and tissue inflammation, and clinical improvement following therapy corresponds to changes in serum, synovial fluid, and synovial tissue cytokine expression, often within hours[37,38]. Thus measurement of plasma cytokine levels is a clinically feasible method of evaluating pathologic mechanisms in synovial tissue.

The immune features of importance in early inflammatory arthritis likely relate to the initial innate inflammatory response to nonspecific stimuli, the development of autoimmunity with breaking tolerance, and the response of the tissue. Interplay between these processes likely occurs through disease evolution in chronic inflammatory arthritis, and the balance of these components will determine the disease outcome. Early untreated RA can be divided into subsets based on cytokine patterns that are likely mechanistically-based, potentially reflecting evolution of these immune processes. Measuring a cytokine profile consisting of pro- and antiinflammatory cytokines, chemokine growth factors, and possibly other mediators has the potential for characterizing these immune features, and may be helpful in understanding the immune process in a given individual, thereby assisting with prognostication or treatment decisions.

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