Mononuclear cell response to enterobacteria and Gram-positive cell walls of normal intestinal microbiota in early rheumatoid arthritis and other inflammatory arthritides

T. Chen[1], M. Rimpiläinen[1], R. Luukkainen[2], T. Möttönen[3], U. Yli-Kerttula[4], R. Saario[5], P. Toivanen[1]

[1]Department of Medical Microbiology, Turku Immunology Center, Turku University, Turku;
[2]Department of Rheumatology, Satalinna Hospital, Harjavalta;
[3]Division of Rheumatology, Department of Medicine, Turku University Central Hospital, Paimio;
[4]Division of Rheumatology, Department of Medicine, Tampere University Hospital, Tampere;
[5]Department of Medicine, Turku University Central Hospital, Turku, Finland

Abstract

Objective

To study whether enterobacteria and Gram-positive bacterial cell walls (BCW) derived from normal intestinal microbiota are involved in the etiopathogenesis of early rheumatoid arthritis (RA).

Methods

Peripheral blood mononuclear cells (PBMC) and synovial fluid mononuclear cells (SFMC) were isolated from patients with early RA (the average duration of 5 months) and the controls (other types of inflammatory arthritis). The mononuclear cell proliferation and tumor necrosis factor-alpha (TNF-) responses to heat-killed Salmonella enteritidis *(SE),* Yersinia enterocolitica *(YE), and* Escherichia coli *(EC), and to Gram-positive BCW derived from four common intestinal indigenous bacteria,* Eubacterium aerofaciens *(EA),* Eubacterium limosum *(EL),* Lactobacillus casei *(LC), and* Lactobacillus fermentum *(LF), and a BCW derived from a pathogen,* Streptococcus pyogenes *(SP) were investigated.*

Results

39% or 56% of patients with early RA showed significant proliferation responses by PBMC or SFMC against enterobacteria, respectively. In other types of arthritis, corresponding figures were 59% or 66%. When BCW were used as antigens, 8.1% or 23% of patients with early RA showed proliferation responses by PBMC or SFMC, respectively. In other types of arthritis the corresponding figures were 7.5% or 35%, respectively. However, TNF- production by SFMC stimulated by EA BCW, SE, YE or EC, was significantly higher in early RA than in other types of arthritis.

Conclusion

These results suggest that SFMC reacting with enterobacteria or BCW exist in some patients with early RA, but also in other types of inflammatory arthritis. Intestinal bacterial agents may play a role in the etiopathogenesis of RA, but the effect appears to be non-specific.

Key words

Bacterial cell wall, early rheumatoid arthritis, immune response, synovial fluid.

T. Chen, MD; M. Rimpiläinen, PhD; R. Luukkainen, MD; T. Möttönen, MD; U. Yli-Kerttula, MD; R. Saario, MD; P. Toivanen, MD.

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Please address correspondence to: Dr. T. Chen, Department of Medical Microbiology, Turku Immunology Center, Kiinamyllynkatu 13, Turku University, FIN-20520, Turku, Finland. E-mail: tonche@utu.fi

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Introduction

It has been suggested that bacteria or their components, such as bacterial cell walls (BCW) derived from normal intestinal microbiota, may play a role in the etiopathogenesis of RA (1,2). This is supported by several lines of observations: (i) The intestinal microbiota of patients with early RA is significantly different from those of controls (3, 4). (ii) Bacterial antigens have been detected using a monoclonal antibody in the macrophages and dendritic cells of the rheumatoid synovium (5). (iii) Mucosal leukocytes may migrate to the inflamed synovium (6-8). (iv) Increased joint complications occur in inflammatory bowel diseases (9). (v) The BCW of certain members of intestinal microbiota are able to induce chronic polyarthritis in experimental animals (10-13). (vi) The genetically manipulated animals may spontaneously develop chronic arthritis under conventional, but not under germ-free conditions (14, 15).

Eubacterium spp. and *Lactobacillus* spp. are Gram-positive rods and among the most common bacterial genera occurring in the normal intestinal microbiota of humans (16, 17). *Streptococcus pyogenes* (SP) is a Gram-positive group A streptococcus (18). It is known that a single i.p. injection of the BCW from *Eubacterium aerofaciens* (EA), *Lactobacillus casei* (LC) or SP induces chronic polyarthritis in susceptible rat strains, whereas BCW derived from *Eubacterium limosum* (EL) or *Lactobacillus fermentum* (LF) do not have this capacity (10-13, 18). Previously the proliferation responses of synovial fluid mononuclear cells (SFMC) to BCW of *Eubacterium* and SP have been observed in RA (19). However, this has not been studied in the patients with early RA.

Here, we have used several enterobacteria and Gram-positive BCW derived from normal intestinal microbiota to study the proliferation responses of peripheral blood mononuclear cells (PBMC) and SFMC in the early RA. The enterobacteria *Salmonella enteritidis* (SE) and *Yersinia enterocolitica* (YE) were included, due to their role in the etiopathogenesis of reactive arthritis (20); *Escherichia coli* (EC) served as a control. The capacity of these different bacterial antigens to induce production of tumor necrosis factor-alpha (TNF-) was also investigated.

Patients and methods

Patients

All studies involving human subjects were approved by the local ethical committee. The patients enrolled were from Satalinna Hospital, Turku University Central Hospital, and Tampere University Hospital. Twenty-eight patients with early RA (duration of the disease < 1 year) and fulfilling the 1987 revised American College of Rheumatology criteria were included (21). Twenty-nine patients with inflammatory joint diseases other than RA served as controls (Table I). In addition, the

Table I. Clinical features of the patients.

	Rheumatoid arthritis		Other arthritides*	
Number of patients	28		29	
Sex (% female)	61		59	
Age,mean (range), years	53	(21-86)	45	(16-74)
Duration, median, months	5	(1-12)	57	(0.1-396)
Rheumatoid factor + (%)	64		17	
Treatment				
NSAID (%)	66		59	
Corticosteroids (%)	18		29	
DMARD (%)	21		18	
Cytotoxic drugs (%)	None		4	

* Other arthritides: 12 reactive arthritis, 7 psoriatic arthritis, 2 ankylosing spondyarthritis, 2 pseudogout, 2 gout, 1 scleroderma, and 3 undifferentiated arthritis (2 self-limited acute knee arthritis, and 1 polyarthritis).

PBMC of 10 healthy blood donors (Finnish Red Cross Blood Transfusion Service, Turku, Finland) were used. The diagnoses of other inflammatory arthritides are based upon standard clinical criteria. Clinical features of the patients are given in Table I.

Cells

Peripheral blood and synovial fluid were collected simultaneously from the patients during diagnostic or therapeutic aspiration. Peripheral blood was also donated by healthy donors. PBMC and SFMC were isolated by density gradient centrifugation with Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden). RPMI-1640 (GIBCO BRL, Paisley, UK) supplemented with 10 mM HEPES, 2 mM L-glutamine, and 10% heat-inactivated pooled human AB serum was used as a culture medium in all experiments.

Antigens and mitogens

Phytohemagglutinin (PHA) was purchased from Difco Laboratories (Detroit, USA). Tuberculin PPD was purchased from Statens Serum Institute (Copenhagen, Denmark). *Yersinia enterocolitica* (YE) O3 (CCUG 8233) and *Escherichia coli* (EC) (ATCC 25922) were obtained from the Culture Collection, University of Gothenburg (Gothenburg, Sweden) and from the American Type Culture Collection (Manassas, VA), respectively. *Salmonella enteritidis* (SE) was isolated from the stool sample of a patient with reactive arthritis. Bacteria were heat-killed at 100°C for 1 hour.

EA (ATCC 25986), EL (ATCC 8486), LC (ATCC 11578), LF (ATCC 14931) and SP (ATCC 10389) were obtained from the American Type Culture Collection (Manassas, VA). The BCW were prepared as described before (22, 23). Briefly, the heat-killed bacteria were disrupted in a Braun MSK cell homogenizer (B. Braun Biotech International, Melsungen, Germany), and were further purified by treatment with RNase A (25 g/mg of CW) and DNase I (2.5 g/mg) and trypsin (25 g/mg) for 4 h at 37°C. All enzymes used were purchased from Sigma Chemical Co. (St. Louis, MO). After enzyme treatments, the BCW were dialyzed against sterile water and lyophilized.

The lyophilized BCW were subjected to sonication for approx. 80 minutes in a Branson Sonifier Model B15 (Smith-Kline Co., Danbury, CT). After sonication, the supernatants were centrifuged at 10,000 g for 30 minutes (Sorvall RC-5C centrifuge, SS-34 rotor). The supernatants from 10,000 g were centrifuged at 100,000 g at 4°C for 60 minutes in a Sorvall OTD-65B ultracentrifuge (Du Pont Co., Wilmington, DE; Ti60 rotor). The pellets from 100,000 g were resuspended in phosphate-buffered saline (PBS) for a 30 s sonication and labeled as 100p60. This 100p60 preparation of different BCW was used in all experiments. It is known that this BCW preparation from certain bacterial strains can induce chronic arthritis in a rat model (22). Finally, BCW solutions were filtered through 0.45 m pore size filters (Millipore S.A. Molsheim, France) and stored at 4°C before use.

The Limulus Amebocyte Lysate assay was used to exclude any possible LPS contamination in our reagents and BCW preparations. E-TOXATE® reagents were purchased from Sigma Chemical Co. The assays of endotoxin gave negative result (< 0.3 EU/ml) for all reagents and CW preparations used.

Proliferation assays

Proliferation assays were performed to study the mononuclear cell responses to heat-killed enterobacteria (SE, YE, and EC) and to BCW of EA, EL, LC, LF, and SP. PHA and PPD were used as controls. Isolated PBMC or SFMC were cultured at a concentration of 1 x 10^5 cells/well in flat-bottom 96-well plates (Costar, Cambridge, MA) with appropriate antigen at 37°C in 5% CO_2 incubator for 6 days. On the day 6 [^{3}H] thymidine was added at 0.2 Ci/well. The cells were incubated for another 18 h, then harvested onto glass fiber filters. The incorporated radioactivity was determined in a liquid scintillation counter. Experiments were performed in triplicate. The results are expressed as mean stimulation indices (SI). Stimulation indices are defined as the proliferation (counts per minute, cpm) induced by an antigen divided by the background proliferation. A significant response is defined as SI >3 and cpm (the [^{3}H] thymidine uptake) > 1,000.

TNF- induction and detection

Cells were stimulated with BCW preparations of EA, EL, LC, LF, or SP, or with heat-killed enterobacteria (SE, YE, or EC) at 37°C for 20 h in flat-bottom 24-well tissue culture plates (Costar, Cambridge, MA). Lipopolysaccharide (LPS) from EC serotype 0127: B8 (Sigma Chemical Co.) was used as a control. Culture supernatants were collected and stored at -70°C prior to the cytokine determination. TNF- concentrations of the supernatants were measured by using ELISA kits from CLB (Amsterdam, The Netherlands). The sensitivity of assays was 40 pg/ml. All cytokine assays were carried out in duplicate.

Statistical analysis

The non-parametric one-way ANOVA with Kruskal-Wallis test was used for multiple comparisons. Thereafter, the Mann-Whitney U-test was used for the comparisons between two groups. To test the differences among the repeated measurements, the non-parametric one way analysis of repeated measurements with Friedman test was used. Thereafter, Wilcoxon signed rank test was used for the paired comparisons. All statistics were done by the STATISTICA 5.0 (97th ed., Statsoft.Inc., Tulsa). Differences were considered statistically significant when $P < 0.05$.

Results

Proliferation responses of PBMC and SFMC to enterobacteria

To elucidate the potential role of enterobacteria in the etiopathogenesis of RA, the proliferation responses of PBMC and SFMC from patients with early RA were studied. Three heat-killed enterobacteria (SE, YE, and EC) were used as antigens. The patients with other types of inflammatory arthritis served as controls. PHA (100 g/ml) and PPD (10 g/ml) were used as mitogen and antigen controls. Significant responses to all three enterobacteria were detected in most of the

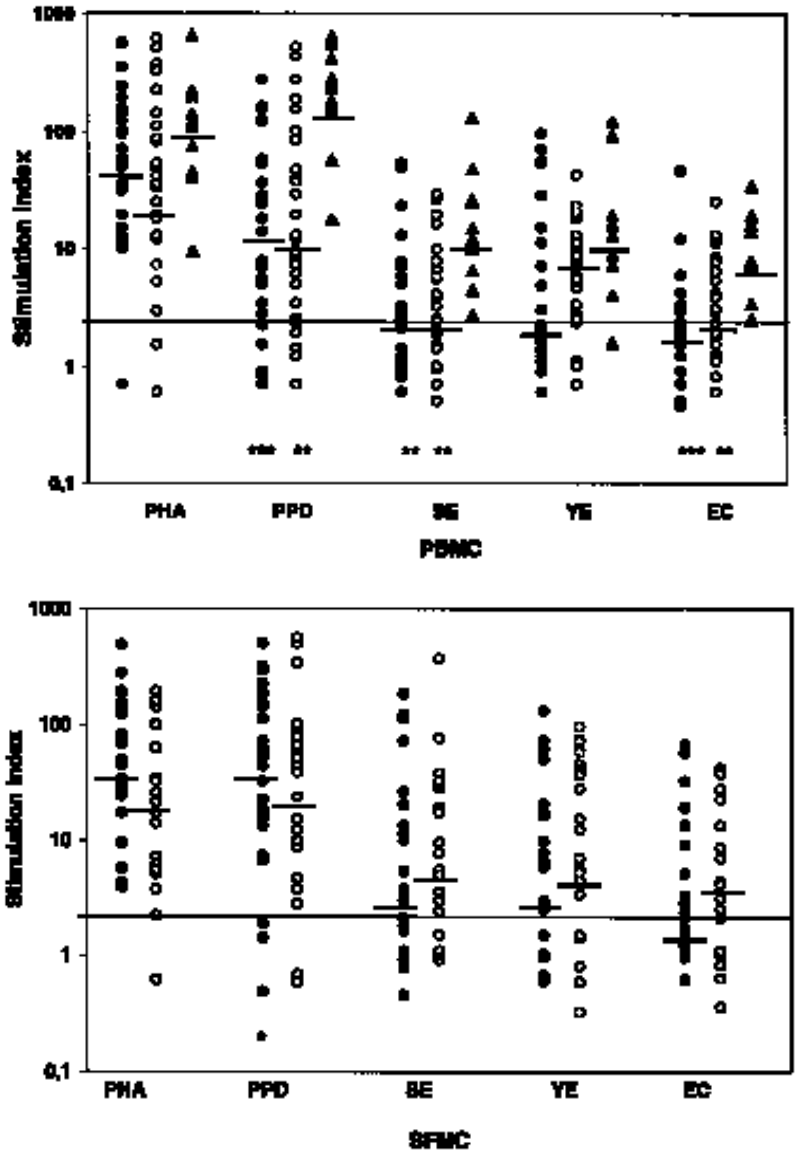


Fig. 1. Proliferation responses of peripheral blood mononuclear cells (PBMC) and synovial fluid mononuclear cells (SFMC) to heat-killed *Salmonella enteritidis* (SE), *Yersinia enterocolitica* (YE), or *Escherichia coli* (EC). Phytohemagglutinin (PHA) and tuberculin purified protein derivatives (PPD) served as controls. = early RA; = other arthritides; = healthy donors. Each symbol represents one individual (some of the symbols are completely overlapping). The median values are indicated by a line and the stimulation index of 3 by a dashed line. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. In the upper panel, the asterisks refer to the comparison of early RA or other arthritides patients to healthy donors; in the lower panel, they refer to the comparison between respective PBMC responses and SFMC responses.

samples from the patients and healthy donors (Fig. 1). Regarding PBMC responses against SE, YE, and EC, patients with early RA or with other types of arthritis showed values of the similar magnitude, although significantly lower than those of blood donors (healthy controls). The same observations were made with using the non-enteric antigen PPD. In PBMC responses, the frequency of patients with significant proliferation responses against enterobacteria was 39% in early RA and 59% in other types of arthritis. For the SFMC responses, the corresponding figures were 56% and 66%, respectively. No significant difference between early RA and other types of arthritis was observed. These data were obtained by using 10^7 bacteria/ml and an incubation time of 6 days, which were found to be optimal. In all the groups studied, significant proliferation responses of both PBMC and SFMC to PHA and PPD were observed in the majority of samples.

Proliferation responses to different concentrations of BCW

To find the optimal concentration of BCW in proliferation responses, PBMC and SFMC from patients with early RA were incubated with different concentrations of BCW of EA, EL, LC, LF, and SP. The optimal concentration of BCW for proliferation assays was observed to be 10 g/ml (Fig. 2), which was used in all further experiments.

Proliferation responses to different BCW in early RA

To study whether patients with early RA have aberrant proliferation responses of PBMC or SFMC to any particular BCW, the BCW from EA, EL, LC, LF, and SP were used. The responses to BCW were observed to be highly variable (Fig. 3). In PBMC responses, the frequency of patients with significant proliferation responses against BCW was 8.1% in early RA and 7.5% in other types of arthritis. For SFMC responses the corresponding figures were 23% and 35%, respectively. No significant difference was observed between early RA and other arthritides. Considerably high proliferation responses of PMBC and SFMC against BCW were observed in some of the patients with early RA or other types of arthritis. However, the same patients usually showed increased responses to two or more antigens. Likewise, no significant difference was observed between patients with early RA and those with other types of arthritides.

TNF- responses induced by different bacterial antigens in early RA

To investigate the potency of bacterial antigens to induce TNF- production, enterobacteria and BCW were incubated with PBMC or SFMC from patients with early RA. Other inflammatory arthritides were used as a comparison group, and LPS as a positive control. The results show that all three enterobacteria (10^7 cells/ml) and the five

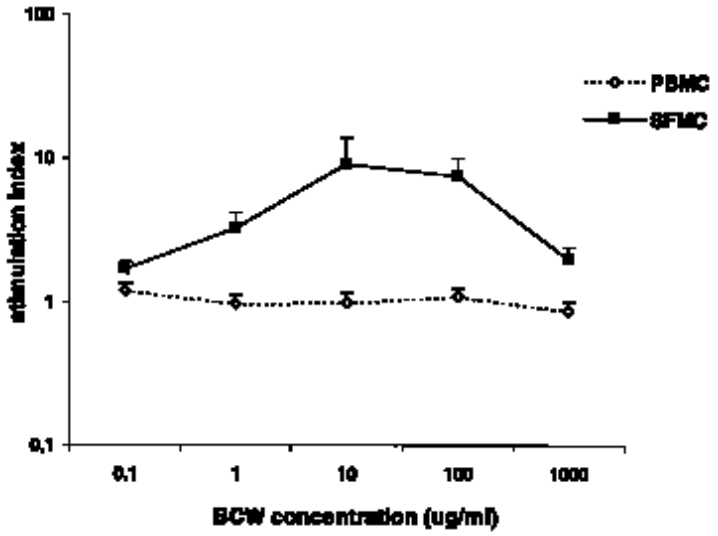
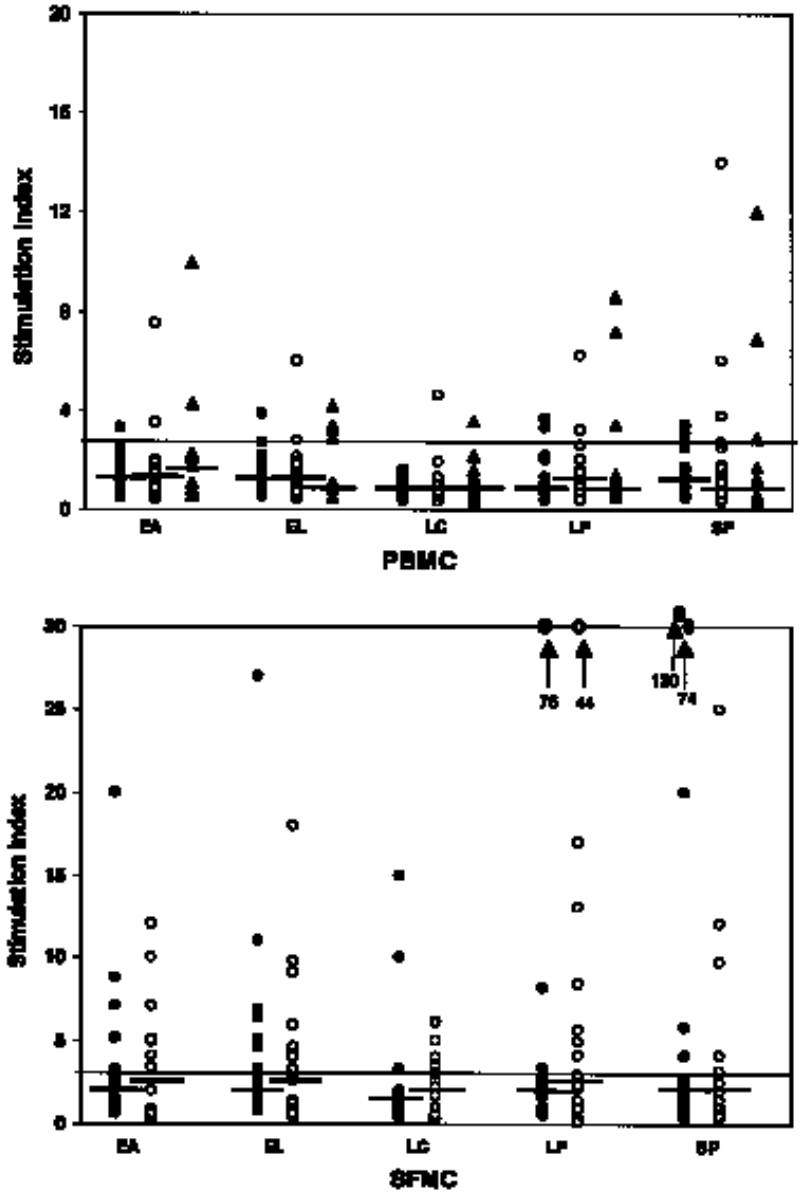


Fig. 2. Proliferation response against Gram-positive bacterial cell walls (BCW) in early rheumatoid arthritis (RA). Paired samples of PBMC and SFMC were incubated with different concentrations of BCW. The results represent mean values ± standard deviations (SD) of 3-7 patients, and are pooled data obtained with BCW from *Eubacterium aerofaciens*, *Eubacterium limosum*, *Lactobacillus casei*, *Lactobacillus fermentum*, and *Streptococcus pyogenes*.



BCW (10 g/ml) stimulate PBMC and SFMC to produce TNF- at least on same level as that induced by LPS (1 g/ml) (Fig. 4). In PBMC responses, no significant difference was observed between early RA and other arthritides (data not shown). Generally the proliferation responses by PBMC were lower than those by SFMC. In contrast to PBMC, SFMC from patients with early RA seemed to produce higher amounts of TNF- than that produced by SFMC from patients with other arthritides; this applies to stimulation by BCW from EA and to stimulation by heat-killed SE, YE and EC (Fig. 4).

Discussion

Previously it has been reported that PBMC and SFMC of RA patients may show significant proliferation responses to *Eubacterium* and *Streptococcus* BCW (19). However, this question has not been addressed in the patients with

Fig. 3. Proliferation responses to different bacterial cell walls (BCW) in early rheumatoid arthritis (RA). BCW (10 g/ml) of *Eubacterium aerofaciens* (EA), *Eubacterium limosum* (EL), *Lactobacillus casei* (LC), *Lactobacillus fermentum* (LF), or *Streptococcus pyogenes* (SP) were incubated with PBMC (upper panel) or SFMC (lower panel) from patients with early RA for 6 days. Samples from patients with other inflammatory arthritides and from healthy donors served as controls. = early RA; = other arthritides; = healthy donors. Each symbol represents one individual. The median values are indicated by a line. The value of 3 for SI is indicated by a dashed line.

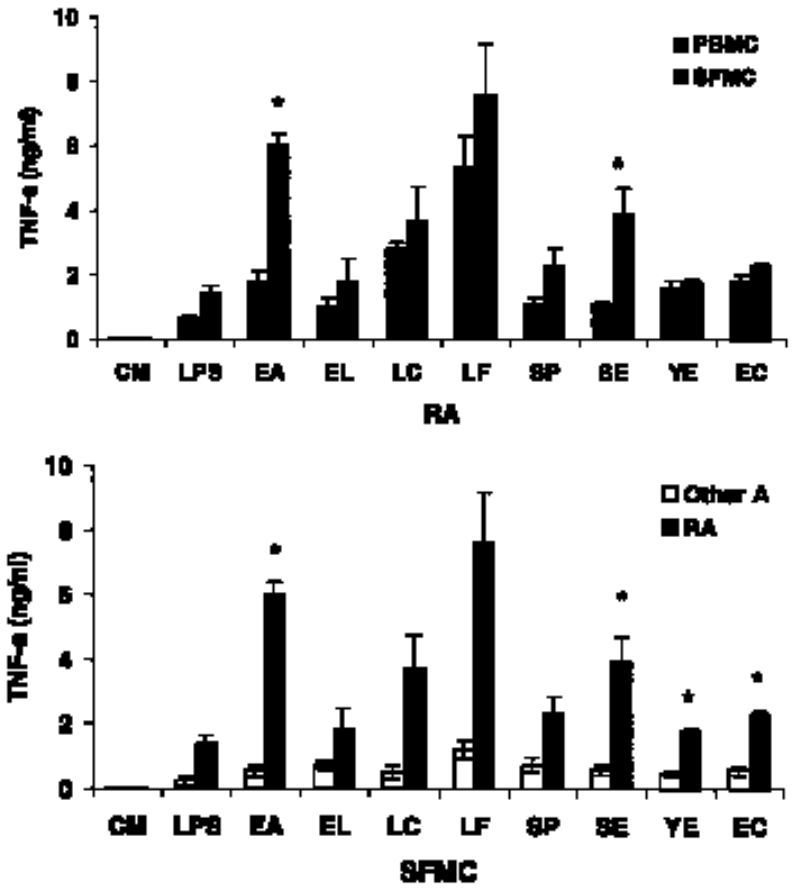


Fig. 4. Tumour necrosis factor-alpha (TNF-) production of PMBC and SFMC from patients with early rheumatoid arthritis (RA) or with other types of arthritis (other A). PBMC and SFMC were incubated with BCW (10 g/ml) of *Eubacterium aerofaciens* (EA), *Eubacterium limosum* (EL), *Lacto - bacillus casei* (LC), *Lactobacillus fermentum* (LF), *or Streptococcus pyogenes* (SP),or with heat-killed *Salmonella enteritidis (SE), Yersinia enterocolitica (YE),* and *Escherichia coli* (EC). CM = culture medium. Lipopolysaccharide (LPS) (1 g/ml) was used for comparison. Each column represents three patients (a mean (SD). * $P < 0.05$. In the upper panel, PBMC responses are compared to SFMC responses in early RA; in the lower panel, SFMC responses in other types of arthritis are compared to those in early RA.

newly developed RA. Several observations suggest that studies on patients with early RA could contribute to our understanding of the etiopathogenesis of RA (24). Given this consideration, we collected PB and SF samples from RA patients as early as possible during their disease history. All of the RA patients studied in the present work had a disease duration of less than 1 year (average 5 months). The diagnoses were confirmed retrospectively. Patients with other inflammatory arthritides were used as a control group. Although several clinical features are comparable between the two groups, a difference in the disease duration (mean of 5 months for early RA vs. 5 years for the control group) is obvious; this was mainly due to the difficulty of recognizing patients with early psoriatic arthritis.

According to our results, increased PBMC responses to heat-killed enterobacteria (SE, YE and EC) were observed in individuals from the three groups studied. However, it is remarkable that significant SFMC responses to these antigens were observed both in the patients with early RA and in those with other types of arthritis. In most cases the same patients showed increased responses to several antigens, probably due to a cross-reaction or bystander-activation. It is also of interest that similar results were observed when a variety of Gram-positive BCW derived from enteric bacteria was used. It is apparent that T cells reactive to some bacterial antigens exist in the SF and PB of patients with early RA, but they are also present in other inflammatory arthritides. Furthermore, the responses of PBMC from early RA patients are significantly lower than the responses of those from healthy blood donors. A lower proliferation response by PBMC against the causative microbes compared to the SFMC responses has also been reported in reactive arthritis, of which the exact underlying mechanism is currently unknown (25, 26). Although a comparison between the patients and healthy individuals is not the focus of the present study, it needs some explanation. For example, it might be that PBMC with reactivity to the bacterial antigens migrate to the inflamed joint tissue, leading to a decreased frequency of the responding cells in the periphery (6, 7, 27). We also observed that in early RA, SFMC proliferation responses against PPD are higher than those shown by PBMC, which is in line with a previous report (28).

The present results indicate that the enterobacteria and BCW used have a capacity to stimulate TNF- production. The level of TNF- induced by these antigens is similar to or higher than that induced by the LPS control. It is of interest that SFMC of RA patients show higher TNF- responses to some bacterial antigens than those from other types of arthritides (Fig. 4). Whether this reflects their general activity or something else cannot be determined at present. These results are in concert with our previous findings, indicating that the human immune system responds to antigens of the intestinal indigenous bacteria by cytokine production (28).

Based on our results, it seems that the T cells reactive with bacterial antigens such as BCW can be found in the SF of patients with early RA. The variability of the proliferation responses did not correlate with the positivity of rheumatoid factor, the previous infection history or the treatments administered (data not shown). Although the significance of these findings is not clear, there are several possibilities. First, bacteria or their components might persist in the

inflamed joint tissue, or be released from a reservoir in other parts of the body either continually or intermittently. Thus, in genetically susceptible individuals the bacterial antigens might be presented by antigen presenting cells, activate T cells and maintain local immune responses. It is known that some indigenous bacteria can translocate from the intestine (29), and bacterial components are detected even in the circulation of healthy individuals (30, 31). This is also supported by studies using PCR or immunohistochemistry (5, 32, 33). Nevertheless, so far no etiological agents have been reproducibly and convincingly identified in the rheumatoid joints. Second, synovial T cells with reactivity to microbial antigens may migrate to the joint tissue from other parts of the body, for example from the mucosa-associated lymphoid tissues. Recently it has been shown that human immunoblasts have dual binding capacity to the mucosa and inflamed synovial endothelium (6). There are also reports suggesting that T cells are continuously recruited into the affected RA joints (27). Finally, those synovial T cells reacting with bacterial antigens may be cross-reactive by molecular mimicry or be activated by a bystander effect (34-37).

In summary, we observed significant proliferation responses of PBMC and SFMC to different enterobacteria and BCW derived from normal intestinal microbiota in some patients with early RA. However, regarding the individual bacterial antigens, such responses seem to be non-specific and similar responses were also found in other inflammatory arthritides. Furthermore, the enterobacteria and BCW used have the capacity to induce TNF- production by both PBMC and SFMC. The TNF- response by SFMC, stimulated by EA BCW, SE,YE, or EC, was significantly higher in early RA than in other types of arthritis. Our results suggest that intestinal bacterial agents might play a role in the etiopathogenesis of RA, but the effect appears to be non-specific.

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