Clin Exp Immunol 1995; 99:303–310

Cytokine production by normal human monocytes: inter-subject variation and relationship to an IL-1 receptor antagonist (IL-1Ra) gene polymorphism

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(Accepted for publication 9 November 1994)

SUMMARY

Monocytes from different individuals show variable cytokine production in response to a variety of stimuli. We wished to determine the sets of conditions (cytokine combinations) that would enable us to demonstrate stable inter-individual differences in the production of IL-1α, IL-1β, IL-1Ra, IL-6 and tumour necrosis factor-alpha (TNF-α) by monocytes. We assessed the ability of a number of recombinant human cytokines (granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFN-γ), TNF-α, IL-4, IL-6, transforming growth factor-beta (TGF-β), IL-10 and IL-1Ra)) to stimulate or inhibit the production of one or more of these monocyte products. GM-CSF was found to stimulate the production of all five of these cytokines in a highly reproducible manner. TNF-α also up-regulated production of IL-1α, IL-1β, IL-1Ra and IL-6 by monocytes, but the variability in the results of cells cultured from the same individuals on different occasions was greater. Other cytokines either stimulated production of only some of the five cytokine products tested, or stimulated the production of some cytokine products while inhibiting production of others. This was especially evident when cytokines were used in combination with GM-CSF: IFN-γ down-regulated production of IL-1Ra while up-regulating the production of IL-1α/β, IL-6 and TNF-α, while IL-4 had the exact opposite effect. Polymorphisms in regions of cytokine genes that affect transcription may account for some of the inter-individual variation in cytokine production. We have shown that a stable estimate of cytokine production phenotype can be obtained when monocytes collected on at least two separate occasions are stimulated by GM-CSF *in vitro*. We have looked for a relationship between IL-1 production and an 86-bp variable repeat polymorphism in intron 2 of the IL-1Ra gene. A less common allele of this polymorphism (allele 2) was associated with increased production of IL-1Ra protein, and also reduced production of IL-1α protein by monocytes.

Keywords IL-1 tumour necrosis factor IL-6 variation polymorphism

INTRODUCTION

Monocyte-derived cytokines such as IL-1 and tumour necrosis factor-alpha (TNF-α) contribute to the pathogenesis of inflammatory diseases such as rheumatoid arthritis (RA). In the absence of infections, cytokines at the inflammatory sites may contribute to up-regulation or down-regulation of cytokine production by monocytes/macrophages. Overproduction of IL-1 and TNF-α is believed to be pathogenic in RA, and production of these mediators seemed to be increased with active disease and decreased after treatment with disease-modifying drugs [1–3]. Higher levels of IL-1 production by monocytes could only be demonstrated in a subset of RA patients [1]. We have observed that monocytes from both normal individuals and RA patients varied considerably in the amount of IL-1 released in response to lipopolysaccharide (LPS) stimulation [4]. This raises the possibility that monocytes may be genetically programmed to produce higher or lower amounts of IL-1 and other cytokines in response to a given stimulus, although this may be masked in RA patients by disease and treatment variables.

Production of cytokines by monocytes must be stable over time in order for genetic association studies to be meaningful. Stable production of IL-1 and TNF-α by LPS-stimulated monocytes was first reported in a limited study of four male subjects tested weekly for 3 weeks [5]. This study attempted to show a correlation between cytokine secretion phenotype and HLA-DR haplotype. HLA-DR antigens are involved with the interaction with LPS on the cell surface, and high or low IL-1

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and TNF-α secretory responses have subsequently been shown to be associated with certain HLA-DR haplotypes [6]. In a larger study (n = 19), Jacob *et al.* [7] demonstrated stable inter-individual differences in TNF-α production in mitogen-stimulated peripheral blood mononuclear cells (PBMC) in male subjects and in post-menopausal women, but not in pre-menopausal women. Endres *et al.* [8] showed that IL-1β production (secreted plus cell-associated) by LPS-stimulated PBMC was stable in 6/6 donors when tested a few days apart, but was stable in only 6/9 donors when tested 25 weeks apart. In a larger study (19 males and 19 females) [9] these authors reported that there was considerable inter-subject variability (extending over two orders of magnitude) in production of IL-1α, IL-1β and TNF-α proteins. Less is known about the variability of production of other monocyte products such as IL-1Ra and IL-6.

LPS is a potent but non-specific monocyte activator, but is of little relevance to the pathogenesis of diseases such as RA. Further, response to LPS may partly depend on the HLA-DR haplotype. Studies with PBMC are also compounded by variable proportions of lymphocytes and monocytes, by lymphocye–monocyte interactions that affect monocyte activation, and by the fact that lymphocytes can produce some of the same cytokines as do monocytes. In this study we have taken the approach of studying purified human monocytes in a defined culture medium in order to study responses to specific cytokine stimuli. Monocytes in the joints of RA patients are probably activated by cytokines produced by a variety of cell sources. Therefore we have tested monocyte production of IL-1α, IL-1β, IL-1Ra, TNF-α and IL-6 in response to a variety of cytokines and cytokine combinations and assessed the inter-subject variability and intra-subject consistency of the responses. We report that granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulated the production of all five cytokines with good intra-subject consistency. IL-1Ra protein production appeared to be regulated independently of IL-1α/β. Combination of GM-CSF with other cytokine stimuli confirmed this observation. Cytokines such as transforming growth factor-beta (TGF-β), IL-4 and IL-10 up-regulated IL-1Ra production but down-regulated production of IL-1α/β. Interferon-gamma (IFN-γ) up-regulated production of IL-1α/β, but down-regulated production of IL-1Ra.

Recently, a number of polymorphisms in human [10–13] and murine [14] cytokine genes have been reported, but their significance in terms of protein production has not yet been determined. Here we report an association between a less common allele of the human IL-1Ra gene polymorphism and increased production of IL-1Ra protein and decreased production of IL-1α protein in GM-CSF-stimulated monocyte cultures.

MATERIALS AND METHODS

Reagents

Recombinant human IFN-γ (2×10^7 U/mg) and recombinant human TNF-α (6×10^7 U/mg) were provided by Boehringer (Ingelheim, Austria), recombinant human GM-CSF by the Schering Corporation (Sydney, Australia), recombinant human IL-1Ra by the UpJohn Company (Kalamazoo, Kalamazoo, MI), recombinant human IL-10 by the DNAX Institute (Palo Alto, CA) and recombinant human IL-6 (specific activity 10^8 U/mL by the 7TD1 hybridoma assay) by Dr Gordon Wong (Genetics Institute, USA). The following recombinant human cytokines were purchased from Boehringer (Sydney, Australia): TGF-β_1 (10^7 U/mg), IL-4 (5×10^5 U/mg), IL-1α (10^7 U/mg) and IL-1β (10^7 U/mg). Reagents were tested for endotoxin contamination using the Limulus lysate assay (E-toxate; Sigma, St Louis, MO) with a chromogenic substrate (LAL, Spectrozyme, American Diagnostica, Sydney, Australia). This assay detected LPS over the range 0·1–1000 pg/ml. rIFN-γ (5 ng/ml), rIL-6 (10 ng/ml), rIL-1Ra (100 ng/ml), rTNF-α (20 ng/ml) and rGM-CSF (10 ng/ml) were estimated to contain 0·02, 0·2, 0·3, 0·5, and 1·2 pg/ml of endotoxin, respectively, from assays of concentrated stock preparations. rIL-4 and rTGF-β_1 at 1 ng/ml contained < 0·01 EU/ml endotoxin according to the manufacturer's product specifications.

Monocyte isolation and culture

Venous blood (50–60 ml) was collected from 36 healthy volunteers (16 male and 20 female), using 0·38% sodium citrate as anti-coagulant. Monocyte isolation and enrichment were achieved by density gradient and counterflow centrifugation steps as previously described [15,16]. Recovery of monocytes was >85% and monocyte enrichment was >80% by the criterion of latex ingestion. Purified monocytes were cultured in liquid suspension in 70 × 11 mm Minisorp tubes (Nunc) which had been sterilized by ethylene oxide. The culture medium was X-Vivo 15 (BioWittaker, Walkersville, MD). Comparisons with RPMI 1640 and with our previous serum-free medium formulation (RPMI 1640 buffered with 20 mM HEPES and supplemented with lactalbumin hydrolysate, bovine insulin, human transferrin and sodium selenite) showed that X-Vivo 15 supported higher production of IL-1Ra protein in cytokine-stimulated cultures while at the same time reducing the background levels of IL-1β and IL-6 production. Background levels of cytokine production by purified monocytes were relatively low and highly variable. It did not appear to be due to LPS contamination of the elutriation buffer or of the culture medium, as there was no background production in unseparated mononuclear cell cultures treated with these media.

Monocytes were cultured at 10^6/ml in a total volume of 0·4 ml in tubes fitted with a loose cap in 5% CO_2 in air in a humidified incubator. After 20 h the tubes were centrifuged at 200 *g* for 10 min and the supernatants harvested. The cells were resuspended in 0·4 ml of fresh culture medium and disrupted by sonication for 10 s at 50 W using a Labsonic 2000 ultrasonic homogenizer (B. Braun Instruments, Sydney, Australia) with a 4-mm titanium needle probe. This was used to estimate the cell-associated IL-1 activity. Levels of cell-associated IL-6 and TNF-α were consistently low (< 0·3 ng/ml) and were not routinely measured. Samples were stored at −20°C for up to 4 weeks before assay.

Cytokine ELISA

Sandwich ELISAs specific for IL-1, IL-1β, IL-1Ra, IL-6 and TNF-α were developed and used as previously described [17]. Briefly, polyclonal sheep antisera for IL-1, IL-1β and IL-1Ra, and polyclonal goat antiserum for IL-6 (provided by Dr S. Poole, National Institute for Biological Standards and Control, Potters Bar, UK) were used to develop sandwich ELISAs for IL-1α, IL-1β, IL-1Ra and IL-6 using the IgG fraction (from

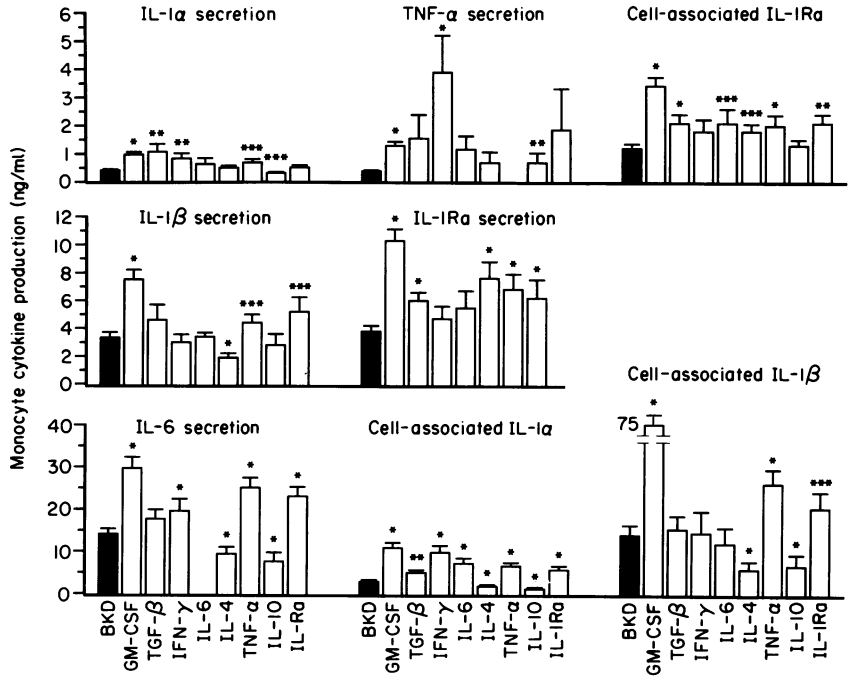

Fig. 1. Production of cytokines by human monocytes relative to background (BKD, ■). Cultures were stimulated with the following recombinant human cytokines: granulocyte-macrophage colony-stimulating factor (GM-CSF; 10 ng/ml), transforming growth factor-beta (TGF-β; 1 ng/ml), IFN-γ (5 ng/ml), IL-6 (10 ng/ml), IL-4 (1 ng/ml), tumour necrosis factor-alpha (TNF-α; 20 ng/ml), IL-10 (10 ng/ml) and IL-1Ra (10 ng/ml). Data from cells from 36 individuals. Cells were collected on two separate occasions, but not all sets of stimuli were used each time. Data bars represent a minimum of 28 cultures. $*P < 0.001$; $**P < 0.01$; *** $P < 0.05$ compared with BKD by Wilcoxon rank sign test.

protein-G affinity chromatography) for capture, and biotinylated IgG of the same fractions as tracer. MoAbs directed against two non-overlapping domains of the TNF-α molecule were used for the TNF-α ELISA, one set for capture and the other set was biotinylated and used as the tracer. Streptavidin-peroxidase (Amersham, Sydney, Australia) binding was amplified using biotinylated anti-streptavidin MoAb (Jackson Immunoresearch, West Grove, PA). TMB substrate (100 mg/ml; Boehringer) was added and the reaction was stopped after 2–5 min with 2 N sulphuric acid. Absorbance at 450 nm was read on a Titertek Multiskan MCC (Flow) and the data were analysed using the PC AutoMate program (Flow).

Results were calibrated with serial dilutions of known quantities of recombinant cytokines. The range of detection was 0·1–40 ng/ml for each assay. The assays were specific for their respective cytokine and also showed no reactivity to any of the other recombinant cytokines used in this study. When calibrated against international reference standards for recombinant cytokines (rIL-1α 86/632; rIL-1β 86/680; rIL-6 88/514; rTNF-α 87/650; rIL-1Ra 92/644; National Institute for Biological Standards and Control), the ELISAs for IL-1α, TNF-α and IL-6 overestimated the readings (by factors of 1·6, 1·8 and 4·2, respectively), and the IL-1β and IL-1Ra ELISAs underestimated the readings (by factors of 1·3 and 3·3, respectively). Variation in quantification within different wells of the same ELISA plate was 8% for IL-6 and IL-1Ra, 12% for IL-1α and TNF-α, and 20% for IL-1β. Inter-assay variation over a 12-month period (using three different reference culture supernatants that had been aliquoted and stored at −20°C) was 15% for IL-1α, 16% for IL-1Ra, 21% for TNF-α, 23% for IL-6 and 28% for IL-1β. In the shorter term (several months) the correlation in the repeated assay of 33 samples was 0·95 ($P < 0.00001$). Cytokine production by cells from the same individual at two different time points was usually measured within the same assay in order to minimize the effect of inter-assay variation of the ELISAs.

PCR analysis of IL-1Ra gene polymorphism

Genomic DNA was extracted from polymophonuclear leucocytes (obtained after Ficoll–diatrizoate centrifugation of whole blood) by proteinase K digestion, phenol-cholorform extraction and ethanol precipitation using an established protocol [18]. The average DNA yield was 470 mg/60 ml blood. DNA (200 ng) was subjected to polymerase chain reaction (PCR) amplification for the 86-bp tandem repeat polymorphism of the IL-1Ra gene in a GeneAmp PCR System 9600 (Perkin Elmer, Sydney, Australia) using the primers as described [10]. Primers were synthesized using a 391 DNA Synthesiser (PCR-Mate, Applied Biosystems, Melbourne, Australia). DNA was denatured at 96°C for 60 s followed by 30 cycles of the following: 94°C 30 s, 60°C 30 s, 70°C 60 s. The final extension was carried out at 70°C for 7 min. PCR products were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. A molecular ladder ranging from 67 bp to 500 bp

(pUC*Hpa*II, Bresatec) was used to determine the size of the PCR fragments.

Statistical analysis

The Wilcoxon signed rank and Spearman's rank correlation tests for dependent samples and the Mann–Whitney test for independent samples were used. Unless otherwise indicated, data are represented as mean ± s.e.m. Analysis of variance after $\log_{10}$ transformation was used to estimate the number of samples (time points) required to derive a stable estimate of the mean.

RESULTS

Cytokine-stimulated monokine production relative to background

The effect of various cytokines and cytokine combinations on the production of IL-1α, IL-1β, IL-1Ra, TNF-α and IL-6 by monocytes from 36 normal donors was studied. Secreted and cell-associated components of IL-1 production were analysed separately making a total of eight products measured. Cytokine production relative to background stimulation is summarized in Fig. 1, and the key observations are as follows: (i) GM-CSF up-regulated the production of all eight monocyte products; (ii) TNF-α also enhanced the production of the other seven monocyte products; (iii) TGF-β up-regulated IL-1α and IL-1Ra production, but had no effect on IL-1β, TNF-α or IL-6 production; (iv) IFN-γ up-regulated the production of IL-1α, IL-6 and especially TNF-α, but had no effect on IL-1β or IL-1Ra production; (v) IL-6 slightly increased production of cell-associated IL-1α and cell-associated IL-1Ra; (vi) IL-4 and IL-10 up-regulated IL-1Ra production but inhibited IL-1α, IL-1β and IL-6 production; (vii) IL-1Ra up-regulated the production of IL-1α, IL-1β and IL-6.

Monokine production in GM-CSF-stimulated cultures modulated by other cytokines

When monocytes were stimulated with GM-CSF, the effect of the addition of some of these other cytokines was slightly different. The results are summarized in Fig. 2, and the main observations are as follows: (i) IFN-γ augmented the secretion of IL-1α, IL-1β, IL-6 and TNF-α, but inhibited the production of IL-1Ra; (ii) TGF-β depressed the production of IL-1α and IL-1β and augmented the production of TNF-α; (iii) IL-4 inhibited IL-1α, IL-1β, IL-6 and TNF-α production, but still augmented IL-1Ra secretion; (iv) IL-6 slightly reduced the amount of cell-associated IL-1α and IL-1β and secreted IL-1Ra induced by GM-CSF; (v) TNF-α and IL-1Ra did not significantly modify GM-CSF-induced monokine production. The inhibitory effect of IFN-γ on GM-CSF-induced IL-1Ra production could be reversed by the addition of TGF-β or IL-4, but not IL-10 (data not shown).

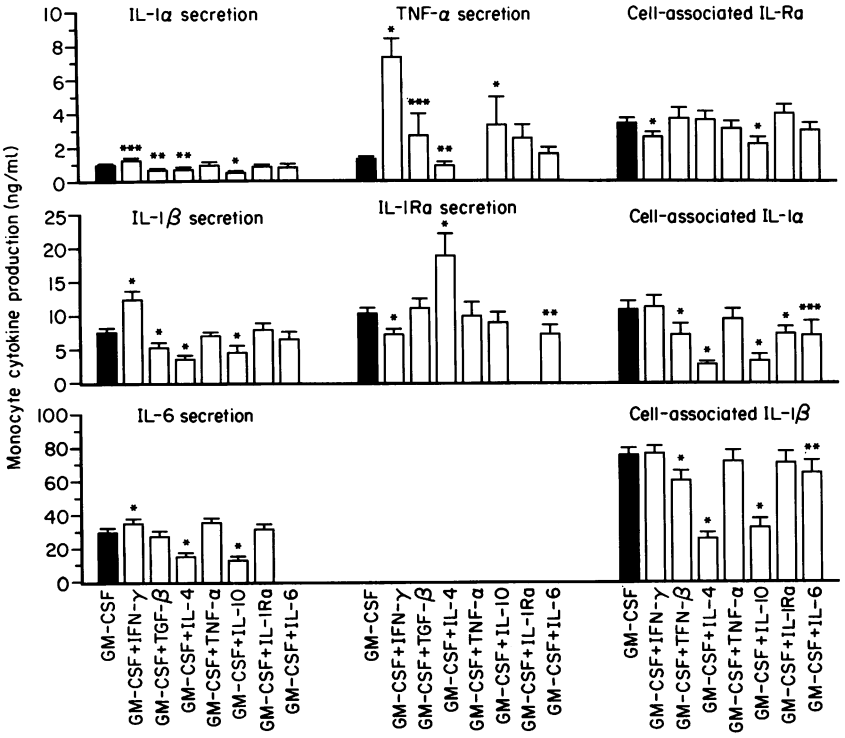

Fig. 2. Production of cytokines by human monocytes relative to: granulocyte-macrophage colony-stimulating factor (GM-CSF; 10 ng/ml). GM-CSF was used alone (■) or combined with one of the following cytokines: IFN-γ (5 ng/ml), transforming growth factor-beta (TGF-β; 1 ng/ml), IL-4 (1 ng/ml), tumour necrosis factor-alpha (TNF-α; 20 ng/ml), IL-10 (10 ng/ml), IL-1Ra (10 ng/ml) and IL-6 (10 ng/ml). Data from cells from 36 individuals. Cells were collected on two separate occasions, but not all sets of stimuli were used each time. Data bars represent a minimum of 26 cultures. $*P < 0.001$; $**P < 0.01$; $***P < 0.05$ compared with GM-CSF alone by Wilcoxon rank sign test.

Table 1. Reproductivity of cytokine secretion in granulocyte-macrophage colony-stimulating factor (GM-CSF)-stimulated monocyte cultures

	Time 1		Time 2			
Cytokine	Median (ng/ml)	Interquartile range	Median (ng/ml)	Interquartile range	Spearman's correlation	P ($n = 34$)
IL-1α	0·7	0·4–1·55	0·95	0·3–2·0	0·8	0·000 001
IL-1β	7·55	5·1–12·0	7·55	4·3–13·7	0·64	0·000 05
IL-1Ra	11·1	5·2–17·2	9·2	4·6–14·5	0·51	0·002
IL-6	24·9	14·5–41·0	26·8	16·6–41·9	0·57	0·0004
TNF-α	1·1	0·5–2·3	1·1	0·3–2·7	0·55	0·0007

Median values of secretion of five cytokine products on two different occasions are compared using Spearman's rank correlation test. Monocytes were cultured at a million per ml in serum-free medium, for 20 h as outlined in Materials and Methods.

Reproducibility of monokine secretion in GM-CSF-stimulated cultures

There was considerable inter-individual variation in monocyte responses to GM-CSF and other cytokine stimuli. In order to determine whether these differences were stable, monocytes from 34 individuals were cultured on two occasions separated by at least 1 month. Reproducibility of monokine secretion in GM-CSF-stimulated cultures is shown in Table 1. The correlations between the two time points were significant for all five products. Analysis of variance showed that the standard error of the estimation of the mean would be 13%, 13%, 15%, 13% and 24%, respectively, for IL-1α, IL-1β, IL-1Ra, IL-6 and TNF-α for a sample size of 2. A sample size of 4 or more would be required to get this standard error down to below 10%.

Reproducibility of monokine secretion in cultures treated with other cytokines

Inter-individual differences in cytokine secretion were less stable in cultures stimulated with other cytokines or cytokine combinations. However, there was still a statistically significant correlation in secretion of some cytokine products measured at two different occasions with some other cytokine stimuli: IL-1α and IL-1Ra secretion in TNF-α-stimulated cultures ($P = 0·02$ and 0·007, respectively); IL-1α secretion in cultures stimulated with GM-CSF and IFN-γ ($P = 0·001$). Analysis of variance showed that the standard error of the estimation of the mean would be in the order of 20% for GM-CSF + IFN-γ-stimulated cultures, 20–30% for TNF-α-stimulated cultures, and > 30% for other cytokine stimuli for a sample size of two.

Reproducibility of cell-associated monokine production

Cell-associated IL-1 production was more variable than IL-1 secretion. The correlation coefficients for cell-associated IL-1α, IL-1β and IL-1Ra production at two time points were 0·23 ($P = 0·18$), 0·35 ($P = 0·04$) and 0·31 ($P = 0·07$), respectively, in GM-CSF-stimulated cultures, 0·27 ($P = 0·13$), 0·33 ($P = 0·06$) and 0·56 ($P = 0·006$), respectively, for GM-CSF + IFN-γ-stimulated cultures, 0·16 ($P = 0·39$), 0·26 ($P = 0·19$) and 0·59 ($P = 0·001$), respectively, for TNF-α-stimulated cultures. Nevertheless, there were good correlations between IL-1 secretion and cell-associated IL-1 production in individual subjects for IL-1α and IL-1Ra products.

GM-CSF appeared to be the best cytokine stimulator of monocyte cytokine production in terms of range of products and reproducibility. The concentration of GM-CSF used in this study (10 ng/ml) was optimal for all five cytokines. Dose response studies showed that this concentration was on the plateau range of the dose response curves for IL-1 (see Fig. 3) and for IL-6 and TNF-α production (data not shown).

High IL-1Ra production and low IL-1α production associated with the uncommon allele of the IL-1Ra gene polymorphism

Monocyte cytokine production was estimated by calculating the mean of the results from two samplings in 34 individuals and the results of a single estimation in a further two individuals. All 36 individuals were typed for the variable length polymorphism in intron 2 of the IL-1Ra gene. The frequency of the alleles A1 (410 bp) and A2 (240 bp) were 0·73 and 0·25, respectively. We found 20 individuals homozygous for the A1

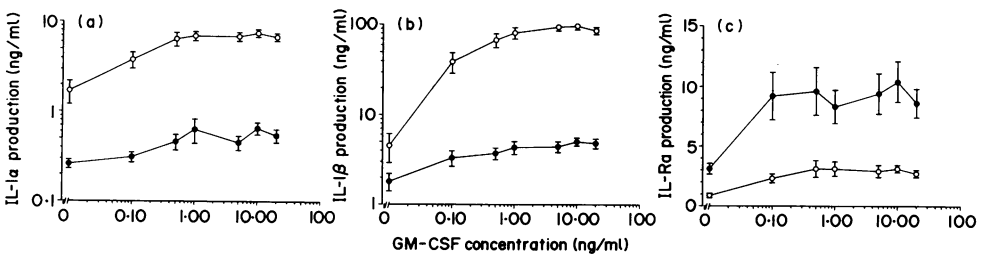

Fig. 3. Monocyte IL-1 production in response to granulocyte-macrophage colony-stimulating factor (GM-CSF). Data from 15 subjects. (a) IL-1α. (b) IL-1β. (c) IL-1Ra. ○, Cell-associated; ●, secreted.

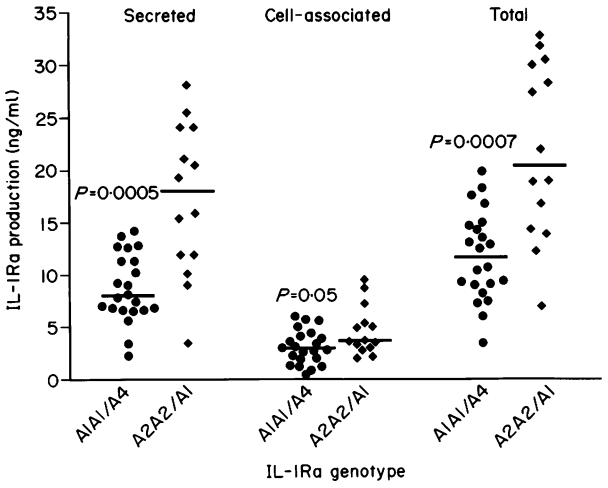

Fig. 4. Production of IL-1Ra protein by human monocytes related to a polymorphism in intron 2 of the IL-1Ra gene. ●, A1A1 or A1A4 genotype; ◆, A2A2 or A2A1 genotype. Horizontal bars indicate medians.

allele, four individuals homozygous for the A2 allele, 10 individuals with the A1A2 genotype, and two individuals with an A1A4 genotype (A4 corresponding to 325 bp). Figures 4 and 5 show that the A2 allele is associated with increased IL-1Ra production and decreased IL-1α production by GM-CSF-stimulated monocytes. Similar associations were found in TGF-β-stimulated cultures (IL-1Ra secretion $P = 0{\cdot}03$; cell-associated IL-1α $P = 0{\cdot}002$; and total IL-1α production $P = 0{\cdot}007$), TNF-α-stimulated cultures (cell-associated IL-1α $P = 0{\cdot}01$; total IL-1α production $P = 0{\cdot}01$), IL-4-stimulated cultures (cell-associated IL-1α $P = 0{\cdot}03$) and GM-CSF + IFN-γ-stimulated cultures (cell-associated IL-1Ra production $P = 0{\cdot}03$; cell-associated IL-1α $P = 0{\cdot}004$; and total IL-1α production $P = 0{\cdot}008$). In contrast, there were no associations between IL-1Ra genotypes and production of IL-1β, IL-6 or TNF-α in GM-CSF-stimulated cultures or cultures stimulated with other cytokines (data not shown).

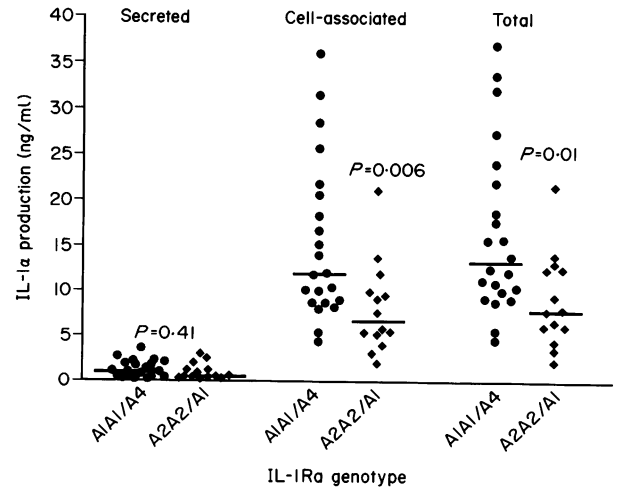

Fig. 5. Production of IL-1α protein by human monocytes related to a polymorphism in intron 2 of the IL-1Ra gene. ●, A1A1 or A1A4 genotype; ◆, A2A2 or A2A1 genotype. Horizontal bars indicate medians.

DISCUSSION

High inter-subject variability in cytokine production by human leucocytes has been commonly observed, but there have been only few studies attempted showing stable production over time [5,7–9]. The variability in cytokine production may be affected by a number of factors, including the cell population studied, the cell culture conditions and the nature of the stimulus. We have previously shown that cytokines such as GM-CSF used alone or in combination with other cytokines are good stimulators of monokine production *in vitro* [17]. Here we report that GM-CSF stimulates monocyte production of the cytokines IL-1α, IL-1β, IL-1Ra, TNF-α and IL-6, in agreement with other published data [19]. Moreover, GM-CSF induces these monokines (at least the secreted components) in a stable, reproducible manner. The high inter-subject variability together with consistent rank order suggests that there may be a genetic basis for the variable cytokine production by monocytes from normal healthy individuals. Such a genetic basis might partly lie in polymorphisms within the parts of the cytokine genes that regulate transcription. A number of polymorphisms in non-structural parts of cytokine genes have been described, but their significance is yet unclear. In order to assess the cytokine protein production phenotype of an individual, one would require a stable expression of product to a given stimulus, and preferably co-ordinate expression of a number of cytokine products in order to test for the specificity of the effects of a given gene mutation.

GM-CSF satisfies these criteria because it stimulates monocytes to produce a number of cytokines (IL-1α, IL-1β, IL-1Ra, TNF-α and IL-6), and the level of production of these cytokines is stable over time in a given individual. GM-CSF is probably an important stimulus for cytokine production in RA joints. Between 0·1 and 1 ng/ml of GM-CSF was sufficient to significantly up-regulate the production of most monocyte cytokine products. GM-CSF levels of up to 1·4 ng/ml have been detected in RA synovial fluid [20], and 0·5–1·5 ng/ml of GM-CSF was produced by RA synovial tissue explants [21]. There are also preliminary data suggesting that monocytes in RA patients have up-regulated expression of GM-CSF receptors [22].

We did not use LPS in this study because it has multiple components that bind a number of cell surface determinants, including CD14 and HLA-DR antigens, potentially adding complexity to the interpretation of variable cytokine production. LPS has recently been shown to induce IL-10 production in monocytes, possibly via TNF-α induction [23], whereas GM-CSF did not appear to induce IL-10 production [19,23]. We have confirmed previous reports that TGF-β, IL-4 and IL-10 may inhibit the production of IL-1α/β and IL-6 while leaving IL-1Ra protein production unaffected or even enhanced [24–28]. We have also shown that IFN-γ not only enhanced production of IL-1α/β (and IL-6 and TNF-α production) but also inhibited production of IL-1Ra in GM-CSF-stimulated monocyte cultures. The exact mechanisms by which IFN-γ

exerts these effects remain to be determined, but up-regulation of GM-CSF receptor expression [29] may be an important component.

Our data support the notion that IL-1Ra and IL-1α/β protein production are differentially regulated. First, in GM-CSF-stimulated cultures we could see a good correlation between IL-1α and IL-1β secretion ($r = 0{\cdot}73$, $P < 0{\cdot}001$), but not between IL-1α/β and IL-1Ra secretion. Second, there were differential effects on IL-1α/β production and IL-1Ra production with different cytokine stimuli. Thus, IL-4, TGF-β, IL-10 and, to a limited extent, IL-6 specifically up-regulated IL-1Ra production. GM-CSF combined with IL-4 was particularly potent at stimulating IL-1Ra production while at the same time inhibiting the production of IL-1α/β as well as TNF-α and IL-6. IL-1Ra production may also be specifically up-regulated by IL-13 [30] which acts in a similar manner to IL-4, as well as by stimuli such as cross-linking of FcRγ or β-glucan receptors [31] and some acute phase proteins [32]. IL-6 has been reported to stimulate some production of IL-1Ra protein by monocytes, but was found to be more potent at stimulating mature macrophage populations [33]. While we could observe some differential effects on the production of all the cytokine products we tested with the wide range of cytokines we used as stimuli, only in GM-CSF-stimulated cultures was the intra-individual variability low enough to be able to obtain a stable estimate of protein production phenotype necessary for genetic studies.

We have used the results of GM-CSF-stimulated cultures to determine the cytokine production phenotype of 36 healthy volunteers who had been genotyped for a variable nucleotide repeat IL-1Ra gene polymorphism [10]. We have shown that a less common allele (allele 2, containing just two repeats) was associated with increased IL-1Ra protein production as well as decreased IL-1α production. There was no association of this allele with the production of IL-1β, IL-6 or TNF-α. The polymorphism in the IL-1Ra gene may be in a regulatory area itself or in linkage disequilibrium with another polymorphism that regulates the transcription of both IL-1Ra and IL-1α genes. The variable repeat sequence has three potential protein binding sites: an IFN-α silencer A, an IFN-β silencer B, and an acute phase response element [10]. The activity of viral enhancer sequences may depend on the number of tandem copies present in the gene [34]. Polymorphism in the IFN response gene in mice has been shown to affect the level of production of a number of cytokines (IFN-α/β, TNF-α and IL-6) after viral infection [35]. The variable tandem repeat polymorphism in intron 2 of the human IL-1Ra gene may affect the activity of enhancer sequences that affect the transcription of the IL-1Ra gene and other cytokine genes.

The association of low IL-1α production with allele 2 of the IL-1Ra gene polymorphism may be a consequence of higher IL-1Ra protein production in these individuals. IL-1α/β appears to up-regulate its own production in a positive feedback loop. IL-1Ra protein can block this feedback loop, since it has been shown to reduce IL-1α/β production by LPS-stimulated cells [36] and to inhibit IL-1β production induced by IL-1α [37]. In our culture system, addition of exogenous IL-1Ra to GM-CSF-stimulated monocytes down-regulated IL-1α production, but did not affect IL-1β production.

In conclusion, we have demonstrated stable cytokine production by monocytes stimulated with GM-CSF *in vitro*. This provides a reliable estimation of cytokine production phenotype suitable for genetic studies. We have illustrated this with the demonstration of an association between allele 2 of a polymorphism in a non-structural area of the IL-1Ra gene and the level of production of IL-1Ra protein. Increased frequency of this allele has been associated with a number of diseases including psoriasis, systemic lupus erythematosus (SLE) and ulcerative colitis [38, 39]. We are currently assessing the frequency of this allele in RA and SLE patient subgroups. This is the first demonstration of the potential functional significance of this polymorphism.

ACKNOWLEDGMENTS

We are grateful to the Schering Company, Genetics Institute, Upjohn and DNAX Institute, USA, and Boehringer-Ingelheim, Germany, for the generous gifts of recombinant cytokines. We are grateful to Dr S. Poole and the National Institute for Biological Standards and Control, UK, for the supply of antisera to human cytokines (IL-1α, IL-1β, IL-1Ra and IL-6) and WHO international cytokine standards. We are grateful to Dr Debbie Rathjen (Peptide Technology, Sydney) for the supply of monoclonal antibodies to human TNF-α. This work has been supported by grants from the Northern Sydney Area Health Service and the Bone and Joint Research Foundation of The University of Sydney.

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