PRODUCTION OF T-CELL CYTOKINES AT THE SINGLE-CELL LEVEL IN PATIENTS WITH INFLAMMATORY ARTHRITIDES: ENHANCED ACTIVITY IN SYNOVIAL FLUID COMPARED TO BLOOD

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SUMMARY

We used a newly developed, sensitive ELISPOT technique in order to estimate the number of cells producing interferon-gamma (IFN-γ) and interleukin-4 (IL-4) in synovial fluid mononuclear cells (SFMC) and peripheral blood mononuclear cells (PBMC) in patients with rheumatoid arthritis (RA) and other inflammatory arthritides, and to correlate the results with clinical and laboratory parameters of disease activity. SFMC and PBMC were cultured either without stimuli or with a standardized dose of phytohaemagglutinin (PHA) for 6 h. Twenty-nine patients, 16 with RA and 13 with other inflammatory joint diseases, were investigated and compared to PBMC from 25 healthy controls. The mean number of IFN-γ-producing cells was 37.1/10^5 plated SFMC (range 0–121.5). The corresponding value for PBMC was 5.1 (0–39). The difference was highly significant (P = 0.0033 for RA patients, P = 0.0050 for non-RA patients and P < 0.0001 for all patients). Forty-five per cent of SFMC samples (range for all samples 0–38.5 SFC/10^5 MNC) and 25% of PBMC samples (0–20.5) exhibited spontaneous IL-4 production, yielding a significant difference for all patients treated collectively (P = 0.021). Although the cells that spontaneously secrete these cytokines are relatively few, quantification of these cells thus shows increased functional T-cell activation and decreased ratio of cells spontaneously producing IL-4 vs IFN-γ in the joint fluid as compared to blood of arthritis patients.

KEY WORDS: ELISPOT, Rheumatoid arthritis, Interferon-γ, Interleukin-4, Synovial fluid.

The inflamed synovial tissue of rheumatoid arthritis (RA) patients encompasses gross accumulations of mostly CD4-positive T lymphocytes. These cells express HLA-DR, a marker of immunological activation [1, 2]. Despite this phenotypic T-cell activation, the question of functional T-cell activation in the rheumatoid joint has been a major point of discussion during the last few years. Many studies that have been performed have failed to detect interferon-gamma (IFN-γ) in rheumatoid joints, either in joint tissue [3–7] or in synovial fluid (SF) [3, 5, 7–11]. Other studies have reported expression of IFN-γ in the rheumatoid joint either at protein [12] or mRNA [13, 14] levels. IL-4 has been reported not to be present in significant amounts in the rheumatoid joint [7, 15]. At the T-cell clonal level, attempts have been made to characterize cells from inflamed joints as belonging to either the Th1 or the Th2 subset. A predominance of Th1 phenotypes has then been described for cells both from RA joints [16, 17] and from joints of patients with reactive arthritides [18–21]. However, in none of these reports has the question concerning whether this Th1 predominance is joint specific or a generalized phenomenon been fully addressed. It is also well known that the T-cell cloning procedure itself might influence which cytokines will eventually be produced by the cloned T cells, as the fate of an unprimed Th0 cell depends on the stimuli encountered in the initial activation steps [22, 23]. A more indisputable way to characterize cytokine production might then be to enumerate and compare numbers of cells spontaneously secreting key Th1 and Th2 cytokines in the joint and in the circulation directly.

The ELISPOT technique for the enumeration of single cytokine secreting cells allows high-sensitivity detection of cytokine production [24, 25]. As the cytokines in the ELISPOT adhere to a solid phase immediately after production, they are probably efficiently prevented from being consumed by the producing cell or other cells in the culture. Therefore, the ELISPOT assay might yield information concerning net production in a more stringent way than ELISA of culture supernatants, in which concomitant consumption of the relevant cytokine or effects of simultaneously produced antagonists may influence the amounts measured. In fact, the consumption of T-cell cytokines such as interleukin-2 (IL-2) has been reported to be considerable in mononuclear cells from the rheumatoid joint SF [26], arguing for the use of technologies measuring net secreted substance.

We have established a variant of the cytokine ELISPOT method in our laboratory utilizing plastic ELISA plates which, compared to the ELISPOT using nitrocellulose bottomed wells, allows the detection of IFN-γ at an earlier time after mitogen stimulation, and also seems to be devoid of an auto-stimulatory effect encountered in the nitrocellulose-based assay [27]. In this paper, we have addressed the questions concerning relative functional T-cell
activation and cytokine production in the SF vs peripheral blood by enumerating single cells spontaneously secreting IFN-γ and IL-4 in patients with chronic inflammatory joint diseases. In addition, parallel cultures stimulated with a T-cell mitogen have been studied as a measure of potential cytokine production in the samples. The results have then been correlated to serological parameters of inflammatory activity, medication, and to clinical variables of disease distribution and disease progression.

PATIENTS AND METHODS

Patients
Sixteen patients (13 women and three men) fulfilling the 1987 ACR classification criteria for RA [28] were investigated. Mean age was 52 yr (range 41–68). In parallel, 13 patients with other diagnoses of inflammatory joint disease were studied: psoriatic arthritis (PsA) (three patients), ankylosing spondylitis (AS) (2), juvenile chronic arthritis (JCA) (1), Felty’s syndrome (FS) (1), primary Sjögren’s syndrome (SS) (1) and other unspecified inflammatory joint disease (5). Mean age for non-RA patients was 40 yr (23–67). The duration of disease varied between 0.5 and 30 yr. Patient characteristics are displayed in Table 1. All patients had knee joint effusions at the time of inclusion in the study and were considered as having active inflammatory joint disease. Informed consent was obtained from all patients before sampling. In 23/30 cases, samples of peripheral blood were analysed in parallel to SF and, in those cases, both samples were collected at the same time. As controls, peripheral blood mononuclear cells (PBMC) from 25 healthy blood donors or laboratory personnel (12 women and 11 men) were used; mean age of the controls was 45 yr (24–70).

Preparation of mononuclear cells
SF and peripheral blood samples were collected in heparinized tubes by aseptic technique at the Department of Rheumatology, Karolinska Hospital, and transported to the nearby laboratory without delay. Separation was started as quickly as possible, and in no case later than 2 h after sampling. Blood samples were diluted in equal amounts of phosphate-buffered saline (PBS) at room temperature, and separated on Ficoll-Hypaque (Pharmacia & Upjohn, Uppsala, Sweden). Following two washings in PBS, cells were suspended in RPMI-1640 (Flow Laboratories, Irvine) supplemented with glutamine, HEPES buffer, penicillin and streptomycin in adequate amounts, and 10% fetal calf serum (FCS; Flow). The FCS batch used throughout the study was devoid of general mitogenic properties [27]. Synovial fluid mononuclear cells (SFMC) were prepared using the same technique as for PBMC, except that the SF was diluted three times in PBS prior to separation. All cell suspensions were adjusted to 10⁶ mononuclear cells (MNC)/ml.

ELISPOT method
The method has been described recently [27]. In brief, primary monoclonal antibodies (1-D1K for IFN-γ and IL4-1 for IL-4; both from Mabtech AB, Stockholm, Sweden) were diluted to 15 µg/ml in PBS and adsorbed to plastic ELISA plates (Nunc Maxisorb, Roskilde, Denmark), 50 µl/well at 4°C in a moist chamber overnight. The coated plastic plates were washed four times in PBS. One hundred microlitres of cell suspension (100 000 MNC/well) were added to duplicate wells, with or without 5 µg/ml of a pre-screened batch of phytohaemagglutinin (PHA; Murex Diagnostics, Dartford). Results obtained using this cytokine ELISPOT method depend on cell density and cell culture volume, and changing these parameters affects the results (data not shown). To make comparisons possible, we therefore kept cell concentrations and cell volumes stable and equal for both cytokines. Plates were placed in a 37°C cell incubator with 5% CO₂ for 6 h. This time was chosen because it made possible the simultaneous collection of data for IFN-γ and IL-4 from both unstimulated and mitogen-stimulated cultures [27]. Secondary biotinylated antibodies (7-B6 for IFN-γ and IL4-2 for IL-4; Mabtech) were diluted in PBS to a concentration of 1 µg/ml. The cell suspension was then flicked off, plates washed four times in PBS, and 50 µl of diluted secondary antibody added to each well. Thereafter, plates were left overnight at 4°C. After three new washings, 50 µl of avidin–alkaline phosphatase (Dakopatts AS, Glostrup, Denmark) were added at 1.2 µg/ml in PBS and left for 2 h at room temperature. The plates were washed three times, 50 µl of a BCIP phosphatase substrate solution (Sigma, St Louis, MO, USA) were added and the plates developed at room temperature for 5 h. The plates were washed in deionized water and left to dry. Spot-forming cells (SFC) were counted using an inverted microscope with a 2x objective and a cross-ruled grid in one of the eyepieces.

Determinations of serum acute-phase reactants C-reactive protein (CRP), orosomucoid, α₁-antitrypsin and haptoglobin, as well as IgM rheumatoid factor (RF), serum albumin and serum immune globulin levels (IgG, IgA, IgM) were made by nephelometry. ELISA was used to measure serum IgA RF levels.

Statistical analyses
For comparisons between variables measured in parallel samples of PBMC and SFMC, Wilcoxon’s signed rank test was used. For unpaired comparisons between different groups of patients, or between patients and healthy controls, Mann-Whitney’s U-test was used. For calculations of correlation between parallel variables in single patients, Spearman’s rank correlation test was performed. To make possible the calculation of IL-4/IFN-γ ratios in PBMC and SFMC samples, a value of 0 (no SFC in any of the duplicate wells) was transformed to 0.25, a value separated from zero, but less than the lowest recognizable positive value (0.5; one SFC in any of two duplicate wells).
TABLE I
Clinical characteristics of the investigated patients

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<th>Duration of disease (months)</th>
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RA, rheumatoid arthritis; PsoA, psoriatic arthritis; AS, ankylosing spondylitis; SS, primary Sjögren’s syndrome; JCA, juvenile chronic arthritis; FS, Felty’s syndrome; UNS, other unspecified joint disease; AUR, auranozin; AZA, azathioprine; BET, betamethasone; CYC, cyclosporin A; DIC, diclofenac; IBU, ibuprofen; KET, ketoprofen; MTX, methotrexate; NAB, nabumetone; NX, naproxen; PEN, penicillamine; PREP, prednisolone; PRO, proresid (podophyllum derivatives); REU, reumacon (podophyllum derivative); SASP, sulphasalazine; SUL, sulindac; TEN, tenoxicam; ND, not done; NR, not recorded; F, female; M, male.
RESULTS

Increased spontaneous IFN-γ and IL-4 production in SFMC as compared to PBMC

Twenty-nine patients were studied with regard to spontaneous and mitogen-induced cytokine production in SFMC, and in 23 of these parallel samples were obtained from peripheral blood. The results are summarized in Table II. In SFMC, a spontaneous production of IFN-γ was generally observed, for RA patients mean 39.4 SFC/10^5 plated SFMC (range 0–121.5), for non-RA patients 34.3 (0.5–102) and for all patients 37.1 (0–121.5). In parallel PBMC samples, the spontaneous production was on average seven times lower; the mean production for RA patients was 6.8 SFC/10^5 plated MNC (range 0–39), for non-RA patients 3.2 (0–16) and for all patients 5.1 (0–39). In almost all cases, the parallel production was higher in SFMC than PBMC, yielding a highly significant difference (P = 0.0033 for RA patients, P = 0.0050 for non-RA patients and P < 0.0001 for all patients; Fig. 1a).

The number of cells spontaneously producing IL-4 in patient samples was lower than for IFN-γ. Only 45% of SFMC samples gave any production (range...
for all samples 0–38.5), as compared to 25% of parallel PBMC samples (0–20.5). Parallel investigations demonstrated a significantly higher production in SFMC as compared to PBMC for all patients studied collectively ($P = 0.021$), although no patient group studied separately showed significant differences.

For PBMC of healthy donors, the mean value for IFN-$\gamma$ was 18.5 SFC/10^5 plated PBMC (range 0–135). Production of IL-4 was only observed in 12% of control PBMC samples (Table II). No differences were observed between patients and controls concerning spontaneous production of IFN-$\gamma$ or IL-4 in PBMC samples.

The 6 h incubation time was set from preliminary kinetic titrations. During the study, it became apparent that for many SFMC samples, the increased size of mitogen-induced IFN-$\gamma$ spots made the number impossible to enumerate reliably, and the results for mitogen-induced IFN-$\gamma$ production have therefore been omitted from Table II. However, the appearance of the ELISPOT well clearly indicated an increased mitogen-driven production of IFN-$\gamma$ in SFMC as compared to parallel PBMC samples. For IL-4, no significant differences were found concerning mitogen-stimulated cytokine production in patient vs control PBMC, or in patient SFMC vs PBMC. No significant difference was noted between the number of mitogen-induced IL-4 spots in SFMC in RA and non-RA patients.

Some variation between duplicate wells was often visible. The coefficient of variation was calculated for duplicate wells expressing >20 spots/well, and was 23% for unstimulated production of IFN-$\gamma$, and 13 and 15% for PHA-stimulated production of IFN-$\gamma$ and IL-4, respectively. As unstimulated production of IL-4 showed very low SFC counts, no coefficient of variation was calculated for this variable.

Decreased spontaneous IL-4/IFN-$\gamma$ ratios in SFMC vs PBMC

When comparing numbers of cells spontaneously secreting IFN-$\gamma$ and IL-4, IFN-$\gamma$ production dominated over IL-4 production in PBMC both from patients ($P = 0.035$ for non-RA patients and $P = 0.0090$ for all patients, but not significant for RA patients only), and from healthy controls ($P = 0.0004$). Thus, an IFN-$\gamma$ predominance seemed to be a general phenomenon in PBMC samples investigated. This dominance of IFN-$\gamma$-production was even more striking in SFMC ($P = 0.0010$ for RA patients, $P = 0.0096$ for non-RA patients and $P < 0.0001$ for all patients). To compare cytokine secretion profiles in PBMC vs SFMC, ratios between numbers of cells spontaneously secreting IL-4 and IFN-$\gamma$ were calculated. The data are displayed in Fig. 2, demonstrating lower values of the IL-4/IFN-$\gamma$ ratio in SFMC as compared to PBMC. This difference reached significance for all patients ($P = 0.014$), but not for RA patients or non-RA patients treated separately. Inverting the ratio gave stronger significance values, but the approach was abandoned due to the low numbers of IL-4-secreting cells. No difference was found between spontaneous IL-4/IFN-$\gamma$ ratios in PBMC of healthy controls and PBMC of either all patients or RA patients only.

Correlation of cytokine SFC to laboratory parameters

When comparing spontaneous or mitogen-stimulated cytokine production in PBMC or SFMC, no correlation was evident with IgM RF levels, IgM RF seropositivity, IgA RF levels, immunoglobulin levels (IgG, IgA or IgM) or acute-phase reactants including CRP. The only exception was a weak positive correlation between serum levels of $\alpha_1$-antitrypsin and numbers of SFMC spontaneously secreting IFN-$\gamma$ ($P = 0.031$). ELISPOT results did not correlate with the erythrocyte sedimentation rate as determined at the time of cell sampling.

Association between mitogen-induced IL-4 production and severe disease

Retrospective characterization of the investigated patients included duration of disease, present and recent medication, number of tender and swollen joints, and evaluation of X-rays. No correlation could be made between cytokine production and

![Fig. 2.—Ratios between the number of cells spontaneously secreting IL-4 and IFN-$\gamma$ in individual samples obtained from 20 patients with rheumatoid joint diseases. Filled symbols represent rheumatoid arthritis patients and open symbols depict patients with other inflammatory joint diseases. Data represent means from duplicate wells. In two cases, lines overlap in the figure.](image)
either duration of disease, the use of any particular drug, or level of steroid medication. There was a correlation between mitogen-induced production of IL-4 in SFMC and number of swollen joints (Fig. 3; $P = 0.049$, $\rho = 0.656$ for RA patients, $P = 0.027$, $\rho = 0.783$ for non-RA patients and $P = 0.010$, $\rho = 0.604$ for all patients) and tender joints ($P = $ not significant, $\rho = 0.608$ for RA patients, $P = 0.040$, $\rho = 0.774$ for non-RA patients and $P = 0.021$, $\rho = 0.558$ for all patients), respectively. No other measure of cytokine production gave such correlations. When corrected for multiple comparisons (tender and swollen joints, respectively), $P$ values for all patients were still significant (swollen joints, $P = 0.020$; tender joints, $P = 0.042$; Bonferroni correction).

**DISCUSSION**

Since the demonstration that T lymphocytes expressing HLA-DR are present in the rheumatoid joint, the inconsistency between the presentation of phenotypic activation markers and the lack of signs of functional activation of T lymphocytes has been an issue of debate. In this study, we demonstrate an average 7-fold increase in numbers of cells producing IFN-$\gamma$ and also a less marked increase in the production of IL-4 in mononuclear cells from the joint fluid as compared to peripheral blood of patients with chronic inflammatory joint diseases. The increased spontaneous production of IFN-$\gamma$ and IL-4 in SFMC as compared to PBMC is a functional finding corresponding to earlier reports of increased phenotypic T-cell activation in arthritic joints. Although our study shows such a parallel functional activation, the absolute number of cytokine-producing cells is small; only in a few cases are more than one out of 1000 MNC true cytokine producers. The functional importance of activity in small fractions of the total pool of MNC in the joint is not definitely known. However, there are data arguing for the functional significance of infrequent active T cells orchestrating local inflammatory reactions. The inflammatory reaction in the rheumatoid joint has much in common with a delayed-type hypersensitivity reaction [29]. In model systems, using limiting dilution techniques, one single T cell is sufficient to initiate a delayed-type hypersensitivity reaction [30]. Recently, in the murine autoimmune experimental allergic encephalomyelitis model of multiple sclerosis, only rare tissue-specific T cells have been detected in inflammatory foci in the central nervous system. These few cells then regulate the influx of both T lymphocytes with irrelevant specificities, as well as non-specific inflammatory cells, to the central nervous system [31]. If an analogous situation were to exist in the rheumatoid joint, the detection of very few functionally active cells might be of major pathogenetic importance.

Earlier studies have reported SF T-cell clones from different arthritides to be mostly of the Th1 subset [16–21]. In analogy with these previous studies, our data on spontaneous cytokine production also disclosed a much higher number of cells producing IFN-$\gamma$ than IL-4 in the joint fluid in almost all patients investigated. In fact, IL-4 production in unstimulated SFMC cultures was only encountered in 47% of the SF samples. However, the IFN-$\gamma$ dominance was not joint specific, but was also evident in PBMC of both patients and healthy controls. We therefore compared ratios between the number of cells spontaneously secreting IL-4 and IFN-$\gamma$ in PBMC and SFMC, and determined this ratio to be significantly lower in SFMC than in PBMC when all patients were analysed. In this study, as in the reports of T-cell clones cited above, an *in vitro* cell culture period is included in the assay procedure. In all assays using *in vitro* cultured cells, the cell activation step itself might bias the outcome of Th1/Th2 determinations [22, 23]. In our ELISPOT study, incubations of 6 h duration were used. The risk of changing any intrinsic Th profile of the individual cells during such short periods can be considered to be small, as development of Th1/Th2 profiles is described to take between 3 and 10 days [32].
in different body compartments could be divergent cellular composition. No parallel investigations on phenotypes of PBMC and SFMC were performed on the samples studied. However, in 12 other paired PBMC/SFMC samples collected during the same period and prepared using the same protocol, flow cytometry analysis showed that the mean percentage of the probable IFN-γ and IL-4 producers (T cells and NK cells) did not differ enough between PBMC and SFMC samples to explain the observed differences in lymphokine production between PBMC and SFMC (CD4+CD3+ T cells in PBMC 52.0% and in SFMC 35.6%; CD8+CD3+ T cells in PBMC 15.0% and in SFMC 29.7%; CD16+CD56+CD3–NK cells in PBMC 12.7% and in SFMC 14.7%). Thus, differences in cellular activation rather than varying cellular composition are the probable explanation for the observed differences in cytokine production between PBMC and SFMC samples.

In this investigation, we determined an association between potential Th2 activity measured as numbers of cells producing IL-4 after a brief and standardized period of mitogen stimulation, and severity of joint disease. These findings were quite unexpected as systemic IL-4 treatment in animal models of arthritis has ameliorated the disease process [33], and as IL-4 has been shown to downregulate production of proinflammatory cytokines in in vitro studies of cells and tissues from RA joints [34, 35]. The present finding concerns potential in vitro production as measured in mitogen-driven cultures as contrasted with spontaneous production, and the pathogenetic significance of these findings is hitherto unknown. Hypothetically, these cells present within SFMC with the potential of IL-4 production might reflect a counter-regulatory mechanism in patients with active disease (many affected joints; Fig. 3).

It is common to all findings presented in this paper that although the significance of the findings is often high, only a very small portion of all cells investigated actually secrete the investigated cytokines. This fact stresses the potential significance of enumerating very small numbers of functionally active cells in the rheumatoid joint. It might be that the T cells orchestrating the joint inflammation only constitute a very minor fraction of the total lymphocyte pool populating the joint, and that future techniques aimed at monitoring the local responses to immunointerventive therapies will have to measure changes in such diminutive fractions.

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