

# The oxidative metabolism of circulating phagocytes in ankylosing spondylitis: determination by whole blood chemiluminescence

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## Abstract

**Objective**—Superoxide anion radicals within the human body are regarded as a major cause of inflammation. However, their role in the pathogenesis of ankylosing spondylitis (AS) has not been well identified. This study aimed at investigating the relation between AS and the oxidative metabolism of phagocytes in whole blood.

**Methods**—24 patients with classic AS were examined to determine their clinical status; complete blood count, erythrocyte sedimentation rate (ESR), and C reactive protein (CRP) were determined, and levels of the superoxide anion radicals in the patients with AS and 21 healthy subjects were assessed by the ultraweak chemiluminescence method. Subsequently, the relation between this disease and phagocytes was examined by using *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) and phorbol-12-myristate-13-acetate (PMA) stimulants.

**Results**—In clinical assessments, patients with AS had abnormally raised serum CRP (>10 mg/l) and ESR (>15 mm/1st h) levels. In contrast with healthy subjects, patients with AS had significantly increased rates of superoxide anion radical production in their whole blood either in the resting state or with either fMLP or PMA stimulation. In addition, chemiluminescence maximum light intensity was significantly higher in patients with AS than in healthy subjects after fMLP or PMA stimulation.

**Conclusions**—Our results suggest that the phagocytes of patients with AS are partly activated in the resting state, and are sensitive to fMLP or PMA stimulation. The priming of phagocytes in the bloodstream is likely to be a causative factor in the onset of AS.

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usually begins in the second or third decade and the prevalence in men is about three times that in women. Although the pathogenesis of AS is yet unknown, a combination of genetic and environmental factors probably plays a part. Several studies suggest that the HLA-B27 gene is present in more than 90% of patients with AS, particularly in North American white people,<sup>1</sup> and raised serum titres of antibodies to certain enteric bacteria, particularly *K pneumoniae*, are common in patients with AS. Based on consistent findings of raised antibodies to klebsiella,<sup>2</sup> and the presence of molecular mimicry between HLA-B27 and *K pneumoniae* nitrogenase,<sup>3</sup> AS can be considered as an arthritic disease following *K pneumoniae* infection in HLA-B27 positive patients.<sup>4</sup> However, the exact role of molecular mimicry in the pathogenesis of AS remains to be determined.

Free radicals exist abundantly in the physiological environment and are unstable. They can damage biological molecules and cause malfunctions and diseases in the human body. Inappropriate release of oxygen free radicals and proteolytic enzymes from activated polymorphonuclear leucocytes (PMNs), both in the bloodstream and synovial fluid of patients with rheumatoid arthritis (RA), is responsible for joint and other tissue damage.<sup>5</sup> AS, like RA, is one of the inflammatory arthritic diseases, and therefore we wish to address the question of whether free radicals might be directly detected in the whole blood of patients with AS.

In our study we used the lucigenin (bis-*N*-methylacridinium nitrate) enhanced chemiluminescence method<sup>6</sup> to assess the content of superoxide anion radicals in the whole blood of both patients with AS and healthy subjects in the presence or absence of *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) and phorbol-12-myristate-13-acetate (PMA). The purpose of this study is to investigate the relation between AS and the oxidative metabolism of phagocytes.

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Ankylosing spondylitis (AS) is an inflammatory disorder of unknown cause that mainly affects the axial skeleton as well as the peripheral joints and extra-articular structures. The entheses, the site of tendinous or ligamentous attachment to bone, is another common site of disease in AS, especially around the area of the spine and pelvis. The most common symptoms are low back pain, morning stiffness, decreased spinal mobility, and even kyphotic deformity, all of which hinder daily activities greatly. AS

Table 1 Clinical characteristics of patients with ankylosing spondylitis

Number	Mean age, years (range)	Mean ESR* (SE) (mm/1st h)	Mean CRP* (SE) (mg/l)
24	35.6 (19-54)	31.2 (6.6)	22.0 (6.5)
		<15 (n = 9)	<10 (n = 14)
		≥15 (n = 15)	≥10 (n = 10)

\*ESR = erythrocyte sedimentation rate; CRP = C reactive protein; n = number of patients.

The normal values for ESR and CRP are <15 mm/1st h and <8 mg/l, respectively.

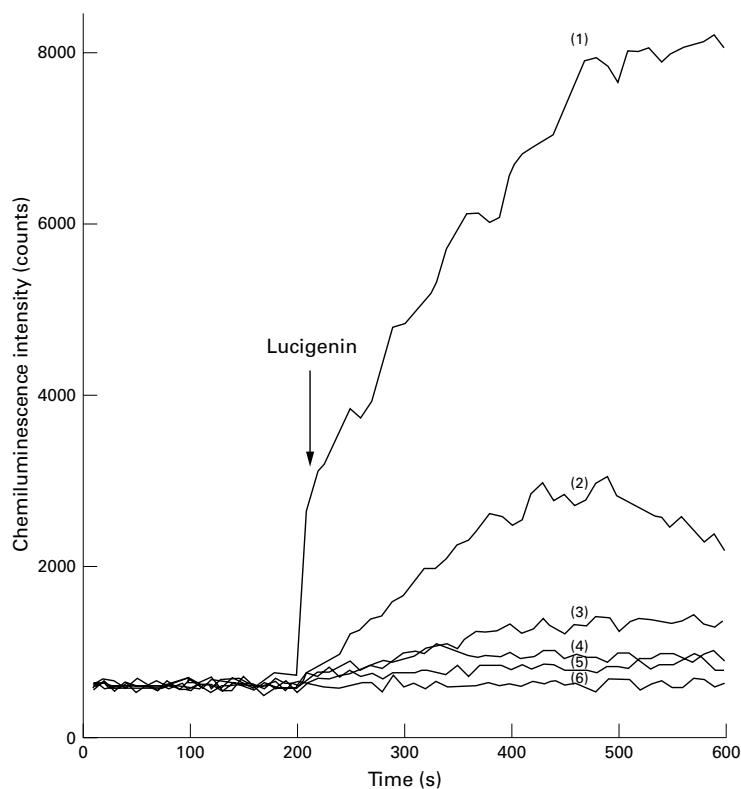


Figure 1 Comparisons of superoxide anion radicals in whole blood between one patient with AS and one healthy subject using the lucigenin enhanced ultraweak chemiluminescence method. 0.2 ml of heparinised blood was mixed with 0.1 ml of phosphate buffered saline (pH 7.4), *N*-formyl-methionyl-leucyl-phenylalanine (fMLP;  $1 \times 10^{-5}$  mol/l) or phorbol-12-myristate-13-acetate (PMA; 10  $\mu$ g/ml) at 37°C. Then, 1 ml lucigenin (0.01 mmol/l) was added 200 seconds later. The levels of superoxide anion radicals in one patient with AS and one healthy subject were assessed by the ultraweak chemiluminescence method. (1) AS + PMA; (2) AS + fMLP; (3) healthy subject + PMA; (4) healthy subject + fMLP; (5) AS only; (6) healthy subject only.

## Patients and methods

### PATIENTS

Samples from 24 male patients with AS (aged 19–54 years, with a mean age of 35.6), who fulfilled the New York criteria<sup>7</sup> for definite diagnosis of AS, were collected from the outpatient clinic of the Department of Orthopaedic Surgery, National Taiwan University Hospital. All these patients were HLA-B27 positive and had not taken non-steroidal anti-inflammatory drugs in the past two weeks. They were compared with 21 healthy subjects (aged 22–47 years, with a mean age of 30.2) selected from the students and teachers of the Medical College, National Taiwan University. Peripheral venous blood from the subjects was mixed with sodium heparin in tubes covered

with aluminium foil and was stored in ice until used for the assay.

### LABORATORY INVESTIGATIONS

Complete blood count, C reactive protein (CRP), and erythrocyte sedimentation rate (ESR) were measured on the same day. The CRP concentrations were determined by the single radial immunodiffusion method of Mancini *et al.*,<sup>8</sup> (normal <10 mg/l) and the ESR levels of patients with AS were measured by the Westergren method (normal <15 mm/1st h).

### DETERMINATION OF SUPEROXIDE ANION RADICALS IN WHOLE BLOOD

The content of superoxide anion radicals in whole blood was measured at 37°C by the ultraweak chemiluminescence method as used by Lu *et al.*<sup>6</sup> In brief, 0.2 ml of heparinised blood was mixed with 0.1 ml of phosphate buffered saline (pH 7.4), fMLP ( $1 \times 10^{-5}$  mol/l), or PMA (10  $\mu$ g/ml). The chemiluminescence intensity (CLI) was measured for 200 seconds at 10-second intervals using the Chemiluminescence Analysing System (Tohoku Electronic Ind. Co, Sendai, Japan). One millilitre of lucigenin (0.01 mmol/l) was added to this mixture when measurement was complete, and then the CLI was recorded continuously every 10 seconds for a total of 400 seconds. The total CLI counts were calculated by deducting the background value (the area of CLI that was equal to three times that of the first 200 seconds) from the total area of CLI (the total area of CLI within 600 seconds). The rate of superoxide anion radical generation was expressed as counts/10 s/10<sup>5</sup> phagocytes. To calculate the value of the chemiluminescence maximal light intensity (CMLI), the average CLI value of the first 200 seconds was subtracted from the highest CLI of the last 400 seconds and the result divided by the phagocyte count.

### STATISTICAL ANALYSIS

The means of superoxide production rates and CMLI in patients with AS and healthy subjects were compared with Mann-Whitney's non-parametric U test. All data were expressed as means (standard error). Significance was set at  $p < 0.05$ .

## Results

In clinical assessments, patients with AS had a raised ESR (>15 mm/1st h) and a high

Table 2 Production of superoxide anion radicals measured by chemiluminescence

	Production rate (counts/10 s/10 <sup>5</sup> phagocytes)		CMLI* (counts/10 s/10 <sup>5</sup> phagocytes)	
	Healthy subjects	Patients with AS	Healthy subjects	Patients with AS
Resting	6.2 (1.5) (0–26.1) (n = 21, age 22–47)	60.9 (18.6) (0–354.7)† (n = 24, age 19–54)	ND	ND
fMLP*	34.9 (5.9) (4.9–62.8) (n = 9, age 23–42)	178.8 (36.9) (9.2–498.2)† (n = 18, age 19–54)	59.8 (11.3) (17.5–131.9)	237.2 (45.0) (17.2–584.2)†
PMA*	105.2 (19.1) (33.6–194.4) (n = 9, age 23–42)	583.0 (112.8) (87.2–2006.3)† (n = 20, age 19–54)	150.2 (25.6) (56.5–282.5)	766.5 (127.1) (139.2–2166.5)†

\*CMLI = chemiluminescence maximal light intensity; fMLP = *N*-formyl-methionyl-leucyl-phenylalanine; PMA = phorbol-12-myristate-13-acetate; n = number of patients; ND = not done.

All results are expressed as mean (standard error) (range).

† $p < 0.01$  compared with healthy subjects by Mann-Whitney U test.

concentration of CRP (>10 mg/l) (table 1). In these patients there was a significant positive correlation between ESR and the rate of superoxide anion radical production in the resting state ( $r = 0.575$ ,  $p = 0.02$ ) or under the stimulation of fMLP ( $r = 0.565$ ,  $p = 0.044$ ). However, no significant correlation between CRP concentrations and the rate of superoxide anion radical production was discovered.

Compared with a healthy subject, a patient with AS had a higher intensity of lucigenin enhanced chemiluminescence, with or without the stimulation of fMLP or PMA, than the controls (fig 1). The rate of superoxide anion radical production in patients with AS was significantly higher than that in healthy subjects both when their blood was in the resting condition or was stimulated by either fMLP or PMA, with average increases of 8.8, 4.1, and 4.5 times, respectively ( $p < 0.01$ ; table 2). These patients also showed a significant increase of CMLI in response to stimulation with either fMLP or PMA ( $p < 0.01$ ; table 2).

### Discussion

Although the pathogenesis of ankylosing spondylitis is still unknown, the activation and production of superoxide anion radicals from phagocytes may play a part in the process. Different results have been reported in studies on superoxide anion radical production from PMNs in patients with AS. No difference, or a small difference in superoxide anion radical production of PMNs between patients with AS and healthy subjects, was shown in response to stimulation with fMLP, PMA, or opsonised zymosan.<sup>9-11</sup> However, the results of this study show that the superoxide anion radical production is significantly higher in patients with AS than in healthy subjects, in the resting state or after stimulation with fMLP or PMA. These results are in agreement with the findings of Wendling<sup>12</sup> and Biasi,<sup>13</sup> which show an increase in the oxidative metabolism of the phagocyte system in AS after fMLP stimulation. These different results might be attributable to the methodology or technology used. In recent years, purified phagocytes have been used to study phagocyte function. However, during the purification of phagocytes, activated and primed phagocytes might be lost, and circulating inflammatory mediators might also be removed from serum. Therefore, their free radical measurements might not reflect the exact state of circulating phagocytes; whereas, direct use of whole blood to measure the phagocyte function would minimise artefacts caused by cell purification, and maintain phagocytes in an *in vivo* physiological state. Furthermore, our method is more sensitive and convenient than the traditional cytochrome *c* assay and can continuously assess the respiratory bursts from activated phagocytes.

The exact role of superoxide anion radicals in the inflammatory damage of AS has not yet been fully elucidated. NADPH oxidase is a key enzyme that can generate superoxide anion radicals in phagocytes. When phagocytes are exposed to fMLP or PMA the components of NADPH oxidase form a functional complex

responsible for the production of superoxide anion radicals.<sup>14</sup> In this paper we show that phagocytes of patients with AS are partly activated in the resting condition, and are sensitive to the stimulation of fMLP or PMA. It is highly likely that the sera of patients with AS might have some factors that increase the sensitivity of PMNs in response to fMLP or PMA. These factors could change in number or binding affinity for surface receptors or activate proteins that were in the upstream signal of NADPH oxidase, thus enhancing the superoxide production by NADPH oxidase.

Increased concentrations of interleukin 6 (IL6) and tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) in patients with AS were described by Gratacos<sup>15</sup> and Tutuncu.<sup>16</sup> In addition, abundant TNF $\alpha$  mRNA was also found in the synovium from inflamed sacroiliac joints.<sup>17</sup> IL6 can induce systemic inflammation and is said to be the major cytokine responsible for the production of acute phase reactants in the patients with AS.<sup>16</sup> TNF $\alpha$  is an inflammatory and priming agent for increased production of reactive oxygen species and matrix metalloproteinases from phagocytes.<sup>17</sup> When phagocytes are primed by TNF $\alpha$ , they become much more sensitive to fMLP or PMA stimulation. In this study we suppose that the phagocytes from patients with AS were primed by TNF $\alpha$  to enhance their responsiveness to fMLP or PMA stimulation and then trigger respiratory bursts. Therefore, the existence of cytokines (such as IL6 and TNF $\alpha$ ) in the blood of patients with AS might be the main cause of the inconsistency between various studies.

In conclusion, the production of superoxide anion radicals in the blood of patients with AS is increased either in the resting or stimulated state. The primed phagocytes in the bloodstream are more easily activated by stimulants such as bacterial peptides. The primed or activated phagocytes migrate from bloodstream to specific inflamed joints where abundant TNF $\alpha$  are found, and they produce large amounts of free radicals and metalloproteinases, leading to joint damage over time. The primed phagocytes may be one of the causative factors in the pathogenesis of ankylosing spondylitis, but further research is required.

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