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Superinduction of interleukin 8 mRNA in activated monocyte derived macrophages from rheumatoid arthritis patients

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Abstract

***Objective*—Synovial inflammation in patients with rheumatoid arthritis (RA) is characterised by the presence of large numbers of highly activated monocytes and macrophages. The importance of these cells in the aethiopathogenesis and prognosis of RA is increasingly recognised. The object of this report is to determine whether monocytes and monocyte derived macrophages of RA patients produce increased cytokine mRNA levels.**

***Methods*—Monocyte derived macrophages from RA patients and healthy controls were cultured either in the absence or presence of lipopolysaccharide. The expression levels of the mRNAs encoding GAPDH, interleukin 1β (IL1β), IL8, and α_2 macroglobulin in these cells were analysed by reverse transcriptase-polymerase chain reaction (RT-PCR).**

***Results*—Activated monocyte derived macrophages from RA patients produce significantly higher IL8 mRNA levels than activated macrophages from healthy controls. By contrast, resting RA and control macrophages produce similar levels of IL8 mRNA. Culturing of activated macrophages in the presence of RA or control sera has no effect on the expression levels of IL8 mRNA. No significant differences between RA and control macrophages were observed in the expression levels of IL1β and α_2 macroglobulin mRNAs.**

***Conclusion*—These data indicate that the increased IL8 mRNA production capacity of RA macrophages upon activation is an intrinsic property of these cells, and is not attributable to factors present in the circulation. Based on these observations, it is postulated that this innate hyperresponsiveness of RA macrophages contributes to the high IL8 levels present in the synovial fluid of rheumatoid joints, and is implicated in the chemotactic gradient leading to the homing of leucocytes to the joints.**

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Rheumatoid arthritis (RA) is a common systemic disease characterised by inflammation of the synovial membrane of diarthrodial joints. Although several risk factors have been identified, the aetiology of RA is essentially unclear. Macrophages and their products are crucial to the inflammation and joint destruction observed in RA.[1] The synovial membrane of healthy joints is coated with a thin layer (lining) of resident macrophages. Experiments in murine arthritis models have shown that removal of the lining macrophages from the joint prevents synovitis,[2] indicating that these macrophages are crucial for the onset of an inflammatory response in the joint. Interestingly, other lining tissues containing resident macrophages, such as bursae, tendon sheaths, pleura, and pericardium also are frequently affected in RA. An early event in the pathogenesis of RA is the infiltration of the synovial membrane by large numbers of highly activated macrophages.[3 4] The degree of monocyte/macrophage infiltration of rheumatoid joints has been reported to correlate with both the activity of the disease and the progression of joint destruction.[5] Activated macrophages produce proinflammatory cytokines, such as tumour necrosis factor α (TNFα) and interleukin 1β (IL1β), which are present in high concentration in the synovial fluid and pannus of rheumatoid joints.[6] Clinical trials have shown that strategies directed against TNFα and IL1 result in significant reduction of arthritis in RA patients,[7 8] which emphasises the important role of these macrophage derived cytokines in RA.

The macrophages present both in inflamed and healthy joints are derived from bone marrow monocyte progenitors. Monocytes from the circulation differentiate into macrophages upon entering extravascular tissues, such as the synovial membrane. Previously, it has been shown that circulating monocytes from RA patients express higher IL8 mRNA levels than those from healthy people.[9] As RA synovial tissue is enriched with large numbers of activated macrophages, it was investigated whether the overexpression of IL8 mRNA is preserved in monocyte derived macrophages from RA patients. Therefore, the expression levels of the mRNAs encoding the chemokine IL8 and the cytokine IL1β (both of which are strongly expressed in RA synovial membranes) were evaluated in both resting and in activated macrophages. In addition, as the expression of IL8 and IL1β may be regulated by circulating factors—including other cytokines, the effects of RA and control sera on macrophage maturation and activation were studied.

Methods

ISOLATION AND CULTURING OF MONOCYTES

Peripheral blood was collected from 10 female patients with established RA (according to the

1987 ARA criteria[10]), aged between 41 and 59 years, disease activity scores[11] ranging between 1.6 up to 4.25, disease duration between 8 and 19 years (average 13 years). All patients were treated with stable doses of methotrexate (7.5 to 15 mg per week) and non-steroidal anti-inflammatory drugs. None of the patients had ever been treated with systemic corticosteroids. The patient population was chosen to be relatively homogeneous, in order to minimise variation because of age, sex, and drugs. The control group consisted of 10 age matched healthy women. To isolate monocytes, heparinised patient blood was diluted 1:1 with phosphate buffered saline (PBS) containing 0.6% Na_3-citrate and layered over a Ficoll cushion. After centrifugation, the interface containing peripheral blood mononuclear cells was collected and washed twice with PBS. Further purification was accomplished by allowing the monocytes to adhere for 45 minutes to plastic culture dishes in RPMI 1640 medium (with glutamax-I, Dutch modification, GIBCO BRL Life Technologies, Breda, the Netherlands) containing 10% pooled human serum. The non-adherent lymphocytes were subsequently removed by washing with PBS. Subsequently, the monocytes (>90%) were cultured for seven days in RPMI 1640 medium supplemented with 10% pooled human serum, 1 mM pyruvate and 50 μg/ml gentamicin (GIBCO BRL). This is a well established method to induce differentiation of monocytes into mature macrophages,[12] which is accompanied by dramatic morphological changes, and with changes in the gene expression pattern, for example, the induction of cathepsin B, MDC and α_2 macroglobulin expression and the suppression of α_1 antitrypsin expression.[13] Cells were treated with 10 ng/ml lipopolysaccharide (LPS, from *E coli* 0111:B4, Sigma, St Louis, USA) as indicated in the figure legends. To minimise interassay variation, culturing was performed using a single batch of serum containing medium (except for the experiment in fig 2 in which different sera were compared), and a single batch of LPS.

SEMI-QUANTITATIVE REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR) ANALYSIS OF GENE EXPRESSION LEVELS

The adhering macrophages were washed with PBS, and total RNA was isolated using Trizol-reagent (GIBCO BRL) according to the manufacturer's instructions. Synthesis of cDNA was performed using 200 ng of total RNA and 100 ng of the oligonucleotide 5'-TTTTTTTTTTTTTVN-3' in a reaction mixture containing 20 μM dNTPs and AMV buffer (Boehringer Mannheim, Almere, the Netherlands). The mixture was incubated for five minutes at 65°C to melt the RNA, and 10 minutes at 37°C to allow the primer to anneal. Subsequently, 2.5 U AMV reverse transcriptase (Boehringer Mannheim) was added and the incubation was continued for one hour at 37°C. Semi-quantitative RT-PCR analysis was performed using 1 μl of this mixture in a 15 μl PCR reaction mixture as previously described.[13] PCR reactions were performed in duplicate using a GeneE thermal cycler (Techne, Cambridge, UK) for various number of cycles (30 seconds at 94°C, one minute at 60°C, 45 seconds at 72°C). The reaction products were analysed on polyacrylamide gels. The following primer pairs were used: glyceraldehyde triphosphate dehydrogenase (GAPDH): 5'-GTGAGGAG-GGGAGATTCAG-3' and 5'-GCATCCTGGGCTACACTG-3' (product size 305 bp, 20 PCR cycles); IL8: 5'-TCTTGCACAAATATTTGATGC-3' and 5'-CCACTGTGCCTTGGTTTC-3' (192 bp, 24 cycles); IL1β: 5'-GGATATGGAGCAACAAGTGG-3' and 5'-ATGTACCAGTTGG-GGAACTG-3' (263 bp, 22 cycles); α_2 macroglobulin: 5'-CAGCAGCAACCATGTCTTG-3' and 5'-TGCAAACTCATCCGTCTCG-3' (147 bp, 22 cycles). The number of PCR cycles was kept to a minimum to maintain a linear amplification of the template.

QUANTIFICATION OF RT-PCR PRODUCTS AND STATISTICAL ANALYSIS

RT-PCR products were quantified using a phosphoimager (BioRad Laboratories, Veenendaal, the Netherlands). The cDNAs used to compare cytokine gene expression in RA and control macrophages were normalised for the expression levels of the housekeeping gene GAPDH. The expression levels were related to the median expression level in the control group, which was set to 1. Statistical analysis was performed using the Mann-Whitney U test.

Results and Discussion

Monocytes cultured in the presence of 10% human serum differentiated into macrophages within seven days. Figure 1A shows that seven day old macrophages from RA patients and control subjects produced similar levels of α_2 macroglobulin mRNA (indicative of fully matured macrophages[13]), which indicates that the rate at which RA macrophages differentiate is similar to that of control macrophages. Analysis of the expression levels of IL1β and IL8 mRNAs revealed no significant differences between RA patients and controls (fig 1A). Next, we analysed whether RA monocyte derived macrophages are prone to higher activation levels than control macrophages in response to an external stimulus, such as LPS. As figure 1B shows, the expression levels of IL1β and IL8 mRNAs in activated macrophages varied considerably between people. However, no significant differences in IL1β mRNA levels between RA and control macrophages were detected. In contrast, the IL8 mRNA levels detected in macrophages from RA patients after stimulation with LPS were significantly higher than in controls (fig 1B, $p<0.01$, Mann-Whitney U test). The median IL8 mRNA expression level in monocyte derived macrophages from RA patients was approximately 3.5-fold higher than in controls. Moreover, each of the RA macrophage preparations analysed showed a higher IL8 mRNA level than the median of the IL8 mRNA levels in control macrophages. Neither in

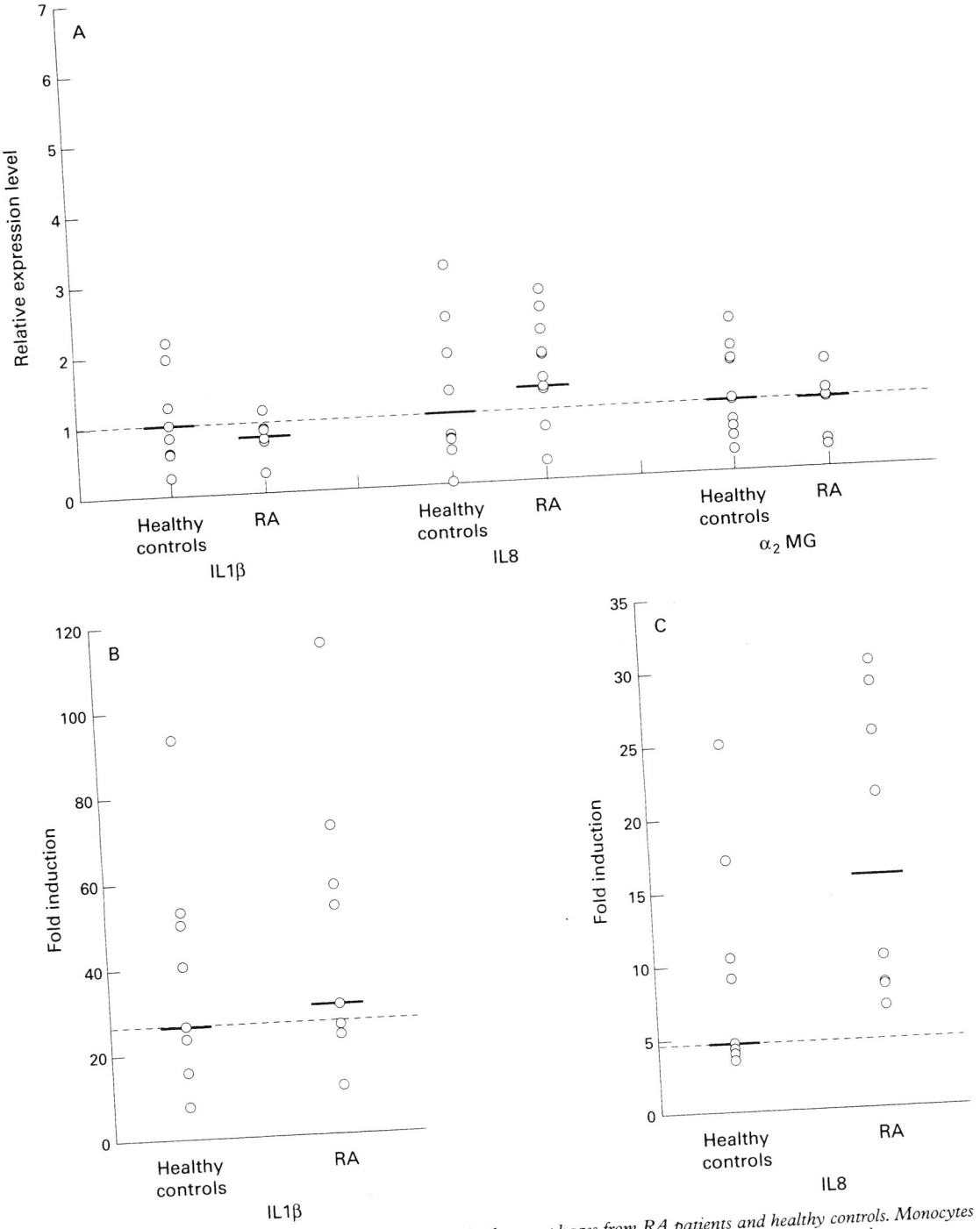

Figure 1 Analysis of gene expression in monocyte derived macrophages from RA patients and healthy controls. Monocytes isolated from heparinised peripheral blood collected from 10 RA patients and 10 healthy controls were allowed to differentiate into macrophages by culturing the cells in medium containing 10% human serum for seven days, after which mRNA expression levels were determined by RT-PCR. The expression levels of the various mRNAs were normalised for GAPDH mRNA levels. The median of each dataset is indicated by horizontal bars. (A) Relative expression levels of mRNAs encoding IL1β, IL8, and α_2 macroglobulin (α_2 MG) in unstimulated macrophages. (B) Induction by LPS of IL1β and IL8 mRNA levels in macrophages stimulated for two hours with 10 ng/ml LPS, as determined by RT-PCR. The differences in IL8 mRNA expression levels between RA and control macrophages are statistically significant ($p<0.01$, Mann-Whitney U test).

unstimulated nor in stimulated macrophages a correlation between the individual expression levels of α_2 macroglobulin, IL1β and IL8 mRNAs was observed in individual macrophage preparations (data not shown). Furthermore, no obvious correlation between disease duration or activity and gene expression levels could be observed. As all RA patients from which peripheral blood monocytes were isolated received methotrexate treatment, it cannot be excluded that this may have contributed to the overexpression of IL8 mRNA in RA monocyte derived macrophages.

However, methotrexate has been shown to exert an adenosine induced anti-inflammatory effect,[14] and treatment of RA patients leads to a reduction of IL1 concentrations in synovial fluid, and decreased IL6 and soluble IL2 receptor levels in the circulation.[15] Therefore, an inhibitory effect on cytokine mRNA expression levels rather than an enhancement as described in this report would be expected

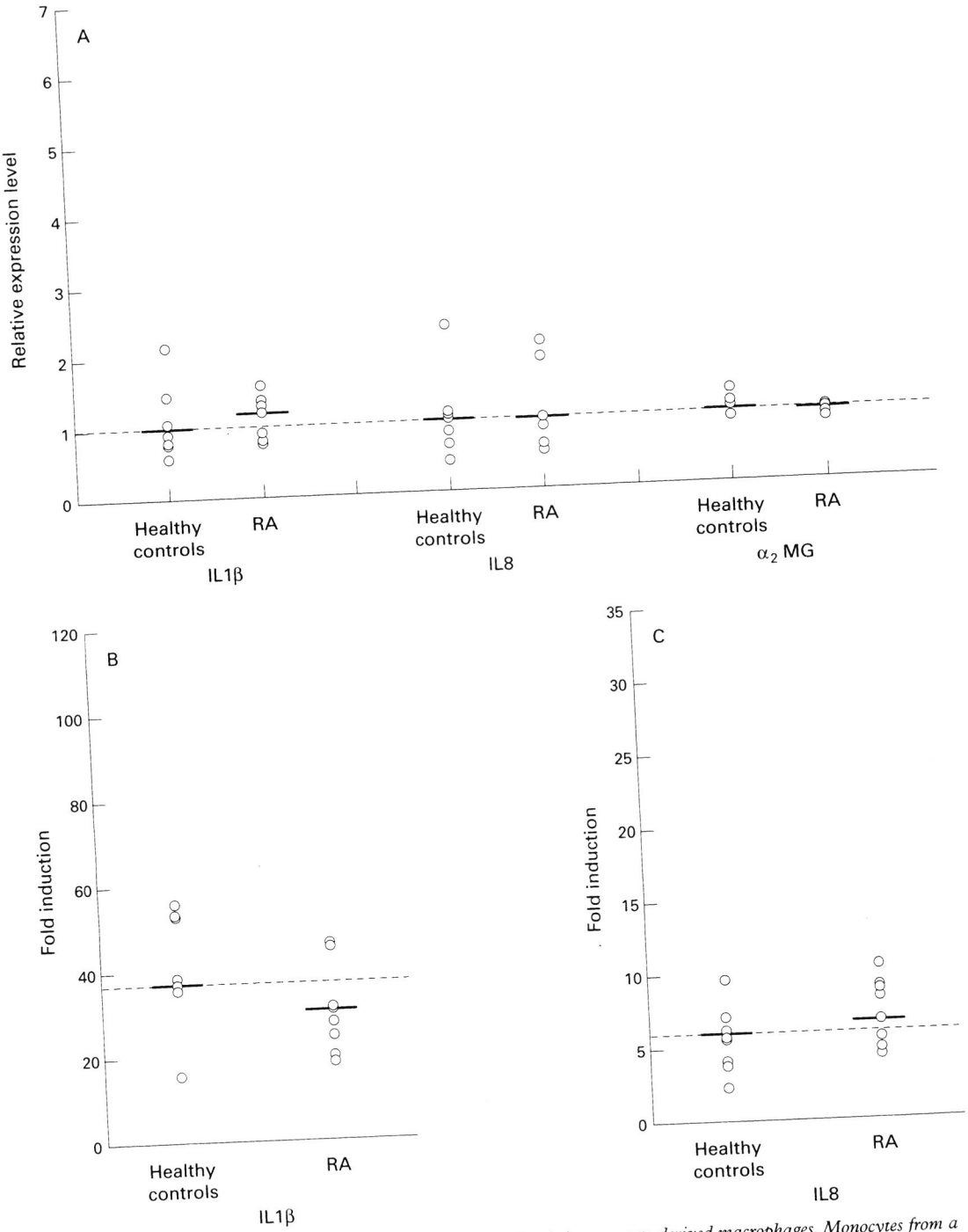

Figure 2 Effect of RA and control sera on mRNA expression levels in monocyte derived macrophages. Monocytes from a healthy donor were cultured in medium containing 10% serum from RA patients (n=10) or healthy controls (n=10). The expression levels of the various mRNAs were determined by RT-PCR and were normalised for GAPDH mRNA levels. See also the legend to figure 1. (A) Relative expression levels of mRNAs encoding IL1β, IL8, and α_2 macroglobulin (α_2 MG) in unstimulated macrophages. (B) Induction by LPS of IL1β and IL8 mRNA levels in macrophages stimulated for two hours with 10 ng/ml LPS. Graphs are presented on the same scales as in figure 1.

from this drug. Furthermore, unstimulated macrophages of RA patients and controls display similar cytokine mRNA levels (fig 1A), it therefore seems unlikely that methotrexate has influenced the results presented in this study. Several cytokines and other soluble factors are present in the circulation of RA patients and it can be hypothesised that these factors modulate monocyte/macrophage differentiation and activation. This was analysed in macrophages from one healthy person that showed intermediate IL1β, IL8, and α_2 macroglobulin mRNA expression levels in the previous experiment. Monocytes isolated from this person were subdivided into 20 aliquots and were subsequently allowed to differentiate into macrophages in the presence of sera from healthy controls (n=10) or RA patients (n=10). Results show that the expression levels of α_2 macroglobulin mRNA were very similar irrespective of the serum tested (fig 2A). Furthermore, there were no significant differences in IL1β or IL8 mRNA levels between groups (that is, sera from RA patients or healthy people) or in unstimulated (fig 2A) or in stimulated (fig 2B) macrophages. These results

indicate that circulating factors are not responsible for the increased IL8 mRNA expression levels observed in stimulated monocyte derived macrophages of RA patients. In addition, this result further substantiates the notion that drugs or drug related compounds present in the serum of RA patients affected the results presented in figure 1B. Our results suggest that the IL8 mRNA hyper-responsiveness is an intrinsic property of these cells. It may be hypothesised that this property is acquired in the bone marrow, which is phenotypically different in RA patients as shown by the increased production rate of $CD14^+$ cells.[16] This would also be in agreement with the observation that RA bone marrow serum contains high levels of IL8 protein.[17] Other possible explanations are that RA monocytes are primed in vivo by direct cell to cell interactions with other circulating cells or endothelial cells, or thus far unidentified genetic factors. In this study, a relatively homogeneous patient population was studied (women, age 41–59 years), to minimise variation because of age and sex. Future experiments should show whether the overexpression of IL8 mRNA in RA monocyte derived macrophages is also present in men and in patients of different age. Furthermore, it will be of interest to analyse the expression levels of other genes expressed in activated macrophages that play an important part in the pathogenesis of RA, such as TNFα.

Macrophages are the predominant source of IL8 in RA synovial tissue, and are probably the main source of the high IL8 levels in RA synovial fluid.[18] IL8 is one of the major neutrophil chemoattractants, and has been shown to be responsible for the large numbers of neutrophils present in synovial fluid of RA patients.[19 20] The results presented in this report lead us to hypothesise that the innate property of activated RA macrophages to produce increased levels of IL8 mRNA is a major determinant of the chemotactic gradient for neutrophils in the rheumatoid joints.

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