

# Determination of oxygen radical production in spondyloarthropathies by whole blood chemiluminescence

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## Abstract

Oxygen derived free radicals are considered to play an important part in the development of inflammation. A whole blood chemiluminescence assay was used to study *N*-formyl-methionyl-leucyl-phenylalanine induced oxygen radical production in subjects with ankylosing spondylitis or previous yersinia arthritis. In luminol enhanced chemiluminescence, the subjects with previous yersinia arthritis showed significantly increased initial activation (at one minute), whereas the subjects with ankylosing spondylitis showed decreased responses at both the initial activation and at peak activation (at two to three minutes). This finding gives credence to the view that, in terms of oxygen radical production, the pathogenesis of yersinia arthritis is different from that of ankylosing spondylitis.

Reactive arthritis and ankylosing spondylitis are both strongly associated with the HLA-B27 antigen.<sup>1</sup> Reactive arthritis, a sterile inflammatory arthropathy, can follow enteric infections by *Yersinia enterocolitica*, salmonellas, shigellas, and campylobacteria.<sup>2-5</sup> In ankylosing spondylitis infectious agents such as klebsiella have been suggested as triggering factors of the disease.<sup>6,7</sup>

The pathogenesis of spondyloarthropathies is unknown, but both an aberrant immune response<sup>8</sup> and inappropriate inflammatory hyperreactivity<sup>9</sup> may be involved. Patients with yersinia arthritis have altered cell mediated and humoral immunity against yersinia.<sup>10,11</sup> Serum samples from patients with acute yersinia arthritis have a stronger opsonic capacity than serum samples from patients with non complicated yersiniosis.<sup>12</sup> The initial activation, but neither the peak response nor the area under the curve, in *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) induced luminol enhanced whole blood chemiluminescence is increased in subjects with previous yersinia arthritis.<sup>13</sup> In patients with previous yersinia arthritis the increased oxygen radical production from purified neutrophils is associated with the severity of the acute disease<sup>14</sup> and the presence of sequelae,<sup>15</sup> whereas in ankylosing spondylitis the neutrophil oxygen radical production is reported to be normal<sup>16</sup> or decreased.<sup>17</sup>

The exact role of oxygen radicals in the inflammatory damage of arthritides is not known.<sup>18</sup> The purpose of this work was to study oxygen radical production in patients with previous yersinia arthritis who had experienced severe or mild acute disease, with or without

sequelae, in patients with ankylosing spondylitis, and in healthy subjects who were HLA-B27 positive or negative. Luminol and lucigenin enhanced responses were determined; the former measures mainly the reaction between hydrogen peroxide and myeloperoxidase,<sup>19</sup> whereas the latter measures mainly superoxide production.<sup>20</sup>

## Patients and methods

### PATIENTS

Twenty five HLA-B27 positive or negative subjects were selected from a follow up study group of patients with yersinia arthritis.<sup>21</sup> The diagnosis of yersinia arthritis was based on the typical clinical symptoms of reactive arthritis<sup>22</sup> and a raised agglutination antibody titre (>1/160) for *Yersinia enterocolitica* (0:3 or 0:9), or a stool culture positive for the organism. The patients with previous yersinia arthritis were grouped according to the HLA-B27 antigen, the clinical features of acute disease, and the presence of sequelae (radiological sacroiliitis, enthesopathies, or iritis) during follow up (table 1). Seven HLA-B27 positive subjects with ankylosing spondylitis, who fulfilled the New York criteria for definite ankylosing spondylitis<sup>23</sup> and who had had predominantly axial inflammation, were selected from patients at the outpatient clinic of the second department of

Table 1 Characteristics of the subject groups

Characteristic	Previous yersinia arthritis	Ankylosing spondylitis	Healthy subjects
Number of subjects	25	7	20
Men/women	9/16	4/3	6/14
HLA-B27 positive-negative	18/7	7/0	10/10
Acute phase of yersinia arthritis severe/mild*	12/13	—	—
Sequelae after yersinia arthritis present/absent	10/15	—	—
Mean age in years (range)	47 (27-69)	41 (32-52)	44 (31-62)
Mean period in years (range) since yersinia arthritis	14 (10-19)	—	—
Mean period in years (range) since symptoms of ankylosing spondylitis	—	12 (3-20)	—
Mean period in years (range) since diagnosis of ankylosing spondylitis	—	6 (2-13)	—
C reactive protein (mg/l)			
<10	22	3	19
10-19	2	3	1
20-22	1	1	—

\*The subjects with severe yersinia arthritis showed at least two, and the subjects with mild yersinia arthritis only one (or none), of the following characteristics: highest erythrocyte sedimentation rate >110 mm/h; highest white blood cell count >10×10<sup>9</sup>/l; presence of extra-articular manifestations (iritis/conjunctivitis, balanitis/pyuria, keratoderma blenorrhagica, or erythema nodosum).

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medicine of Helsinki University Central Hospital (table 1). The subjects had not taken non steroidal anti-inflammatory drugs. At the time of this study the subjects with ankylosing spondylitis or previous yersinia arthritis had no or few acute musculoskeletal symptoms and their serum concentrations of C reactive protein were normal or only slightly raised (table 1). The healthy control subjects (table 1) were HLA-B27 positive or negative healthy laboratory and hospital staff.

#### STUDY DESIGN

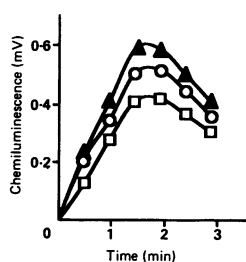
Blood samples from four HLA-B27 positive (with previous yersinia arthritis with sequelae, with previous yersinia arthritis without sequelae, with ankylosing spondylitis, or healthy) and two HLA-B27 negative (with previous yersinia arthritis or healthy) subjects were taken and tested on a single day.

#### BLOOD SAMPLES

Peripheral venous blood was mixed with preservative free heparin, 10 U/ml of blood, and the neutrophil count and haemoglobin concentration were determined.<sup>24</sup>

#### DETERMINATION OF CHEMILUMINESCENCE

Luminol enhanced chemiluminescence of whole blood samples was measured at 37°C.<sup>24</sup> In brief, 100 µl of blood was mixed with 200 µl of luminol (Fluka, Buchs, Switzerland) (100 µg/ml) and 100 µl of fMLP (Sigma Chemical, St Louis, MO, USA) (10<sup>-5</sup> mol/l), or phosphate buffered saline, and the reaction was observed continuously for three minutes in an LKB-Wallac Luminometer 1250 with a chart recorder. In a study of 50 healthy adults by this assay,<sup>24</sup> both fMLP and phosphate buffered saline induced chemiluminescence showed a peak response at two to three minutes. The one minute value representing the rate of increase in the initial activation<sup>24</sup> and the peak response at two to three minutes<sup>24</sup> (fig 1) were used in the statistical analysis. To determine the lucigenin enhanced response, lucigenin (Sigma) was dissolved in Dulbecco's buffered saline at a concentration of 1 mmol/l and used instead of the luminol solution. The reaction was observed



**Figure 1** Luminol enhanced N-formyl-methionyl-leucyl-phenylalanine induced chemiluminescence as a function of time. Geometric means of the subject groups described in table 1 are shown. Previous yersinia arthritis ( $\blacktriangle$ ), ankylosing spondylitis ( $\square$ ), and healthy control subjects ( $\circ$ ). The one minute activation representing the initial activation and the peak response at two to three minutes were used in the statistical analysis.

for three minutes and the peak response, recorded in millivolts, was calculated from the curve obtained.

The arithmetic mean of triplicate measurements was calculated and then converted to be equivalent to the chemiluminescence response of  $2.5 \times 10^9$  neutrophils per litre in 135 g/l haemoglobin with a correction factor based on both the neutrophil count and the haemoglobin concentration.<sup>24</sup> The correction factor is based on an experimental study with cell mixtures<sup>24</sup> in which the correlation of the neutrophil count with chemiluminescence was positive and linear, whereas the correlation of the haemoglobin concentration with chemiluminescence was negative and linearity was attained by logarithmic transformation. The corrected chemiluminescence values are presented.

#### STATISTICS

The significance of the difference between the groups was evaluated by the analysis of variance; we evaluated the effect of (1) previous yersinia arthritis and HLA-B27 among subjects with previous yersinia arthritis and healthy subjects; (2) clinical features among subjects with previous yersinia arthritis; (3) ankylosing spondylitis among subjects with ankylosing spondylitis and healthy subjects; and (4) type of spondyloarthropathy among HLA-B27 positive subjects who had ankylosing spondylitis, had had yersinia arthritis, or who were healthy. The significance of the difference between the subgroups with different clinical features of previous yersinia arthritis was evaluated by the Mann-Whitney U test. On the basis of the analysis of the frequency distributions of all the parameters measured, logarithmic transformation of the data was performed before the analysis of variance and the geometric mean and standard error of the mean were used in the presentation of the data.

#### Results

##### YERSINIA ARTHRITIS AND THE HLA-B27 ANTIGEN

The subjects with previous yersinia arthritis ( $n=25$ ) had higher responses than the healthy subjects ( $n=20$ ) for all the parameters analysed, except in the phosphate buffered saline induced one minute responses (table 2); the association

**Table 2** Whole blood chemiluminescence in subject groups characterised in table 1. Results expressed in millivolts as geometric mean ( $-1$  SEM;  $+1$  SEM)

Subjects	Number of subjects	Luminol enhanced				Lucigenin enhanced
		fMLP one minute	fMLP peak	PBS one minute	PBS peak	fMLP peak*
Yersinia arthritis	25	0.42 (0.39; 0.45)	0.65 (0.60; 0.70)	0.05 (0.04; 0.06)	0.12 (0.11; 0.14)	1.49 (1.41; 1.57)
HLA-B27 positive	18	0.38 (0.35; 0.41)	0.61 (0.56; 0.68)	0.04 (0.03; 0.05)	0.11 (0.10; 0.13)	1.46 (1.37; 1.56)
HLA-B27 negative	7	0.54 (0.47; 0.63)	0.73 (0.61; 0.88)	0.06 (0.05; 0.07)	0.16 (0.13; 0.19)	1.55 (1.40; 1.72)
Sequelae present	10	0.42 (0.37; 0.47)	0.66 (0.56; 0.77)	0.03 (0.02; 0.04)	0.11 (0.09; 0.13)	1.43 (1.29; 1.60)
Sequelae absent	15	0.40 (0.37; 0.45)	0.64 (0.58; 0.71)	0.06 (0.05; 0.07)	0.14 (0.12; 0.16)	1.52 (1.44; 1.61)
Severe arthritis	12	0.48 (0.44; 0.53)	0.71 (0.64; 0.79)	0.04 (0.03; 0.05)	0.13 (0.11; 0.15)	1.67 (1.56; 1.78)
Mild arthritis	13	0.37 (0.33; 0.41)	0.59 (0.52; 0.67)	0.06 (0.05; 0.07)	0.11 (0.09; 0.13)	1.33 (1.24; 1.44)
Ankylosing spondylitis	7	0.27 (0.24; 0.31)	0.43 (0.38; 0.49)	0.03 (0.02; 0.04)	0.08 (0.07; 0.10)	1.32 (1.25; 1.39)
Healthy subjects	20	0.34 (0.32; 0.37)	0.57 (0.53; 0.60)	0.05 (0.04; 0.07)	0.11 (0.09; 0.13)	1.33 (1.23; 1.44)
HLA-B27 positive	10	0.38 (0.35; 0.42)	0.62 (0.57; 0.67)	0.05 (0.04; 0.07)	0.13 (0.10; 0.16)	1.29 (1.18; 1.42)
HLA-B27 negative	10	0.31 (0.27; 0.35)	0.52 (0.48; 0.56)	0.05 (0.04; 0.06)	0.09 (0.07; 0.12)	1.37 (1.24; 1.52)

Abbreviations: fMLP=N-formyl-methionyl-leucyl-phenylalanine; PBS=phosphate buffered saline

\*The one minute responses were almost the same in all groups (data not shown).

was significant in the luminol enhanced fMLP induced one minute responses ( $p=0.028$ ; table 3, fig 2A).

When the effect of the presence of the HLA-B27 antigen was analysed in subjects with yersinia arthritis and healthy subjects the effect of the presence of HLA-B27 was not statistically significant (table 3). However, the healthy subjects with HLA-B27 ( $n=10$ ) had higher responses than those without HLA-B27 ( $n=10$ ) (table 2), although the difference was not statistically significant.

#### CLINICAL FEATURES OF YERSINIA ARTHRITIS

The subjects who with previous severe yersinia arthritis ( $n=12$ ) showed higher responses in all the parameters analysed than those who with previous mild yersinia arthritis ( $n=13$ ), except for the one minute responses in phosphate buffered saline induced chemiluminescence (table 2); the difference was significant in lucigenin enhanced peak responses ( $p=0.009$ ; table 3).

When the subgroups with different clinical features of previous yersinia arthritis were compared the subjects with mild arthritis with sequelae present showed low responses in all the parameters analysed (table 4, fig 2B). In the luminol enhanced fMLP induced peak

responses, the difference between the subgroup with mild arthritis with sequelae present and the subgroup with severe arthritis with sequelae present was significant ( $p<0.05$ ; table 4). In the phosphate buffered saline induced one minute responses the difference between the

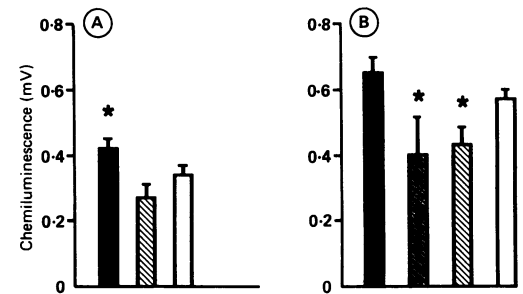


Figure 2 Comparison of luminol enhanced N-formyl-methionyl-leucyl-phenylalanine induced chemiluminescence between the subject groups described in table 1. Previous yersinia arthritis (■), previous yersinia arthritis with mild acute arthritis and sequelae (▨), ankylosing spondylitis (▧), healthy control subjects (□). In the analysis of the one minute values (A) previous yersinia arthritis was associated with significantly increased chemiluminescence responses. In the analysis of peak values (B) both ankylosing spondylitis and previous yersinia arthritis with mild acute arthritis and sequelae were associated with significantly decreased chemiluminescence responses. Each column represents the geometric mean of the group and each bar one standard error of the mean. Statistical significance is indicated by asterisks (for the exact statistical analysis, see tables 2-4).

Table 3 Analysis of variance of whole blood chemiluminescence responses

Subjects	Degrees of freedom	Luminol enhanced								Lucigenin enhanced			
		fMLP one minute				fMLP peak				PBS one minute		PBS peak	
		F	p	F	p	F	p	F	p	F	p		
Yersinia arthritis and HLA-B27													
Yersinia arthritis	1	5.313	0.028*	2.185	0.143	0.010	0.885	1.165	0.289	1.864	0.176		
HLA-B27	1	0.425	0.525	0.017	0.937	0.247	0.628	0.016	0.867	0.431	0.522		
Combined	1	6.290	0.015*	2.331	0.131	1.803	0.184	3.483	0.069	0.001	0.935		
Within	41												
Clinical features of yersinia arthritis													
Sequelae absent or present	1	0.552	0.472	0.285	0.605	3.478	0.073	2.648	0.115	2.786	0.107		
Severe or mild arthritis	1	3.240	0.083	2.807	0.105	0.032	0.837	1.519	0.230	8.334	0.009†		
Combined	1	2.150	0.154	5.373	0.030*	4.128	0.052	7.208	0.013*	1.833	0.188		
Within	21												
Ankylosing spondylitis													
Ankylosing spondylitis or healthy	1	2.241	0.144	5.073	0.031*	1.391	0.248	1.059	0.314	0.012	0.878		
Within	25												
Type of spondyloarthropathy in HLA-B27 positive subjects													
Yersinia arthritis, ankylosing spondylitis, or healthy	2	2.622	0.087	2.777	0.076	0.915	0.414	1.307	0.284	0.908	0.416		
Within	30												

Abbreviations: fMLP=N-formyl-methionyl-leucyl-phenylalanine; PBS=phosphate buffered saline

\* $p<0.05$ .

† $p<0.01$ .

Table 4 Whole blood chemiluminescence in subjects with previous yersinia arthritis grouped according to the severity of acute arthritis and the presence of sequelae. Results expressed in millivolts as geometric mean (-1 SEM; +1 SEM)

Subjects	Number of subjects	Luminol enhanced				Lucigenin enhanced	
		fMLP one minute	fMLP peak	PBS one minute	PBS peak	fMLP peak	
Mild arthritis/ sequelae present	3	0.29 (0.21; 0.40)	0.40 (0.31; 0.52)*	0.02 (0.01; 0.03)†	0.05 (0.05; 0.06)‡	1.04 (0.86; 1.27)	
Mild arthritis/ sequelae absent	10	0.42 (0.38; 0.47)	0.66 (0.58; 0.76)	0.07 (0.06; 0.09)	0.15 (0.13; 0.18)	1.44 (1.35; 1.54)	
Severe arthritis/ sequelae present	7	0.50 (0.46; 0.55)	0.81 (0.71; 0.93)	0.04 (0.03; 0.06)	0.14 (0.11; 0.17)	1.64 (1.48; 1.82)	
Severe arthritis/ sequelae absent	5	0.44 (0.37; 0.54)	0.59 (0.50; 0.69)	0.04 (0.02; 0.07)	0.11 (0.09; 0.13)	1.70 (1.55; 1.86)	

Abbreviations: fMLP=N-formyl-methionyl-leucyl-phenylalanine; PBS=phosphate buffered saline

\*Mild arthritis/sequelae present v severe arthritis/sequelae present;  $p<0.05$ .

†Mild arthritis/sequelae present v mild arthritis/sequelae absent;  $p<0.05$ .

‡Mild arthritis/sequelae present v mild arthritis/sequelae absent, mild arthritis/sequelae present v severe arthritis/sequelae present, mild arthritis/sequelae present v severe arthritis/sequelae absent;  $p<0.05$  in all instances.

subgroup with mild arthritis with sequelae present and the subgroup with mild arthritis with sequelae absent was significant ( $p < 0.05$ ; table 4). In the phosphate buffered saline induced peak responses the difference between the subgroup with mild arthritis with sequelae present and each of the other three subgroups was statistically significant at the 5% level (table 4).

#### ANKYLOSING SPONDYLITIS

The subjects with ankylosing spondylitis ( $n=7$ ) had lower responses than the healthy subjects ( $n=20$ ) in all the parameters measured (table 2): the difference was significant in luminol enhanced fMLP induced peak responses ( $p=0.031$ ; table 3, fig 2B).

#### COMPARISON OF THE TYPE OF SPONDYLOARTHROPATHY

When the responses of HLA-B27 positive subjects were analysed among subjects with ankylosing spondylitis ( $n=7$ ), with previous yersinia arthritis ( $n=18$ ), or who were healthy ( $n=10$ ) the difference between the subjects with ankylosing spondylitis and subjects with yersinia arthritis was significant in luminol enhanced fMLP induced one minute ( $p < 0.05$ ) and peak responses ( $p < 0.05$ ) in the post hoc analysis of variance (Newman-Keuls test).

#### Discussion

The results of this study indicate that subjects with previous yersinia arthritis show an increase of whole blood chemiluminescence; this agrees with the previous findings by luminol enhanced chemiluminescence induced by fMLP or opsonised zymosan.<sup>13</sup> The increase of chemiluminescence in subjects with previous yersinia arthritis was not associated with the HLA-B27 antigen, which supports the view that other factors may be involved in neutrophil responses in spondyloarthropathies.<sup>25</sup>

The increase in chemiluminescence was more evident in the subjects with previous severe yersinia arthritis (tables 2 and 3), which agrees with the results obtained with purified neutrophils.<sup>14</sup> The initial activation in luminol enhanced chemiluminescence induced by fMLP,<sup>26, 27</sup> and the lucigenin enhanced chemiluminescence<sup>28</sup> are considered to associate with extracellular oxygen radical production. It is possible that vigorous extracellular oxygen radical production, measured in this study by the initial activation of fMLP induced luminol enhanced chemiluminescence and by the peak of lucigenin enhanced chemiluminescence, contributes to the acute inflammatory damage in patients with yersinia arthritis. Yersinia and salmonella antigens have been demonstrated in the phagocytes of synovial fluid from patients with reactive arthritis.<sup>29, 30</sup> Yersinia may persist in the host<sup>31</sup> and possibly stimulates the phagocytes for prolonged periods of time. It has been reported that etretinate, a known antioxidant,<sup>32</sup> normalised superoxide formation by neutrophils and ameliorated markedly severe reactive enteroarthritis triggered by *Salmonella infantis*.<sup>33</sup>

In contrast, in ankylosing spondylitis we noted that the luminol enhanced chemiluminescence responses to fMLP were decreased (table 2), in accordance with a previous study on opsonised zymosan induced chemiluminescence.<sup>17</sup> Ankylosing spondylitis has been previously associated with a normal neutrophil superoxide formation induced by fMLP,<sup>16</sup> phorbol myristate, or calcium ionophore,<sup>17</sup> or a decreased neutrophil superoxide formation induced by opsonised zymosan<sup>17</sup> determined by a cytochrome *c* reduction assay. In this study superoxide formation in ankylosing spondylitis, determined by the lucigenin enhanced chemiluminescence test, was decreased, but the decrease was not statistically significant. Decreased oxygen radical production is further supported by the finding that in subjects with ankylosing spondylitis the oxygen consumption of neutrophils was decreased when the cells were stimulated by opsonised zymosan but not by phorbol myristate or by calcium ionophore.<sup>17</sup> The aberration in oxygen radical production may be increased by a membrane receptor modification or by a disorder in the transduction of the activating signal as suggested by El Abbouyi *et al.*<sup>17</sup>

The opposite types of aberrations in oxygen radical production may reflect differences in the pathogenesis of yersinia arthritis and ankylosing spondylitis. Yersinia arthritis is characterised by an acute onset after an enteric infection, whereas ankylosing spondylitis develops insidiously without an obvious triggering infection.<sup>34</sup> Interestingly, in this study the subjects with previous yersinia arthritis who had had mild acute arthritis and sequelae during the follow up, with a clinical picture resembling that of ankylosing spondylitis, had low responses in the peak values of fMLP induced luminol enhanced chemiluminescence in a similar manner to the subjects with ankylosing spondylitis.

The pathogenesis of spondyloarthropathies is probably multifactorial<sup>35</sup> and may involve aberrations in humoral and cellular immunity<sup>9-12, 36, 37</sup> and increased permeability of the gut.<sup>38, 39</sup> The results of this study indicate that oxygen radical production from stimulated neutrophils, tested here concurrently, is increased in subjects with previous yersinia arthritis and decreased in subjects with ankylosing spondylitis. The mechanisms underlying these aberrations and whether the aberrations have different roles in the pathogenesis of seronegative spondyloarthropathies remain to be studied.

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