Synovial fluid lymphocyte proliferation in response to crude microbial antigens is not useful as a diagnostic test to specifically indicate a bacterial cause of arthritis

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Abstract

Objective
To determine the role of lymphocyte proliferation assay of synovial fluid mononuclear cells (SFMC) with whole fraction bacteria in the diagnosis of reactive arthritis (ReA) or arthritis of unknown origin.

Methods
We stimulated SFMC of 52 unselected patients who consecutively presented in our rheumatology outpatient clinic with the following diagnoses: ReA (n = 8), rheumatoid arthritis (RA) (n = 16), ankylosing spondylitis (AS) (n = 6), osteoarthritis (OA) (n = 5), psoriatic arthritis (PsA) (n = 5) and arthritis of varying origin (AVO) (n = 12) and peripheral blood MC (PBMC) of 10 healthy controls with arthritogenic (Y. enterocolitica, S. enteritidis, C. trachomatis) and non-arthritogenic (E. coli, K. pneumoniae, S. pyogenes, C. albicans) bacteria/mitogens and Tetanus toxoid. T cell proliferation was measured in a standard [3H] Thymidine uptake assay.

Results
In all groups of patients tested, SFMC could be stimulated both by arthritogenic and non-arthritogenic bacteria. So-called specific responses were observed in patients with ReA, but also in RA and AS.

Conclusion
Our findings show that a lymphocyte proliferation assay with SFMC with whole fraction bacteria is not an adequate diagnostic tool to confirm bacterial involvement in inflammatory arthritis.

Key words
Synovial fluid mononuclear cells, bulk lymphocyte proliferation assay, reactive arthritis.
Proliferation assay and reactive arthritis / W. Kaluza et al.

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Received on April 14, 1999; accepted in revised form on August 26, 1999.

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Introduction

Reactive arthritis (ReA) (1) is defined as an aseptic arthritis complicating infection of the gastrointestinal or genitourinary tract with *Yersinia enterocolitica*, *Salmonella enteritidis*, *Shigella flexneri*, *Campylobacter jejuni* or *Chlamydia trachomatis*. The disease typically presents as an asymmetric oligoarthritis, mainly confined to the lower limbs and in some patients in conjunction with spondyloarthropathic features such as spine involvement (2, 3). There is a strong association with the HLA-class I molecule B27 (4).

A major problem in daily practice is that although clinical criteria for the diagnosis of ReA have been proposed (3), there are only few specific laboratory tests to confirm this diagnosis or to identify a bacterial trigger in arthritis of as yet unknown origin. Bacterial cultures from the affected joints are sterile by definition, and bacterial DNA detection in the synovium by polymerase chain reaction (PCR) has potential as a diagnostic technique only for *C. trachomatis* (5), but not for enterobacteria (6). Since in ReA the extraarticular infection has usually resolved by the time of the first arthritic presentation, isolation of the arthritis-inducing bacteria from the site of the primary infection is unsuccessful in most cases (1, 7). Furthermore, the initial infection may be asymptomatic. Bacteria-specific antibodies have a high prevalence in the healthy population and are therefore not generally useful in the diagnosis of ReA (8, 9).

ReA is generally considered to be a T cell-driven disease, with an important pathogenetic role of both MHC class II restricted CD4+ T cells, and MHC class I, namely HLA-B27 restricted CD8+ T cells with specificity for bacterial antigens (10-13). The cloning of such bacteria-responsive synovial T cells has led to the detailed characterisation of epitope specificities, HLA restriction patterns and cytokine effector functions. It has therefore been discussed whether the bulk lymphocyte proliferation assay (LPA) of synovial fluid mononuclear cells (SFMC) might also be useful as a diagnostic test for the underlying bacterial trigger. Ford and colleagues were the first to raise the hypothesis that SFMC, in contrast to peripheral blood mononuclear cells (PBMC), might indicate the cause of arthritis (14, 15). Consecutively, other groups also demonstrated SFMC proliferation that was higher to the ReA triggering bacteria than to control antigens (10, 16, 17), but the specificity of these findings was questioned (18, 19) and strong lymphocyte responses to various bacteria were also detected in rheumatic diseases other than ReA (20-23). However, so-called bacteria- or virus-specific synovial T cell proliferations were rather interpreted as an indicator of a probable microbial etiology of the arthritides studied.

The aim of the present study was to evaluate the significance of SFMC proliferation to various bacteria in an unselected population of patients presenting with synovial effusions, including patients with ReA. Our working hypothesis was that the bulk synovial lymphocyte proliferation assay would not show disease- and bacteria-specific responses that could allow the diagnosis of bacteria-induced synovial inflammation. The antigens used were arthritogenic bacteria such as *S. enteritidis*, *Y. enterocolitica*, and *C. trachomatis*, other bacteria which are believed to be associated with certain spondyloarthropathies (*K. pneumoniae*, *S. pyogenes*), but more importantly, also other common infectious antigens not known to be associated with arthritis (*C. albicans*, *E. coli*, tetanus toxoid).

Our data confirm previous work from our group (18) in that care should be taken in the interpretation of synovial fluid T cell responses to microbial antigens as diagnostic tools in arthritis.

Materials and methods

Patients

Samples of synovial fluid (SF) were obtained from 52 patients consecutively presenting with synovial effusions at the Rheumatology outpatient clinic at the University of Mainz, in whom routine punctures of joint effusions were performed for diagnostic or therapeutic reasons. Nineteen female and 33 male patients were studied (mean age 43.6 years, range 15-80 years). Eight patients were diagnosed to have reactive arthritis (ReA) according to the diagnostic criteria proposed by the consensus group of
the Third International Workshop on Reactive Arthritis (3), 16 patients had rheumatoid arthritis (RA) according to the 1987 revised criteria of the American College of Rheumatology (ACR) (24), 6 had peripheral arthritis in established ankylosing spondylitis (AS), 5 had osteoarthritis (OA), and 5 had psoriatic arthritis (PsA).

The patients with reactive arthritis all presented with asymmetric oligoarticular arthritis mainly of the lower limbs. Five of them had ReA following diarrhoea; the infectious agent was isolated from the stool and confirmed by Widal testing (2 cases of *S. enteritidis*, and 3 of *Y. enterocolitica*), respectively. Three patients presented with the typical triad of Reiter’s syndrome (arthritis, urethritis, conjunctivitis); in one case *C. trachomatis* could be isolated from a urethral smear, in the other cases the infectious agent could not be isolated. The remaining group of 12 patients in this study were classified as having arthritis of varying origin (AVO), specifically: Lyme arthritis; arthritis following infection with Rubella virus; sarcoidosis; septic arthritis following infection with *C. albicans*, *P. aeruginosa* or *E. coli*; bursitis olecrani of unknown origin; Crohn’s disease; or Behçet’s disease. Peripheral blood samples were included as controls from 5 patients with arthritis as well as from 10 healthy blood donors (6 males and 4 females, mean age 40.7 years, range 23-79 years) without a history of enteritis or urethritis during the last months.

Mononuclear cell separation

SFMC and PBMC were separated from heparinised SF or PB by standard Ficoll-Hypaque gradient centrifugation, washed 5 times and resuspended in RPMI-1640 (Gibco, Karlsruhe, Germany) containing 2 mM L-Glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated fetal calf serum (FCS). The MNC were cryopreserved in 10% heat-inactivated fetal calf serum containing 2 mM L-Glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated fetal calf serum (FCS). The MNC were cryopreserved in 10% heat-inactivated fetal calf serum containing 2 mM L-Glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated fetal calf serum (FCS).

Antigens

*S. enteritidis*, *Y. enterocolitica* 03, *C. albicans*, *K. pneumoniae*, *E. coli* and *S. pyogenes* were patients’ isolates provided by the Institute of Medical Microbiology, University of Mainz. They were grown in LB medium and harvested in the late exponential phase. The heat-killed and phosphate buffered saline (PBS) washed lysates were aliquoted and stored at -20°C. After being tested in dose-response proliferation assays with PBMC and SFMC to find the optimal final antigen concentration, the antigen preparations were diluted in assay medium (supplemented RPMI-1640 plus 10% of heat inactivated pooled human AB serum and ciprofloxacin at 12 µg/ml) (11, 18). Tetanus toxoid was as a classical T cell stimulating recall antigen and was purchased from Boehringer Ingelheim, Germany. *Chlamydia trachomatis* reticulate bodies, serovar K were kindly provided by PD Dr. J. Wollenhaupt, Department of Rheumatology, Medizinische Hochschule Hannover, Germany.

The following final protein concentrations of bacterial antigens were used: *Y. enterocolitica* 03 5 µg/ml; *S. enteritidis* 2.2 µg/ml; *E. coli* 8 µg/ml; *K. pneumoniae* 5 µg/ml; *C. albicans* 20 µg/ml; *S. pyogenes* 7 µg/ml; Tetanus toxoid: 1 µg/ml, and *C. trachomatis* 40 µg/ml.

[3H] Thymidine uptake proliferation assay for PBMC and SFMC

All experiments were performed in triplicate in 96-well microtitre plates (Nunc, Roskilde, Denmark) with 5 x 10⁴ MC in 100 µl of assay medium per well. Antigens were added at the concentration described above, the mitogen phytohemagglutinine (PHA-P, Difco Labs, Detroit, USA) was used at a concentration of 1 µg/ml as a positive control in each test. The cells were incubated for 5 days at 37°C in 5% CO₂ in a humidified atmosphere. On day 5 [3H] thymidine was added at 0.2 µCi/well. The cells were incubated for another 18 hours, then harvested onto glass fibre filters, and the incorporated radioactivity was determined in a liquid scintillation counter.

Statistical analysis

Proliferation results were expressed as stimulatory indices (SI). SI were determined by dividing the [3H] thymidine uptake (counts per minute, cpm) of antigen-stimulated cells by the [3H] thymidine uptake (cpm) of control cells without antigen (medium control). Positive responses were defined by an SI of > 5 and by a minimum absolute count of 3000 cpm for the antigen in question. In all cases, the SI of the response to PHA had to be > 5. According to the criteria published by Sieper *et al.* (21, 22, 25), the results of the proliferation assays were classified as follows: (1) antigen-specific response (response to a single antigen preparation exceeded the SI of the other antigens by at least twice); (2) negative response (SI < 5 to all antigens tested); and (3) unspecific response (SI > 5 to 2 or more antigens).

For the statistical analysis, Spearman’s correlation coefficient, the χ² test for independence and the Wilcoxon test for independent groups were used, as appropriate.

Results

Lymphocyte proliferation assay of SFMC in patients with inflammatory arthritis

SF proliferation responses of 52 consecutive patients with inflammatory arthritis of varying origin and osteoarthritis were analysed. In all patients the PHA control showed stimulation indices of > 5; the responses to the different bacterial antigens showed considerable differences from patient to patient even within one group of rheumatic diseases.

As shown in Figure 1, there was no significant difference in the level of stimulation between patients with ReA and other inflammatory arthritides. The SFMC of all patient groups (apart from patients with psoriatic arthritis) could be stimulated by so-called “arthritogenic” agents. The proliferative responses to *C. trachomatis* were low in the SFMC of all patients tested. Statistical analysis did not reveal a correlation between the level of stimulation by a certain antigen and any of the groups tested.

Analysis of specific T cell proliferation to different agents

We analysed the response of SFMC to the different bacterial antigens according to the above cited criteria of antigen specificity (21,22,25). Only 13% of patients with ReA showed specific re-
Fig. 1a. Y. enterocolitica

Fig. 1b. S. enteritidis

Fig. 1c. K. pneumoniae

Fig. 1d. E. coli

Fig. 1e. C. trachomatis

Fig. 1f. S. pyogenes

Fig. 1g. C. albicans

Fig. 1h. Tetanus

Fig. 1. Summary of all lymphocyte proliferation assays (stimulation index, SI) in response to different bacterial antigen preparations. Data are given as medians (D), maximum and minimum proliferation ( ) and quartiles 3-1 ( ) (50% of all data). RA = rheumatoid arthritis, AS = ankylosing spondylitis, OA = osteoarthritis, ReA = reactive arthritis, PsA = psoriatic arthritis, AVO = arthritis of varying origin, con = PBMC of healthy control donors. The stimulation index on the y-axis is expressed on a logarithmic scale; the maximum value shown on this scale is adapted to the maximum value measured.
responses at all (Table I), whereas 25% of the patients with RA and 36% of the patients with AS showed a specific response. In a control experiment using PBMC of healthy controls, a specific response could be induced in 25% of the donors.

Specific responses were induced by *S. pyogenes* (3 cases of RA), *Y. enterocolitica* (1 case of RA, 1 case of AS, and 1 healthy control), *S. enteritidis* (1 case of AS), and *C. albicans* (2 healthy controls).

In the group of ReA patients, only one patient with post-enteritic ReA showed a specific response to *S. enteritidis* (Fig. 2, ReAc), the stimulatory response to *S. enteritidis* was high, but not specific. Another case of stool culture-proven Yersinia-induced ReA (Fig. 2, ReAb) showed even higher responses to *S. enteritidis* than to *Y. enterocolitica*. Notably, in the peripheral blood of 3 of the 10 healthy controls, specific T-cell responses to *Y. enterocolitica* and *C. albicans* (2 cases) could be induced.

**Table I. Percentages of specific, non-specific, and negative synovial fluid mononuclear cell responses to bacterial antigens in the different groups of patients.**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Specific response (%)</th>
<th>Non-specific response (%)</th>
<th>Negative response (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactive arthritis (n = 8)</td>
<td>13</td>
<td>74</td>
<td>13</td>
</tr>
<tr>
<td>Rheumatoid arthritis (n = 16)</td>
<td>25</td>
<td>70</td>
<td>5</td>
</tr>
<tr>
<td>Ankylosing spondylitis (n = 6)</td>
<td>36</td>
<td>48</td>
<td>16</td>
</tr>
<tr>
<td>Osteoarthritis (n = 5)</td>
<td>0</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>Psoriatic arthritis (n = 5)</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Arthritis of varying origin (n = 12)</td>
<td>0</td>
<td>78</td>
<td>22</td>
</tr>
<tr>
<td>PBMC of healthy controls, n = 10</td>
<td>25</td>
<td>75</td>
<td>0</td>
</tr>
</tbody>
</table>

A specific response was defined as a response to a single antigen preparation which exceeded the stimulation index (SI) of the other antigens by at least a factor of two; a non-specific response showed an SI > 5 to two or more antigens; a response was classified as negative if the SI was less than 5 in all the antigens tested.

**Discussion**

 Despite the availability of improved clinical criteria for the diagnosis of ReA (3) we are still lacking valid laboratory tests which allow the identification of a supposed bacterial trigger of ReA or of arthritis of unknown origin.

In the past, several papers have argued for a role of the SFMC lymphocyte proliferation assay in the identification of a bacterial etiology in ReA (10, 14, 15), pauciarticular juvenile chronic arthritis (21) and undifferentiated oligoarthritis (22, 25). In the latter studies, crude bacterial antigen preparations of so-called arthritogenic bacteria were used, whereas other presumably "irrelevant" bacteria were often not included as controls.

**Fig. 2.** Examples of 3 individual cases of SFMC proliferation assays in patients with ReA. In patient ReAa (Yersinia-induced ReA), the response to Salmonella was stronger than the response to Yersinia. In patient ReAb (enterogenic ReA with an unknown trigger) there was a specific response to *S. enteritidis*. In patient ReAc (Salmonella-induced ReA), the response to Salmonella exceeded the response to other bacterial antigens, but was not specific.
We (18) and others (26) have already questioned the specificity of the SFMC proliferation assay in previous papers. A prior work of our group has now been extended with respect to the number of patients and microbial antigens tested. Furthermore, we have used published criteria (21, 22, 25) for the definition of specific, unspecific or negative lymphocyte responses. In the present study of 52 consecutive unselected patients, our results further support the concept that the lymphocyte proliferation test is an inappropriate tool for the etiologic diagnosis of inflammatory arthritis.

The most important finding in this paper was that “specific” SF lymphocyte proliferations, according to definitions from the literature, to any of the bacterial antigen preparations used here were found in more patients with RA (25%) or AS (36%) than in patients with known ReA (only 13%). This finding is not in contrast with the findings of other groups (20,21), who also found specific responses to bacterial or viral antigens in the synovial fluids of patients with inflammatory arthritis other than ReA (for example in RA), but our conclusions are different. It was speculated that such proliferative responses might point to a microbial origin of arthritis in patients that had up to then been classified to suffer from undifferentiated oligoarthritis (22) or pauciarticular juvenile oligoarthritis (21), or - an even more provocative thesis - that synovial lymphocytes could indicate bacterial agents that might cause some cases of RA (20).

However, what can be the reason for specific or dominant synovial lymphocyte responses to crude bacterial antigen preparations, if the bacterial antigen itself is not present in the joint? First, in patients with previous or ongoing gut inflammation, for example in patients with ReA and other spondyloarthropathies (27), bacterium-primed gut mucosa derived T-cells could circulate and home to the synovial membrane, where they might recognize a cross-reactive antigenic epitope of cartilaginous origin. Studying the proliferative responses of PBMC and SFMC in a patient with enteropathic arthritis in Crohn’s disease, van den Broek et al. (28) were able to demonstrate significant proliferations of SFMC to the cell walls of both gram-positive and gram-negative bacteria and to various cartilage antigens. Homing receptors such as the integrin α4β7 which are known to be involved in the specific homing of T lymphocytes to the intestinal lamina propria and to the Peyrer’s patches are also expressed by T cells in the synovial membrane (29). By this gut-synovium homing mechanism, during the course of which the target antigen does not necessarily have to be present in the synovium, pre-activated memory T-cells with specificity for ubiquitous gut-derived bacterial antigens could migrate to the joints. It has also been shown that the probability of detecting a specific synovial lymphocyte proliferation in ReA gradually decreases to only 23% in the more chronic stages of disease (19), which may also be due to the immigration of other pre-activated memory T-cells. Second, the T-cell/macrophage ratio in the joint might be responsible for the antigen-induced proliferative responses (30), and suppressor activity of macrophages could diminish the resulting SI (19). Third, cross-reactivity between antigens of different bacteria or between bacteria and self structures is a common phenomenon. The widely observed SFMC response to gram-negative bacteria in both enterobacteria-induced ReA and in juvenile chronic arthritis (23) is often due to the recognition of the common enterobacterial GroEL homologue, a 60kD heat shock protein. Other immunodominant target antigens from Y. enterocolitica, the 19kD urease β subunit and the 13kD ribosomal protein L2 (31, 32), are also known to show substantial homologies with other enterobacteria and, as recently demonstrated for the 19kD protein, may exhibit structures similar to a chlamydial immunodominant antigen, the histone-like protein Hc1 (33). Such cross-reactivity between enterobacteria may explain why in our study we found a marked proliferative response to S. enteritidis in a patient with proven Yersinia-induced ReA without a history of Salmonella infection.

Interestingly, a recent publication (34) has shown that there is no statistical correlation between the presence of a positive synovial lymphocyte proliferative response and the presence of C. trachomatis DNA in patients with sexually acquired ReA or in controls, a finding which is explained by the authors as a down-regulation of T-cell responses by Chlamydia at the site of arthritis where chlamydial antigen can persist. This study supplies further evidence against the diagnostic use of synovial lympho-
cyte proliferation. Another result of our study was the finding that PBMC of both healthy controls and patients with RA, AS and septic arthritis showed marked and, in some cases, even “specific” responses to crude bacterial antigen. In the past, a comparative investigation of the PBMC and SFMC of patients with ReA had provided data that responses with specificity for the triggering agent could only be seen in the joint, but not in the peripheral blood (10, 15). In contrast, it is now becoming clear that the outcome of PBMC responses to bacterial antigens is largely dependent on the timepoint at which the blood is drawn in the course of disease. PBMC responses in patients with acute ReA seem to be lower than in those who have already overcome the disease and are in remission (35). In a detailed limiting dilution analysis, a lower frequency of antigen-specific T-cells was shown in patients with acute Yersinia-induced ReA as compared to patients with uncomplicated enteritis or patients after remission of ReA (36). Similar results have also recently been published in patients with ReA, undifferentiated oligoarthritis and spondyloarthropathy (37). The authors found that the corresponding response in the peripheral blood appeared 2-4 weeks later than in the synovial fluid. Hence, it appears that the systemic answer to the infectious agent in ReA may only be delayed but not significantly reduced as compared to the synovium.

In summary, the present study has shown that the lymphocyte proliferation assay with whole fraction (crude) bacteria in SFMC is neither a sensitive nor a specific tool to identify the infectious agent in ReA. Moreover, care should be taken in the interpretation of dominant bacteria specific synovial lymphocyte responses as an indication of a bacterial cause in arthritis of as yet unknown origin. In future studies, emphasis should be placed on the identification of further specific surface epitopes of arthropathogenic bacteria which directly drive the CD4 T-cell response in ReA, such as the 19kD and 13kD Yersinia-derived target proteins (31, 32), the chlamydial 57 kD and 18 kD target antigens (33), or the Chlamydia HSP 60 molecule (38). Furthermore, the characterisation of bacteria-derived HLA-B27 binding peptides may link the pathophysiological concepts of ReA with more useful diagnostic tools.

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