Production of intracellular and extracellular interleukin-1α and interleukin-1β by peripheral blood monocytes from patients with connective tissue diseases

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Abstract
An enzyme linked immunosorbent assay (ELISA) was used to estimate the production of intracellular and extracellular interleukin-1 (IL-1) α and β by peripheral blood monocytes from 26 patients with various connective tissue diseases (CTD), including 19 with systemic lupus erythematosus, four with progressive systemic sclerosis, two with mixed connective tissue disease, and one with Sjögren’s syndrome. Monocytes obtained from patients with CTD with serum antibodies to nuclear ribonucleoprotein (nRNP) released significantly higher concentrations of extracellular IL-1α and IL-1β, whereas intracellular IL-1α and IL-1β production was similar to that by monocytes from patients with CTD without antibodies to nRNP. Furthermore, the concentrations of extracellular IL-1α correlated significantly with those of extracellular IL-1β. There was no significant correlation between the concentrations of extracellular and intracellular IL-1α, and those of extracellular and intracellular IL-1β, indicating that synthesis and secretion of IL-1 by human monocytes may be two distinct biological events. It seems that enhanced extracellular release of both IL-1α and IL-1β contributes to the excessive anti-nRNP production in CTD.

Interleukin-1 (IL-1) is a cellular product with diverse amplifying effects on immunological cell reactions.1, 2 Interleukin-1 was originally identified and subsequently routinely measured by a mouse thymocyte mitogenesis assay, in which IL-1 increased the proliferation of thymocytes in response to suboptimal concentrations of T cell mitogens such as phytohaemagglutinin.3, 4 Cytotoxic agents mask IL-1 activity, however, whereas other substances, such as mitogens, can mimic or synergise with IL-1 on target cells. Moreover, measurement of IL-1 in clinical samples is complicated by the presence of inhibitory substances in preparations,5 and the presence of interleukin-2 (IL-2) in samples gives rise to false positive results. A potentially ideal solution to these problems lies in adaptation of an enzyme linked immunosorbent assay (ELISA) technique.

Complementary DNA cloning, protein purification, and sequencing studies have shown the presence of two distinct types of IL-1 molecules: an acidic form (pl 5)—IL-1α and a neutral form (pl 7)—IL-1β.6 These two proteins are distantly related to the primary sequence levels in humans and have similar biological functions.7 Recently, specific monoclonal and polyclonal antibodies against IL-1α or IL-1β have become available. Here we describe a sandwich ELISA for IL-1α and IL-1β which uses a rabbit polyclonal antibody and murine monoclonal antibody, both of which recognise human IL-1α and IL-1β respectively. We used an ELISA to examine the production of intracellular and extracellular IL-1α and IL-1β by peripheral blood monocytes from patients with connective tissue diseases (CTD).

Materials and methods
Cytokines
Recombinant IL-1α (rIL-1α) was donated by Dai-nippon Pharmaceutical Co (Osaka, Japan).8 Recombinant IL-1β (rIL-1β) was donated by Dr Y Hirai (Otsuka Pharmaceutical Co, Tokushima, Japan).9 Recombinant IL-2, rIL-6, and recombinant interferon-γ were obtained from Genzyme. Recombinant IL-4 was obtained from Boehringer Mannheim.

Antibodies to IL-1α and IL-1β
The ELISA for IL-1α or IL-1β used a sandwich technique with a rabbit polyclonal antibody to rIL-1α (Dai-nippon Pharmaceutical Co) or rIL-1β (donated by Dr Y Hirai) and a mouse monoclonal antibody to rIL-1α (Dai-nippon Pharmaceutical Co) or rIL-1β (donated by Dr Y Hirai). An immunoglobulin G fraction was isolated from the rabbit serum using staphylococcal protein A-Sepharose (Pharmacia Fine Chemicals, Hounslow, UK).

ELISA for IL-1α and IL-1β
For measurement of IL-1α or IL-1β by an ELISA microtitre plates (Immunoplate I, Nunc, Denmark) were first coated (16 hours, 4°C) with protein A-Sepharose purified rabbit polyclonal antibodies to rIL-1α or rIL-1β (100 µl per well of a solution of 10 µg/ml antibody in TRIS-HCl, pH 7.6, 0.15 M). The plates were then washed with a washing buffer (0.015 M TRIS-HCl, pH 7.6, 0.135 M NaCl, 0.05% Tween 20) and treated with an incubating buffer (10 mg/ml bovine serum albumin and 0.05% Tween 20 in TRIS-HCl) for 60 minutes at 37°C to block any remaining active sites. The wells were emptied and serial dilutions of rIL-1α or rIL-1β and
IL-1 samples in an incubating buffer were added, followed by incubation at 4°C for 16 hours. After extensive washing, 100 μl of mouse monoclonal antibodies to rIL-1α or rIL-1β in an incubating buffer was added to each well. After 60 minutes at 37°C the plates were washed and incubated with biotin conjugated rabbit anti-mouse IgG (EY Laboratories, 60 minutes at 37°C), followed by washing and addition of 100 μl peroxidase labelled streptavidin-biotin (diluted 1/1000). After another washing cycle the plates were inverted and shaken dry, and 100 μl of freshly prepared substrate-chromogen solution containing o-phenylenediamine 0-4 mg/ml and 0.14% hydrogen peroxide in 0.05 M acetate buffer (pH 4.5) was added to each well. The plates were incubated for 20 minutes at room temperature. Enzymatic reactions were inhibited by addition of 100 μl of 2.5 M sulphuric acid. Colorimetric measurements were taken with an automatic ELISA reader (SLT-210; Labinstruments) using a 486 nm filter. Assays were carried out in duplicate and the concentration of IL-1α or IL-1β was read from a standard curve constructed with known concentrations of standard rIL-1 preparations.

PATIENT SELECTION
Thirteen normal control subjects (nine women, four men; mean age 32 years) were selected from the medical and technical staff at the National Medical Center. The groups of patients included 19 (17 women, two men; mean age 39 years) with systemic lupus erythematosus (SLE), defined by the American Rheumatism Association criteria, four (three women, one man; mean age 47 years) with progressive systemic sclerosis, two (both women; mean age 55 years) with mixed connective tissue disease, and one (a woman; aged 56 years) with Sjögren's syndrome. The groups were not matched for age and sex and the disease duration and activity varied among the patients. No patient was receiving non-steroidal anti-inflammatory drugs. Steroids were not given on the day of the test. Serum antibodies to nuclear ribonucleoprotein (nRNP) were assessed by passive haemagglutination methods. Nine patients (six with SLE, one with progressive systemic sclerosis, and two with mixed connective tissue disease) were positive for serum antibodies to nRNP. Serum antibodies to double stranded (ds) DNA in the IgG class were assessed by the ELISA method. Five patients (three with SLE, one with progressive systemic sclerosis, and one with Sjögren's syndrome) were positive for serum antibodies to dsDNA.

MONOCYTE CULTURE
Mononuclear cells were obtained from freshly heparinised blood from patients with CTD and healthy adult volunteers by centrifugation on a Ficoll-Hypaque gradient, and resuspended at a concentration of 1 x 10⁶ cells/ml in RPMI 1640 and 10% heat inactivated fetal calf serum. Aliquots (1 ml) were allowed to adhere to multwell plastic plates, 24 x 17 mm (Linbro, Hamden, CN), in a humidified incubator with 5% CO₂ at 37°C. After 90 minutes the non-adherent cells were removed by washing thoroughly with Hank's balanced salt solution. The purity of the monocytes was over 90% by non-specific esterase staining. The adherent monocytes were further incubated in aliquots of 1 ml RPMI 1640–10% fetal calf serum with or without lipopolysaccharide (Difco, Detroit, MI; 1 μg/ml). The supernatants of peripheral blood monocytes were collected after 20 hours of incubation, centrifuged at 900 g for 10 minutes, and kept frozen at −20°C. The remaining monolayers were covered with 1 ml of fresh RPMI 1640–10% fetal calf serum and frozen at −20°C. After thawing the cells were resuspended with fresh RPMI 1640-10% heat inactivated fetal calf serum, sonicated, and frozen again at −20°C until tested for IL-1α and IL-1β.

RESULTS
STANDARD ELISA FOR IL-1α AND IL-1β
As shown in fig 1 the sandwich ELISA using a combination of rabbit polyclonal antibodies and mouse monoclonal antibodies to IL-1α or IL-1β could efficiently detect IL-1α concentrations over the range 0.125 ng/ml to 8 ng/ml and IL-1β concentrations over the range 2 ng/ml to 128 ng/ml. The specificity of the assay was verified by the fact that IL-1β was not detected by the IL-1α assay and vice versa. Furthermore, the ELISA system showed no cross reactivity with other recombinant cytokines, such as IL-2, IL-4, IL-6, and interferon-γ.

EXTRACELLULAR AND INTRACELLULAR PRODUCTION OF IL-1α OR IL-1β
The mean (SE) concentrations of extracellular IL-1α released by monocytes from patients with SLE (n=19) and other CTD (n=7), and from normal subjects (n=13) were 1.7 (0.7) ng/ml, 7.1 (2.7) ng/ml, and 1.5 (0.4) ng/ml (fig 2A) respectively. The mean extracellular release of IL-1α by monocytes from patients with other CTD was significantly greater than that by monocytes from patients with SLE (t=2.78, p<0.02) and that by monocytes from normal subjects (t=2.81, p<0.02). Furthermore, the mean (SE) extracellular release of IL-1α by monocytes from the nine patients with CTD (SLE + other CTD) with serum antibodies to nRNP (7.8 (2.2) ng/ml) was significantly greater
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than that by monocytes from the 17 patients with CTD without antibodies to nRNP (0-8 (0-1) ng/ml, t=4-25, p<0-001).

The mean (SE) concentrations of intracellular production of IL-1α by monocytes from patients with SLE (n=19) and other CTD (n=6), and normal controls (n=13) were 7-7 (2-8) ng/ml, 14-0 (5-2) ng/ml, and 6-7 (2-7) ng/ml (fig 2B) respectively.

The mean (SE) concentrations of extracellular IL-1β released by monocytes from patients with SLE (n=19) and other CTD (n=7), and from normal subjects (n=13) were 14-3 (6-2) ng/ml, 31-5 (8-9) ng/ml, and 8-6 (3-0) ng/ml (fig 2C) respectively. The mean extracellular release of IL-1β by monocytes from patients with other CTD was significantly greater than that by monocytes from normal subjects (t=3-01, p<0-01). Furthermore, the mean (SE) extracellular release of IL-1β by monocytes from the nine patients with CTD with serum antibodies to nRNP (44-1 (10-8) ng/ml) was significantly greater than that by monocytes from the 17 patients with CTD without antibodies to nRNP (5-6 (2-0) ng/ml, t=4-46, p<0-001).

The mean (SE) concentrations of intracellular production of IL-1β by monocytes from patients with SLE (n=19) and other CTD (n=6), and normal controls (n=13) were 111-4 (39-5) ng/ml, 362-3 (127-2) ng/ml, and 436-2 (167-2) ng/ml (fig 2D) respectively. The mean intracellular production of IL-1β by monocytes from patients with other CTD was significantly greater than that by monocytes from patients with SLE (t=2-55, p<0-02).

RELATION BETWEEN EXTRACELLULAR AND INTRACELLULAR PRODUCTION OF IL-1α OR IL-1β
Figure 3 shows the relation between extracellular production of IL-1α and IL-1β; a significant correlation was found (r=0-97, p<0-001). This finding suggests an associated excretion of IL-1α and IL-1β. On the other hand, there was no correlation between intracellular production of IL-1α and IL-1β (r=0-317, p>0-05), between extracellular and intracellular production of IL-1α (r=0-290, p>0-05), or extracellular and intracellular production of IL-1β (r=0-242, p>0-1). These findings indicate that synthesis and secretion of IL-1 by monocytes may be two distinct biological events.

EXTRACELLULAR AND INTRACELLULAR PRODUCTION OF IL-1α OR IL-1β BY LIPOPOLYSACCHARIDE STIMULATED MONOCYTES
The mean (SE) concentrations of extracellular IL-1α and IL-1β released by lipopolysaccharide stimulated monocytes were significantly greater than those produced by unstimulated monocytes (extracellular IL-1α: 5-87 (1-51) ng/ml v 1-91 (0-65) ng/ml, p<0-01; extracellular IL-1β: 28-59 (8-13) ng/ml v 11-31 (3-31) ng/ml, p<0-01), whereas there was no difference between the mean (SE) concentrations of intracellular IL-1α and IL-1β produced by lipopolysaccharide stimulated and unstimulated monocytes (intracellular IL-1α: 9-05 (4-21) ng/ml v 6-88 (2-22) ng/ml; intracellular IL-1β: 226-69 (57-40) ng/ml v 173-13 (46-51) ng/ml (fig 4).
IL-1β by their monocytes after culture for only 20 hours. These findings also indicate the presence of similar immunological abnormality among patients with CTD irrespective of disease entity, suggesting a close association between the increased extracellular IL-1 production and the vigorous autoantibody production in patients with CTD. Antibodies to dsDNA, however, which are also representative autoantibodies found in patients with SLE, were not associated with extracellular or intracellular IL-1 production (data not shown).

Alcocer-Varela et al have shown that the production of IL-1 by lipopolysaccharide stimulated monocytes from patients with untreated scleroderma was akin to that of their normal matched controls, but that unstimulated monocytes from some patients with scleroderma released IL-1 activity spontaneously into their supernatants. Furthermore, Shore et al have reported that monocytes from patients with rheumatoid arthritis who had a recent onset of their disease, or exacerbation of existing rheumatoid arthritis, had enhanced spontaneous IL-1 secretion. In contrast, patients with SLE have been found to have defective production of IL-1 by lipopolysaccharide or phorbol myristate acetate stimulated monocytes. Whicher et al examined the ability of stimulated leucocytes from patients with various CTD to produce leucocytic endogenous mediator (IL-1) in vitro and showed that its production was decreased significantly below normal in progressive systemic sclerosis, SLE, rheumatoid arthritis, and mixed connective tissue disease. Our study, however, showed that the stimulation of monocytes with lipopolysaccharide resulted in a significant increase of extracellular secretion of both IL-1α and IL-1β in patients with CTD as well as in normal subjects.

Our study also showed a significant correlation between extracellular production of IL-1α and IL-1β, suggesting an associated excretion of the two molecular forms of IL-1. No significant correlation was found between extracellular and intracellular IL-1α or IL-1β production, confirming that synthesis and secretion of IL-1 by monocytes are two distinct biological events. Furthermore, we confirmed that the α type of IL-1 is a minor part of IL-1 both in extracellular IL-1 (α/β=1/5-9) and intracellular IL-1 (α/β=1/25-2), as previously reported.

The results of the ELISA did not correlate well with those of the biological assay (data not shown). It seems likely that the biological assay is complex and may be affected by other cytokines, such as IL-6. A minor disadvantage of the ELISA, which is common in such immunochemical assays, is the possibility of detecting biologically inactive fragments. In addition, it was noted, because of the basic principle of the assay, that the results might be affected by rheumatoid factors present in samples to be measured.

In conclusion, this assay provides a rapid, automated method, suitable for general use, which may be used to measure IL-1 and to help in establishing the type of IL-1 present in various preparations. Establishment of dis-
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criminative ELISAs for IL-1α and IL-1β may offer a useful tool for exploring the role of these polypeptides in the pathogenesis of various CTD.

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