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# Peptidoglycan from sterile human spleen induces T-cell proliferation and inflammatory mediators in rheumatoid arthritis patients and healthy subjects

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## Abstract

*Objectives.* Peptidoglycan (PG), a component of Gram-positive bacteria, may be involved in rheumatoid arthritis (RA) because of its ability to induce production of proinflammatory cytokines, to induce arthritis in rodents, and its presence in antigen-presenting cells in RA joints.

*Methods.* In the present study, physiologically relevant PG was able to induce T-cell proliferation in peripheral blood and synovial fluid samples of RA patients, but the magnitude of the response did not differ from that of cells from healthy subjects. In addition, production of cytokines associated with RA (interleukins (IL)-1$\beta$, IL-6, IL-8, IL-10, IL-12 and tumour necrosis factor $\alpha$) and of the matrix metalloproteinase, gelatinase B (MMP-9), was induced in blood and synovial fluid cultures of RA patients.

*Conclusion.* The fact that PG, which can be found in synovial tissues of RA patients is able to induce the production of inflammatory mediators supports the hypothesis that PG plays a role in the pathogenesis of RA by influencing the inflammatory microenvironment of the joint.

KEY WORDS: Peptidoglycan, Rheumatoid arthritis, Cytokines, Matrix metalloproteinases, T cells, Bacteria, Gut.

Rheumatoid arthritis (RA) is a chronic inflammatory disease with unknown aetiology. For many years, the most attractive concept of the pathogenesis of RA has been the model of autoimmunity, in which autoreactive T cells play an important role. The association of susceptibility to RA and disease outcome with HLA-DR4/DR1 antigens [1, 2] has been regarded as the strongest argument for the T-cell-dependent nature of the disease. Moreover, T cells present in the inflamed joints consist predominantly of the primed CD45RO subset, expressing early and late activation antigens [3–5]. The importance of interactions between T cells and antigen-presenting cells (APC), as shown by the expression of CD40L, CD28 and CTLA4 by T cells and of B7-1 (CD80), B7-2 (CD86) and CD40 by APC in the synovium, has also focused attention on the role of APC in RA [6–10]. A crucial role of APC in the pathogenesis of RA is suggested by the high levels of expression and production of the proinflammatory cytokines interleukin 1$\beta$ (IL-1$\beta$), interleukin 6 (IL-6) and tumour necrosis factor $\alpha$ (TNF-$\alpha$), produced by monocytes/macrophages, which affect fibroblasts, chondrocytes and lymphocytes in the blood, synovial fluid and synovial tissue of RA patients [6–11]. This is supported by the promising results with anti-TNF-$\alpha$ or anti-TNF receptor therapies in RA patients [12] and the development of spontaneous arthritis in TNF-$\alpha$ mice [13, 14]. Furthermore, the matrix metalloproteinase gelatinase B, the expression of which is regulated by cytokines and other inflammatory mediators, has been detected in the synovial fluid of RA patients [15].

The (auto)antigen(s) leading to chronic stimulation of T cells and/or macrophages are still unknown. In reactive arthritis, the triggering antigens are thought to be microbes that cause infections of the urogenital tract and the gut, such as *Chlamydia trachomatis*, *Yersinia* and *Salmonella* species [16, 17]. Antigens of these bacteria have been detected in synovial cells [18–20],

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and T cells specific for these antigens have been found in the synovium [21, 22]. Because RA is a chronic disease, it may be that the bacterial load in the intestine, with which we are in lifelong close contact, is important in the induction and perpetuation of RA. This is supported by the observation that some patients with Crohn's disease and ulcerative colitis, both of which are intestinal autoimmune diseases, may suffer from arthritis [23, 24]. We and others have hypothesized previously that peptidoglycan (PG) plays a role in the pathogenesis of RA [25–28]. PG is the major component of the cell wall of Gram-positive bacteria and is composed of long sugar chains of alternating *N*-acetyl glucosamine and *N*-acetyl muramic acid residues, which are interlinked by peptide bridges, resulting in a large complex macromolecular structure [29]. PG isolated from the anaerobic bacterium *Eubacterium aerofaciens*, which is present in numbers over $10^9$/g human faeces, induces severe and chronic arthritis in rats [30].

Furthermore, PG could be detected in APC in synovial tissues of RA patients by the use of a specific antibody [31, 32]. PG has also been isolated from sterile normal human spleen using biochemical methods ($PG_{spleen}$) [33, 34]. *In vitro* analysis showed that the latter PG fraction is able to induce production of the proinflammatory cytokines TNF-$\alpha$, IL-1 and IL-6 [34], which are crucially involved in the pathogenesis of RA [11]. Furthermore, it has been shown that PG isolated from human spleen is able to bind to the CD14 receptor and to elicit T-cell proliferation of CD4- and CD8-positive $\alpha\beta$TCR T cells after proteolytic processing (I.A. Schrijver *et al.*, submitted for publication). Previously, T-cell responses against peptidoglycan–polysaccharide complexes (PG-$PS_{faeces}$) isolated from human faeces have been analysed in peripheral blood and synovial fluid of RA patients. In this report, only low responses to PG were found [35]. In the present study we used $PG_{spleen}$ because it reflects the properties of PG present in human tissues, it is structurally more intact than PG-$PS_{faeces}$ and it has a 10- to 100-fold greater biological activity [34].

To further delineate the role of PG in the pathogenesis of RA, we determined the influence of PG fractionated from sterile human spleen on T-cell proliferation and the production of inflammatory mediators. Therefore, we determined the production of the proinflammatory cytokines IL-1$\beta$, IL-6, interferon $\gamma$ (IFN-$\gamma$) and TNF-$\alpha$, the immunoregulatory cytokine IL-10, the chemokine IL-8 and the matrix metalloproteinase gelatinase B in peripheral blood of RA patients and control subjects and in synovial fluid samples from RA patients after stimulation with PG.

## Patients and methods

### *Patients*

*Blood samples.* Heparin blood samples of 29 RA patients fulfilling the American College of Rheumatology criteria [36] were obtained from the Zuiderziekenhuis Hospital Rotterdam. Patients received no immunosuppressive medication except for five patients receiving low-dose prednisone (up to 10 mg/day) or methotrexate (up to 30 mg/week). RA disease activity at the time of blood sampling was determined using the disease activity score by an experienced rheumatologist [37]. This was calculated with the formula $0.56\sqrt{\text{TJC}} + 0.28\sqrt{\text{SJC}} + 0.7 \log \text{ESR} + 0.014\ \text{GH}$, where TJC is the number of painful joints (out of 28), SJC is the number of swollen joints (out of 28) and GH is the general health score on a visual analogue scale (0–100). Twenty-seven blood samples of healthy donors were a kind gift from the Department of Epidemiology of the University Hospital of Rotterdam. Ten blood samples were obtained from healthy volunteers. Characteristics of the donors of blood samples are shown in Table 1. All blood samples were processed within 2–5 h after collection.

*Synovial fluid samples.* Synovial fluid samples of an inflamed joint of 14 RA patients were obtained from the Zuiderziekenhuis in Rotterdam (Table 1). Synovial fluid was collected in heparin tubes. Paired blood and synovial fluid samples were collected from 10 of these patients.

### *Proliferation assays*

Peripheral blood mononuclear cells (PBMC) of all healthy donors and RA patients and synovial fluid mononuclear cells (SFMC) of 14 RA patients were isolated from heparinized blood using Ficoll Hypaque (Pharmacia, Uppsala, Sweden). Cells ($2 \times 10^5$ cells in 100 $\mu$l) were cultured in 96-well plates in the presence of 100 $\mu$l PG isolated from human spleen containing 0.70 $\mu$g muramic acid [34], PG-$PS_{faeces}$ containing 70 $\mu$g/ml muramic acid [38], 2 $\mu$g/ml lipopolysaccharide (LPS) (Sigma, St Louis, MO, USA) or 6 Lf/ml tetanus toxoid (TT) as a control recall protein antigen (RIVM, Bilthoven, The Netherlands). Cells and antigens were diluted in RPMI (Biowittaker, Verviers, Belgium) + 10% heat-inactivated human serum. All antigens were analysed in triplicate and concentrations given in the Results section are final concentrations. The cells were cultured for 7 days, the last 8 h in the presence of tritiated thymidine (Amersham, Pharmacia Biotech, Little Chalfont, UK). The cells were then harvested and the incorporated radioactivity was counted in a liquid scintillation counter. The proliferative response was expressed as counts per minute (c.p.m.) or stimulation index (SI), the ratio of mean c.p.m. in the presence and absence of antigen. When $SI > 3$, it was concluded that proliferation had occurred.

### *Induction of cytokine release in whole blood, whole synovial fluid, PBMC or SFMC after stimulation with PG*

Cytokine induction was performed in 20 blood and nine synovial fluid samples of RA patients and in six to 10 healthy control blood samples using a method described before [34]. RPMI 1640 (Biowittaker) [12 $\mu$l,

TABLE 1. General features of the donor groups

| Group | Source | Sex (M/F) | Age (yr)[a] | Duration (months)[a] | Disease activity score[a] |
|---|---|---|---|---|---|
| Healthy control | Blood | 12/21 | 60 (25–84) | n.a. | n.a. |
| RA patient | Blood | 9/21 | 58 (30–82) | 101 (8–360) | 5.0 (1.6–7.8) |
| | Synovial fluid | 3/11 | 63 (46–82) | 66 (8–180) | 5.7 (3.4–7.4) |

[a]Mean (range).
n.a. = not applicable.

containing 90 ng/ml PG (muramic acid) or 25 ng/ml LPS (Sigma)] was added to a polypropylene tube containing 11.25 $\mu$l blood, synovial fluid, PBMC or SFMC. The mixtures were incubated for 4 or 17 h at 37°C in a 5% carbon dioxide atmosphere. Then 375 $\mu$l RPMI was added to each tube and the mixtures were centrifuged for 10 min at 400 *g*. The supernatants were stored at − 20°C until analysis for cytokines and gelatinase B.

*Determination of cytokines*

Cytokine production was measured by commercial capture ELISA. In a pilot study of 10 healthy donors, IL-1$\beta$, IL-4, IL-6, IL-8, IL-10, IL-12p40, TNF-$\alpha$, IFN-$\gamma$ and GM-CSF were measured in culture supernatants of 4- and 17-h supernatants. IL-4 and GM-CSF were undetectable in the supernatants. After LPS stimulation, IL-1$\beta$, IL-6, IL-10, IL-12 and TNF-$\alpha$ reached their highest levels after 4 h of stimulation and IL-8 and IFN-$\gamma$ after 17 h. Therefore IL-1$\beta$, IL-6, IL-10, IL-12p40 and TNF-$\alpha$ production was measured in the 4-h supernatants and IL-8 and IFN-$\gamma$ in the 17-h supernatants. ELISA was performed according to the manufacturer's guidelines (Biosource, Fleurus, Belgium). Briefly, polystyrene microtitre wells (Immuno Maxisorp; Nunc, Roskilde, Denmark) were coated overnight at room temperature with monoclonal anti-cytokine antibodies followed by washing (0.9% NaCl) and 2 h of blocking (phosphate-buffered saline/bovine serum albumin 0.5%). Freshly thawed supernatants of the cell cultures and recombinant human cytokine standards were incubated in duplicate for 2 h in the presence of a biotinylated second anti-cytokine antibody, followed by washing steps, poly-streptavidin–horseradish peroxidase (CLB, Amsterdam, The Netherlands) and enzyme substrate (TMB peroxidase; KPL, Gaithersburg, MD, USA). Optical density was measured at 450 nm.

*Determination of gelatinase B activity*

Gelatinase B (MMP-9) was measured by two methods in the supernatants obtained after 4 h of stimulation. First, the quantitative gelatine zymography method was used. This is a densitrometric method that, by inclusion of appropriate standards, discriminates inducible gelatinase B (MMP-9) from constitutive gelatinase A [15, 39]. Quantitative determination of gelatinase activity was achieved by computerized image analysis using two-dimensional scanning densitometry. Gelatinase activity was expressed in scanning units representing the scanning area under the curve, which is an integration ratio that takes into account both brightness and width of the substrate lysis zone. Second, the gelatinolytic activity that resulted from the balance between enzymes and enzyme inhibitors (the so-called protease load) was measured in the samples using fluorescence-activated gelatin conversion. Both methods have been described in detail previously [15, 39, 40].

*Statistical analysis*

Statistical analysis was performed using the Mann–Whitney method to analyse differences in PG-induced proliferation and the production of effector molecules between RA patients and healthy subjects. The Wilcoxon signed rank test method was used to determine whether PG was able to induce cytokine production. Linear regression was performed to determine relationships of disease activity and disease duration with T-cell proliferation and cytokine production. $P$ values $< 0.05$ were considered significant.

## Results

*Proliferation of PBMC after stimulation with PG*

To examine whether proliferation of T cells in response to PG was increased in RA patients compared with healthy subjects, 29 RA patients and 33 age-matched controls were examined for the proliferative response, to $PG_{spleen}$ and PG-$PS_{faeces}$. LPS and TT were used as positive controls. The results show (Fig. 1) that, after 7 days of culture, T-cell proliferation was elicited by $PG_{spleen}$ in 21 of the 29 RA patients. In 33 healthy donors, 25 reacted against $PG_{spleen}$. The median SI value was 6.9 for RA patients and 6.7 for healthy subjects. The proliferative response to PG-$PS_{faeces}$ was lower than that to $PG_{spleen}$ despite the 100-fold higher concentration used. The proliferative responses to LPS and TT were lower in RA patients than in healthy donors, but this difference did not reach significance.

*Proliferation of SFMC after stimulation with PG*

Synovial fluid was obtained from 14 RA patients. Mononuclear cells were isolated from both samples using Ficoll. In only two of the synovial fluid samples could a proliferative response to $PG_{spleen}$ be measured (Fig. 2). After stimulation with PG-$PS_{faeces}$, LPS and TT, the number of patients responding was 0, 3 and 2 out of 14 respectively. When the proliferative responses of 10 samples of synovial fluid mononuclear cells of the four analysed antigens were compared with those of paired peripheral blood cells, responses were

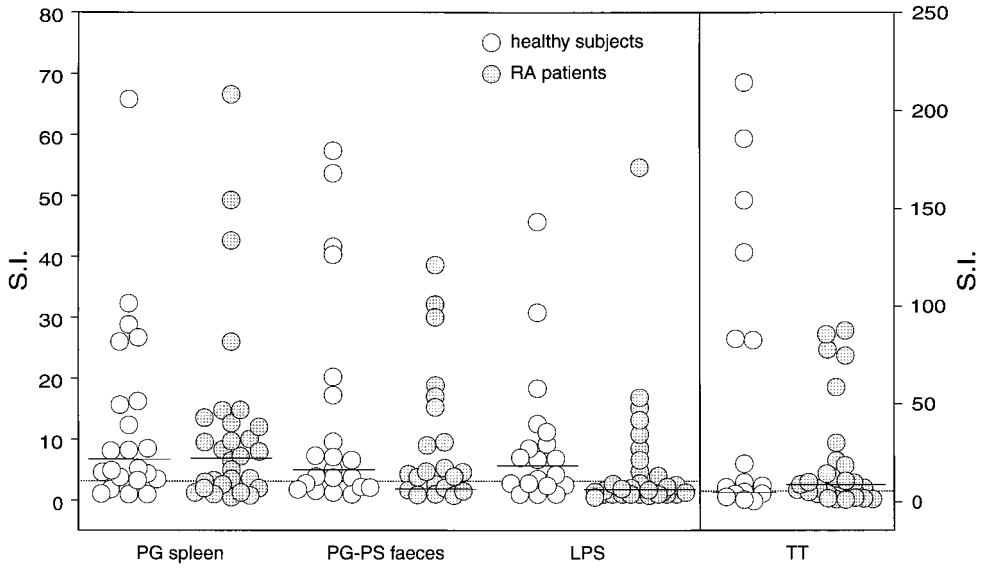


FIG. 1. PG isolated from sterile human spleen induces proliferation of PBMC in both RA patients and healthy subjects. PBMC isolated from 29 RA patients and 20 healthy controls were incubated for 7 days with $PG_{spleen}$, $PG_{faeces}$, LPS or TT. Results are expressed as the SI. No differences were observed between RA patients and healthy controls after stimulation with either PG source. LPS- and TT-induced proliferation were decreased in RA patients, but these effects did not reach significance. The horizontal dotted line indicates the cutoff point for proliferation (SI = 3).

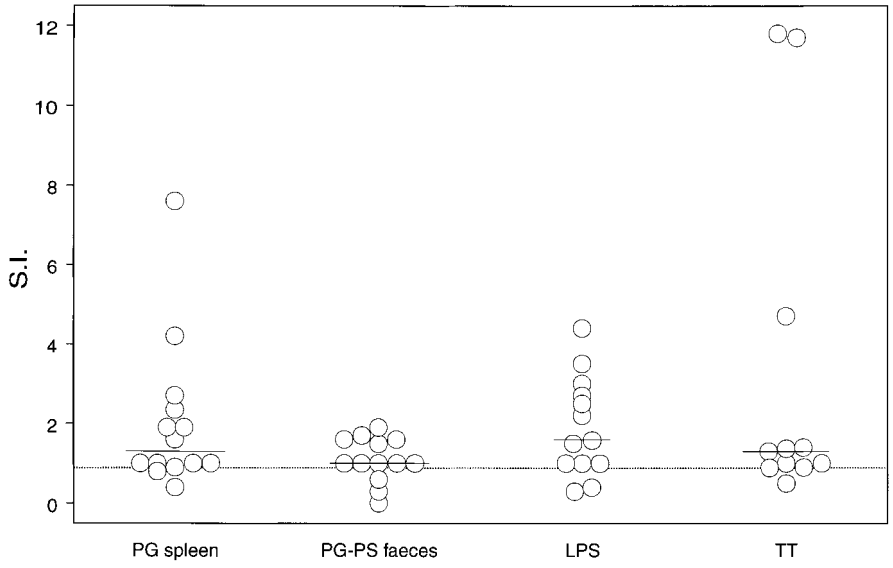


FIG. 2. $PG_{spleen}$ induces proliferation of RA SFMC. SFMC isolated from 14 RA patients were cultured for 7 days with $PG_{spleen}$, $PG_{faeces}$, LPS and TT. Results are expressed as the SI. Proliferation of SFMC of two patients was observed after $PG_{spleen}$ stimulation. After stimulation with $PG_{faeces}$, LPS and TT, the numbers of patients responding were 0, 3 and 2 out of 14 respectively. The horizontal dotted line indicates the cutoff point for proliferation (SI = 3).

significantly lower despite the use of identical cell numbers and stimulation conditions (data not shown).

### *Relationships of proliferation with disease activity and disease duration*

Correlation analysis was performed to determine the possible relationships between proliferation and RA disease activity or disease duration as scored at the time of sampling. No correlations were found for any of the four antigens examined.

### *Cytokine production in peripheral blood after stimulation with $PG_{spleen}$*

To investigate whether PG is able to stimulate production of the cytokines involved in RA, the production of IL-1$\beta$, IL-6, IL-8, IL-10, IL-12, IFN-$\gamma$ and TNF-$\alpha$ was measured after whole-blood cell stimulation of 20 RA

samples and 10 healthy donor samples. The samples were stimulated with $PG_{spleen}$ and LPS served as a positive inducer. Results of RA and healthy donor samples stimulated with medium and $PG_{spleen}$ are shown in Fig. 3.

These results show that, without stimulation, concentrations of all analysed cytokines were higher for RA patients than for healthy subjects. Because this higher concentration was mostly due to five to seven high-responding patients, the difference between control subjects and RA patients was only significant for IL-10.

In healthy subjects, $PG_{spleen}$-induced production of IL-1$\beta$, IL-6, IL-8, IL-10, IL-12 and TNF-$\alpha$ was significantly increased compared with spontaneous production. In the RA patients a significant increase in the production of IL-1$\beta$, IL-6, IL-8, IL-10 and TNF-$\alpha$ was observed.

Because cytokine levels in unstimulated samples of RA patients were very high, probably due to cytokines already present in the plasma, mononuclear cells were isolated to measure newly secreted cytokine. Table 2 shows that, after control stimulation with medium alone, all cytokine levels were low except IL-8. After PG stimulation a significant increase was observed for all cytokines except IL-12 and IFN-$\gamma$. After stimulation with LPS, similar results were observed (data not shown).

### *Cytokine production in synovial fluid after stimulation with $PG_{spleen}$*

Cytokine production was induced and measured in supernatants of whole synovial fluid mononuclear cells and of isolated mononuclear cells from nine RA patients. The results show that all cytokines analysed were present in the synovial fluid. After stimulation with PG, the production of IL-1$\beta$, IL-6, IL-8 and TNF-$\alpha$ was increased (Table 3).

When mononuclear cells were isolated, high concentrations of IL-6, IL-8 and TNF-$\alpha$ were measured without further stimulation, indicating that activated mononuclear cells produced these cytokines spontaneously. After PG stimulation, IL-1$\beta$, IL-8, IL-10 and TNF-$\alpha$ were produced at significantly higher levels, indicating that PG can increase the production of these cytokines in SFMC.

For nine patients, paired samples of peripheral blood and synovial fluid were analysed. Concentrations of the different cytokines in unstimulated samples were similar in peripheral blood and synovial fluid except for IL-10, IFN-$\gamma$ and IL-12, for which concentrations were higher in synovial fluid than in peripheral blood; however, these differences were not significant (data not shown). After stimulation with LPS, similar results were observed (data not shown).

### *Gelatinase B production in whole blood and synovial fluid after stimulation with $PG_{spleen}$*

Quantitative analysis of gelatinase B production showed that, in blood cells, gelatinase B concentration was higher, although not significantly, so in RA patients than in healthy subjects, suggesting a higher spontaneous release of gelatinase B in the peripheral blood of RA patients (Fig. 3). Furthermore, it was shown that PG induces production of gelatinase B. In blood samples of healthy subjects this induction was significant (Fig. 3). In synovial fluid samples the induction of gelatinase B by PG did not reach significance (Table 3). Analysis of the kinetics of the dose-dependent induction of gelatinase B by PG showed that gelatinase B was induced after 4 h of incubation (data not shown). To determine whether this induction resulted in an increase in gelatinase activity, all samples were also analysed by fluorescence activated substrate conversion. None of these samples showed net gelatinase B activity.

### *Relationships of cytokine production with disease activity and disease duration*

To examine the possible relationships between (PG-induced) cytokine and gelatinase production and RA pathology, linear regression was performed for cytokine production separately both for peripheral blood and synovial fluid and for disease activity and disease duration. Although cytokine production by unstimulated cells showed a positive relationship with disease activity in both blood and synovial fluid samples with most cytokines, in particular with IL-6 and TNF-$\alpha$, no significant correlation was observed (data not shown).

## Discussion

The present study shows that PG fractionated from sterile human spleen is able to induce T-cell proliferation in peripheral blood and synovial fluid of RA patients, and that PG is able to induce production of the inflammatory mediators IL-1$\beta$, IL-6, IL-8, IL-10 and TNF-$\alpha$ as well as gelatinase B in peripheral blood and synovial fluid cells. No differences were observed between the T-cell proliferation and cytokine production induced by PG in RA patients and healthy subjects.

In about 75% of RA patients and control donors, proliferation of PBMC against PG was observed. This indicates that there is no apparent disturbance of specific T-cell proliferation in response to PG in RA patients compared with healthy donors. The lower proliferative response against PG-$PS_{faeces}$ in both groups, even with a 100-fold higher concentration, supports the importance of intact PG structure in the induction of T-cell proliferation, because $PG_{spleen}$ contains intact peptide bridges, which are almost absent in PG-$PS_{faeces}$. There is also no indication that, at the site of inflammation, proliferation after stimulation with PG contributes to pathogenesis, because proliferation was observed in only two of the 14 RA patients analysed. Because T-cell proliferation in synovial fluid mononuclear cells is very low after stimulation with the positive control stimuli LPS and TT, it is possible that the method used was not optimal. The mean ratio of APC (i.e. monocytes/macrophages) to T cells was 1 : 1.4 in the synovial fluid, as shown by morphology analysis of cytospins of the synovial fluid cells (data not shown).

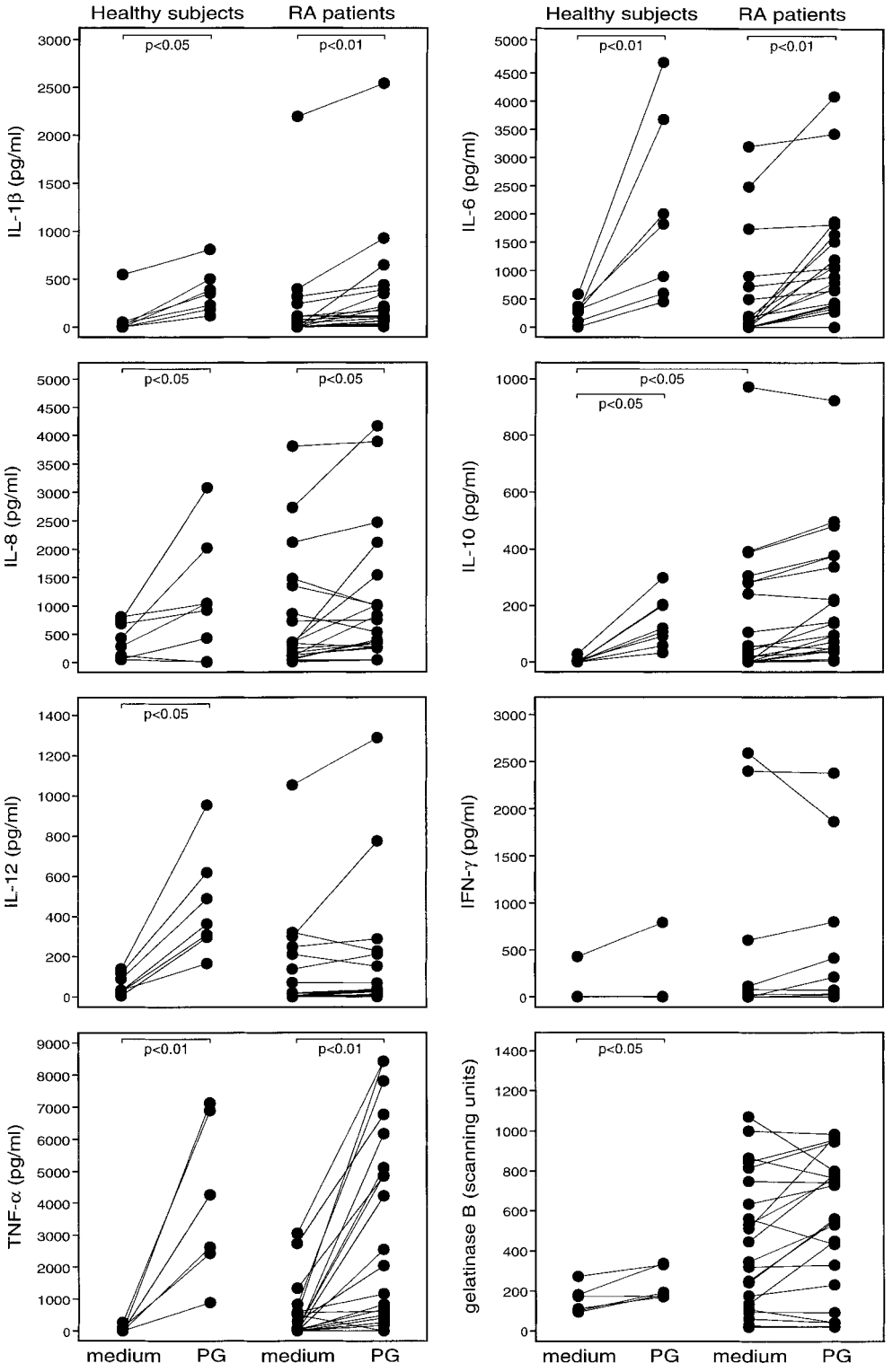


FIG. 3. $PG_{spleen}$ induces cytokine production in healthy subjects and RA patients. Whole blood cell samples of 20 RA patients and 10 healthy controls were stimulated or not stimulated with $PG_{spleen}$. Production of IL-1$\beta$, IL-6, IL-8, IL-10, IL-12, TNF-$\alpha$, and IFN-$\gamma$ was assessed by ELISA. Levels of all cytokines were higher in unstimulated samples of RA patients compared with healthy controls; the difference was significant for IL-10. $PG_{spleen}$ was significantly induced production of IL-1$\beta$, IL-6, IL-8, IL-10 and TFN-$\alpha$ in both healthy subjects and RA patients. IL-12 was significantly induced only in healthy subjects.

In peripheral blood this ratio was approximately 1 : 5, implying that many more potentially responsive T cells were present in the wells. Another explanation for the lack of synovial fluid T-cell proliferation is that most synovial fluid T cells, despite the expression of CD45RO, are hyporesponsive upon stimulation by anti-CD3 and anti-CD28 [41]. A defect in T-cell receptor-mediated signalling at the level of tyrosine phosphorylation has been suggested [42].

Cytokine induction by PG was measured using two different cell populations: whole blood or synovial cells and isolated mononuclear cells. The whole blood cell culture technique has proved to be quite reproducible [43–45] and it closely reflects the *in vivo* situation. However, because in some RA patients the cytokine concentrations present in unstimulated samples were very high, further stimulation of cytokine production by PG was difficult to examine. Therefore, we also used Ficoll-purified mononuclear cells to measure newly secreted cytokines, and cytokines already present in the plasma were not measured.

Both in blood and synovial fluid of RA patients, higher levels of IL-6, IL-8, IL-10, IL-12, IFN-$\gamma$ and TNF-$\alpha$ were present in unstimulated samples compared with healthy subjects. This is consistent with earlier reports [11, 46, 47]. Production of IL-1$\beta$, IL-6, IL-8, IL-10 and TNF-$\alpha$ was increased after $PG_{spleen}$ stimulation in both healthy donors and RA patients by cells in peripheral blood and synovial fluid. An increase in IL-12 after induction by $PG_{spleen}$ was only found in whole blood cell stimulations of healthy subjects. No production of IFN-$\gamma$ induced by $PG_{spleen}$ could be demonstrated. This is not surprising as IFN-$\gamma$ is produced mainly by T cells, and T-cell stimulation by $PG_{spleen}$ reached its highest level after 7 days of culture instead of 17 h. In accordance with this, cytokine production measured in PBMC stimulations of healthy donors at day 7 showed that high amounts of IFN-$\gamma$ (137–399 ng/ml) were produced, but no IL-4 (data not shown). In two out of 14 synovial fluid samples, no induction of TNF-$\alpha$, IL-6 and IL-1 could be observed. The cells of these patients were also refractory to stimulation with PG of LPS. In one patient, this might have been a result of the use of steroids. The use of prednisone and methotrexate by some of the patients also resulted in lower cytokine production by the blood cells compared with patients not using these drugs, but these differences were not significant.

Gelatinase B is detected in synovial fluid [15] and is expressed in the synovial tissues of RA patients [48, 49], where it plays an important role in connective tissue destruction by degrading components of the extracellular matrix [50]. This study shows that $PG_{spleen}$ is able to induce production of this enzyme. Whether this enzyme is induced directly or indirectly through IL-8 or TNF-$\alpha$ for example, which are known to induce gelatinase B [15, 51, 52], has to be analysed further using specific antibodies. The kinetic analysis of gelatinase B expression is in favour, however, of a direct effect of PG. The lack of net gelatinase B activity in the samples can be explained by the presence of natural and possibly induced inhibitors of gelatinase B activity [53].

To determine if the biological properties of PG are directly involved in the pathogenesis of RA, possible relationships of cellular proliferation and cytokine/gelatinase B production with disease activity and disease duration was analysed using correlation analysis. For most cytokines there was a trend for higher cytokine production to be positively related to disease activity

TABLE 2. $PG_{spleen}$ induces production of cytokines by peripheral blood mononuclear cells of RA patients

| Cytokine | Medium | $PG_{spleen}$ |
|---|---|---|
| IL-1$\beta$ | 3 (0–233) | 217 (13–1763)** |
| IL-6 | 30 (0–1418) | 1602 (84–4118)** |
| IL-8 | 2938 (372–3962) | 3293 (567–3779)* |
| IL-10 | 2 (0–42) | 80 (0–280)** |
| IL-12 | 0 (0–444) | 1 (0–2470) |
| IFN-$\gamma$ | 0 (0–0) | 0 (0–0) |
| TNF-$\alpha$ | 13 (0–7002) | 2864 (0–13 263)** |

Data are median cytokine production (pg/ml) with range in parentheses.
*$P < 0.05$; **$P < 0.01$.

TABLE 3. $PG_{spleen}$ induces production of proinflammatory mediators in synovial fluid (SF) samples

| | Medium | | $PG_{spleen}$ | |
|---|---|---|---|---|
| Cytokine | Whole SF | SF mononuclear cells | Whole SF | SF mononuclear cells |
| IL-1$\beta$ | 9 (0–59) | 7 (0–14) | 36 (0–556) | 121 (65–168)* |
| IL-6 | 789 (0–9452) | 133 (2736–3453) | 1286 (0–9443) | 496 (444–2761) |
| IL-8 | 498 (34–1295) | 3003 (2736–3453) | 1530 (960–3570) | 3438 (3233–3866)* |
| IL-10 | 49 (0–733) | 2 (0–16) | 150 (0–678) | 86 (32–133)* |
| IL-12 | 320 (0–2543) | 0 (0) | 348 (0–2918) | 0.5 (0–1.5) |
| INF-$\gamma$ | 0 (0–8089) | 0 (0) | 0 (0–8132) | 0 (0–12) |
| TNF-$\alpha$ | 279 (0–3638) | 952 (0–4080) | 2147 (0–9099)* | 8668 (6182–15 697)* |
| Gelatinase B | 719 (612–2122) | n.d. | 607 (70–2003) | n.d. |

Data are median cytokine production (pg/ml) and median gelatinase B production (scanning units) with range in parentheses.
n.d. = not done.
*$P < 0.05$.

and negatively related to disease duration. These trends did not reach significance.

There remains the question of what role PG plays in the pathogenesis of RA. The main indication for a role of PG in RA is the presence of PG in APC in synovial tissues [32], suggesting that bacterial antigens, ubiquitously present in this mucosa, gain access to the joints, probably carried by phagocytic APC in the bloodstream [54]. The distribution of PG might be increased by ultrastructural gut lesions [55] or a reduced level of protection of the mucosal surface provided by antibodies against PG [56]. Although this study did not identify disturbances in the T-cell response against PG, the results do show that PG is able to induce production of effector molecules that are crucially involved in RA. On the basis of these results, we hypothesize that PG is involved in the pathogenesis of RA by inducing cytokines and gelatinase B. First, the production of these cytokines and gelatinase B can lead to destruction of the joint tissue. Secondly, cytokines might allow autoreactive T cells to overcome self-tolerance to certain autoantigens. Recent studies have shown that various infectious and proinflammatory agents, such as LPS, a functional PG analogue, are capable of reversing the tolerant state of $CD4^+$ [57] and $CD8^+$ [58] T cells in the presence of specific (auto)antigens by the production of cytokines stimulating T-cell proliferation and survival [59, 60]. Thus, inflammatory activity within the RA joint may be dependent on factors governing the redistribution of PG to non-mucosal sites. Therapeutic intervention restricting the access of PG to non-mucosal sites may therefore be of clinical benefit to patients. This can be examined using *in vivo* bacterial translocation models.

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## References

1. Reveille JD. The genetic contribution to the pathogenesis of rheumatoid arthritis. Curr Opin Rheumatol 1998;10:187–200.
2. Auger I, Roudier J. HLA-DR and the development of rheumatoid arthritis. Autoimmunity 1997;26:123–8.
3. Potocnik AJ, Kinne R, Menninger H *et al*. Expression of activation antigens on T cells in rheumatoid arthritis patients. Scand J Immunol 1990;31:213–24.
4. Mizokami A, Eguchi K, Kawakami A *et al*. Increased population of high fluorescence 1F7 (CD26) antigen on T cells in synovial fluid of patients with rheumatoid arthritis. J Rheumatol 1996;23:2022–6.
5. Tak PP, Hintzen RQ, Teunissen JJ *et al*. Expression of the activation antigen CD27 in rheumatoid arthritis. Clin Immunol Immunopathol 1996;80:129–38.
6. Opdenakker G, Van Damme J. Cytokine-regulated proteases in autoimmune diseases. Immunol Today 1994;15:103–7.
7. Sfikakis PP, Via CS. Expression of CD28, CTLA4, CD80, and CD86 molecules in patients with autoimmune rheumatic diseases: implications for immunotherapy. Clin Immunol Immunopathol 1997;83:195–8.
8. Summers KL, O'Donnell IL, Williams LA, Hart DN. Expression and function of CD80 and CD86 costimulator molecules on synovial dendritic cells in chronic arthritis. Arthritis Rheum 1996;39:1287–91.
9. MacDonald KP, Nishioka Y, Lipsky PE, Thomas R. Functional CD40 ligand is expressed by T cells in rheumatoid arthritis. J Clin Invest 1997;100:2404–14.
10. Verwilghen J, Lovis R, De Boer M *et al*. Expression of functional B7 and CTLA4 on rheumatoid synovial T cells. J Immunol 1994;153:1378–85.
11. Feldmann M, Brennan FM, Maini RN. Role of cytokines in rheumatoid arthritis. Annu Rev Immunol 1996;14:397–440.
12. Feldmann M, Brennan F, Paleolog E, Taylor P, Maini RN. Anti-tumor necrosis factor alpha therapy of rheumatoid arthritis. Mechanism of action. Eur Cytokine Netw 1997;8:297–300.
13. Keffer J, Probert L, Cazlaris H *et al*. Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis. EMBO J 1991;10:4025–31.
14. Butler DM, Malfait AM, Mason LJ *et al*. DBA/1 mice expressing the human TNF-alpha transgene develop a severe, erosive arthritis: characterization of the cytokine cascade and cellular composition. J Immunol 1997;159:2867–76.
15. Opdenakker G, Masure S, Grillet B, Van Damme J. Cytokine-mediated regulation of human leukocyte gelatinases and role in arthritis. Lymphokine Cytokine Res 1991;10:317–24.
16. Sieper J, Braun J, Brandt J *et al*. Pathogenetic role of Chlamydia, Yersinia and Borrelia in undifferentiated oligoarthritis. J Rheumatol 1992;19:1236–42.
17. Wuorela M, Granfors K. Infectious agents as triggers of reactive arthritis. Am J Med Sci 1998;316:264–70.
18. Granfors K, Jalkanen S, Lindberg AA *et al*. Salmonella lipopolysaccharide in synovial cells from patients with reactive arthritis. Lancet 1990;335:685–8.
19. Hammer M, Zeidler H, Klimsa S, Heesemann J. Yersinia enterocolitica in the synovial membrane of patients with Yersinia-induced arthritis. Arthritis Rheum 1990;33:1795–800.
20. Taylor-Robinson D, Gilroy CB, Thomas BJ, Keat AC. Detection of Chlamydia trachomatis DNA in joints of reactive arthritis patients by polymerase chain reaction. Lancet 1992;340:81–2.
21. Hermann E, Fleischer B, Mayet WJ, Poralla T, Meyer zum Buschenfelde KH. Response of synovial fluid T cell clones to Yersinia enterocolitica antigens in patients with reactive Yersinia arthritis. Clin Exp Immunol 1989;75:365–70.
22. Gaston JS, Life PE, Granfors K *et al*. Synovial T lymphocyte recognition of organisms that trigger reactive arthritis. Clin Exp Immunol 1989;76:348–53.
23. De Vos M, De Keyser F, Mielants H, Cuvelier C, Veys E. Review article: bone and joint diseases in inflammatory bowel disease. Aliment Pharmacol Ther 1998;12:397–404.
24. Orchard TR, Wordsworth BP, Jewell DP. Peripheral arthropathies in inflammatory bowel disease: their articular distribution and natural history. Gut 1998;42:387–91.
25. Hazenberg MP, Klasen IS, Kool J, Ruseler-van Embden JG, Severijnen AJ. Are intestinal bacteria involved in the etiology of rheumatoid arthritis? Review article. APMIS 1992;100:1–9.

26. Hazenberg MP. Intestinal flora bacteria and arthritis: why the joint? Scand J Rheumatol 1995;101(Suppl.):207–11.
27. Gaston JS. The involvement of the gut in the pathogenesis of inflammatory synovitis. Br J Rheumatol 1995;34:801–2.
28. Midtvedt T. Intestinal bacteria and rheumatic disease. Scand J Rheumatol 1987;64(Suppl.):49–54.
29. Schleifer KH, Kandler O. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol Rev 1972;36:407–77.
30. Severijnen AJ, van Kleef R, Hazenberg MP, van de Merwe JP. Chronic arthritis induced in rats by cell wall fragments of Eubacterium species from the human intestinal flora. Infect Immun 1990;58:523–8.
31. Melief MJ, Hoijer MA, Van Paassen HC, Hazenberg MP. Presence of bacterial flora-derived antigen in synovial tissue macrophages and dendritic cells. Br J Rheumatol 1995;34:1112–6.
32. Kool J, De Visser H, Gerrits-Boeye MY *et al*. Detection of intestinal flora-derived bacterial antigen complexes in splenic macrophages of rats. J Histochem Cytochem 1994;42:1435–41.
33. Hoijer MA, Melief MJ, van Helden-Meeuwsen CG, Eulderink F, Hazenberg MP. Detection of muramic acid in a carbohydrate fraction of human spleen. Infect Immun 1995;63:1652–7.
34. Schrijver IA, Melief MJ, Eulderink F, Hazenberg MP, Laman JD. Bacterial peptidoglycan polysaccharides in sterile human spleen induce proinflammatory cytokine production by human blood cells. J Infect Dis 1999;179:1459–68.
35. Klasen IS, Melief MJ, Swaak TJ, Severijnen AJ, Hazenberg MP. Responses of synovial fluid and peripheral blood mononuclear cells to bacterial antigens and autologous antigen presenting cells. Ann Rheum Dis 1993;52:127–32.
36. Arnett FC, Edworthy SM, Bloch DA *et al*. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.
37. van Gestel AM, Haagsma CJ, van Riel PL. Validation of rheumatoid arthritis improvement criteria that include simplified joint counts. Arthritis Rheum 1998;41:1845–50.
38. Hazenberg MP, Pennock-Schroder AM, Wensinck F, Van de Merwe JP. Effect of a soluble bacterial carbohydrate fraction on the viscosity of intestinal contents in healthy subjects and patients with Crohn's disease. Eur J Clin Invest 1989;19:61–4.
39. Masure S, Proost P, Van Damme J, Opdenakker G. Purification and identification of 91-kDa neutrophil gelatinase. Release by the activating peptide interleukin-8. Eur J Biochem 1991;198:391–8.
40. St-Pierre Y, Desrosiers M, Tremblay P, Esteve PO, Opdenakker G. Flow cytometric analysis of gelatinase B (MMP-9) activity using immobilized fluorescent substrate on microspheres. Cytometry 1996;25:374–80
41. Maurice MM, Nakamura H, van der Voort EA *et al*. Evidence for the role of an altered redox state in hyporesponsiveness of synovial T cells in rheumatoid arthritis. J Immunol 1997;158:1458–65.
42. Maurice MM, Lankester AC, Bezemer AC *et al*. Defective TCR-mediated signaling in synovial T cells in rheumatoid arthritis. J Immunol 1997;159:2973–8.
43. Swaak AJ, van den Brink HG, Aarden LA. Cytokine production in whole blood cell cultures of patients with rheumatoid arthritis. Ann Rheum Dis 1997;56:693–5.
44. Swaak AJ, van den Brink HG, Aarden LA. Cytokine production (IL-6 and TNF alpha) in whole blood cell cultures of patients with systemic lupus erythematosus. Scand J Rheumatol 1996;25:233–8.
45. Nerad JL, Griffiths JK, Van der Meer JW *et al*. Interleukin-1 beta (IL-1 beta), IL-1 receptor antagonist, and TNF alpha production in whole blood. J Leukoc Biol 1992;52:687–92.
46. Steiner G, Tohidast-Akrad M, Witzmann G *et al*. Cytokine production by synovial T cells in rheumatoid arthritis. Rheumatology 1999;38:202–13.
47. Cush JJ, Splawski JB, Thomas R *et al*. Elevated interleukin-10 levels in patients with rheumatoid arthritis. Arthritis Rheum 1995;38:96–104.
48. Ahrens D, Koch AE, Pope RM, Stein-Picarella M, Niedbala MJ. Expression of matrix metalloproteinase 9 (96-kd gelatinase B) in human rheumatoid arthritis. Arthritis Rheum 1996;39:1576–87.
49. Grillet B, Dequeker J, Paemen L, Van Damme B, Opdenakker G. Gelatinase B in chronic synovitis: immunolocalization with a monoclonal antibody. Br J Rheumatol 1997;36:744–7.
50. Okada Y, Naka K, Kawamura K *et al*. Localization of matrix metalloproteinase 9 (92-kilodalton gelatinase/type IV collagenase = gelatinase B) in osteoclasts: implications for bone resorption. Lab Invest 1995; 72:311–22.
51. Pugin J, Widmer MC, Kossodo S *et al*. Human neutrophils secrete gelatinase B in vitro and in vivo in response to endotoxin and proinflammatory mediators. Am J Respir Cell Mol Biol 1999;20:458–64.
52. Saren P, Welgus HG and Kovanen PT. TNF-alpha and IL-1beta selectively induce expression of 92-kDa gelatinase by human macrophages. J Immunol 1996;157:4159–65.
53. Zucker S, Hymowitz M, Conner C *et al*. Measurement of matrix metalloproteinases and tissue inhibitors of metalloproteinases in blood and tissues. Clinical and experimental applications. Ann N Y Acad Sci 1999;878:212–27.
54. Lehtonen L, Eerola E, Oksman P, Toivanen P. Muramic acid in peripheral blood leukocytes of healthy human subjects. J Infect Dis 1995;171:1060–4.
55. Porzio V, Biasi G, Corrado A *et al*. Intestinal histological and ultrastructural inflammatory changes in spondyloarthropathy and rheumatoid arthritis. Scand J Rheumatol 1997;26:92–8.
56. Schrijver IA, De Man YA, Melief M-J *et al*. Reduced systemic IgG levels against peptidoglycan in rheumatoid arthritis (RA) patients. Clin Exp Immunol 2001;123:140–6.
57. Rocken M, Urban JF, Shevach EM. Infection breaks T-cell tolerance. Nature 1992;359:79–82.
58. Ehl S, Hombach J, Aichele P *et al*. Viral and bacterial infections interfere with peripheral tolerance induction and activate CD8 + T cells to cause immunopathology. J Exp Med 1998;187:763–74.
59. Vella AT, McCormack JE, Linsley PS, Kappler JW, Marrack P. Lipopolysaccharide interferes with the induction of peripheral T cell death. Immunity 1995;2:261–70.
60. Segal BM, Klinman DM, Shevach EM. Microbial products induce autoimmune disease by an IL-12-dependent pathway. J Immunol 1997;158:5087–90.