Inhibition of polymorphonuclear leucocyte functions in vivo by Yersinia enterocolitica lipopolysaccharide

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SUMMARY A single intravenous injection of 5 μg of Yersinia enterocolitica lipopolysaccharide (LPS) inhibits rabbit polymorphonuclear leucocyte (PMN) chemotaxis, enzyme secretion, and respiratory burst activation in response to partially purified rabbit C5a and leucotriene B4 (LTB4). Respiratory burst activation is also inhibited in response to platelet activating factor (PAF). In contrast, all these responses to n-formyl-methionyl-leucyl-phenylalanine (FMLP) remain unaltered. This LPS does not modulate PMN activation in vitro or activate the respiratory burst. Thus Y enterocolitica LPS acts in vivo by inhibiting PMN responses to endogenous mediators of inflammation. This inhibition presumably impairs the elimination of pathogens and might, therefore, provide favourable conditions for induction by bacteria of further immunological consequences.

Key word: chemotaxis.

Reactive arthritis, an HLA-B27 associated disease, is usually triggered by an infection caused by a variety of micro-organisms.1 2 Most of the organisms known to induce reactive arthritis are Gram negative and thus share a common chemical component—lipopolysaccharide (LPS)—in their cell wall. LPS is a potent inflammatory agent causing fever, leucopenia/leucocytosis, activation of the complement and kinin cascades, changes in the coagulation cascade, and also release of prostaglandins and leucotrienes.3-9

In vitro, LPS is known to act as an immunomodulator affecting the function of lymphocytes, monocytes, and polymorphonuclear leucocytes (PMNs).10 The in vivo effects of LPS have been extensively studied in experimental animals. Intra-articularly injected LPS induces joint inflammation with PMN migration and arthralgia.11 When administered systemically, LPS has been reported to produce microscopic inflammatory changes in guinea pig joints.12 LPS is also able to induce anterior uveitis in experimental animals.13 After studying LPS induced uveitis, Rosenbaum proposed that LPS could serve as a common determinant between all the organisms inducing reactive arthritis.14 Recent evidence on the character of persisting IgA antibodies during yersinia triggered arthritis supports a possible role for LPS in reactive arthritis as well.15 16 Granfors and coworkers have shown that most of the persisting antibodies are directed against yersinia LPS.17 In inflammation PMNs have a crucial role because of their ability to migrate to the site of inflammation, to release lysosomal enzymes, and to produce harmful free radicals and superoxide anions in response to several endogenous mediators of inflammation.18-21 In the case of bacterial infections these functions are appropriate; but in response to a ‘false’ stimulus, as is probably the case in reactive arthritis, they are destructive to the host’s own tissues.

We have earlier reported the inhibitory effects of Escherichia coli LPS on the functions of rabbit PMNs.22 23 In studying reactive arthritis, however, E coli organisms may be inappropriate investigative tools because they have not, in contrast with many other Gram negative bacteria, been reported to induce arthritis. Moreover, the in vivo effects of E coli LPS may be modulated by natural antibodies.10 24 In light of the recent suggestion of a significant role for LPS in reactive arthritis we have in this present work investigated the LPS of an arthritogenic bacterial organism, to which natural
antibody mechanisms would not be a consideration. Here we have tested the effects of *Y enterocolitica* LPS on PMN function in vivo and in vitro.

**Materials and methods**

**ANIMALS**

New Zealand white rabbits, both sexes, weighing approximately 2 kg, were used. They were housed at the Center for Experimental Animals at Turku University and fed standard laboratory food.

**LPS**

Highly purified LPS from *Y enterocolitica* (List Biological Laboratories, Campbell, Ca, USA) was dissolved in sterile, pyrogen free saline and stored in aliquots at −20°C. A sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)\textsuperscript{15} showed clearly bands representing the lipid A and core as well as numerous repeating bands representing the polysaccharide chain, indicating the LPS to be of smooth type.

**ADMINISTRATION OF LPS**

Each rabbit was injected with 5 µg LPS into the marginal ear vein. Blood for the separation of PMNs, as described below, was collected 24 hours later.

**SEPARATION OF PMNs**

PMNs were separated from rabbit arterial blood anticoagulated with acid citrate dextrose. The blood was mixed with 6% dextran T-500 at a ratio of 4:1, and the erythrocytes were left to sediment at 1 g for 25 minutes at room temperature. The supernatant containing the PMNs was layered over 56% Percoll (Pharmacia, Uppsala, Sweden) and centrifuged for 15 minutes at 200 g. The pellet was suspended in 0-83% ammonium chloride solution for seven minutes to lyse the remaining erythrocytes. PMNs were washed twice with cold phosphate buffered saline (PBS). Finally, in preparation for use in the chemiluminescence assay PMNs were resuspended in Hanks's balanced salt solution (HBSS) supplemented with 0-25% bovine serum albumin (BSA, Armour Pharmaceutical Co, Eastbourne, England) at a concentration of 2 × 10^6 cells/ml. For chemotaxis experiments PMNs were resuspended in HBSS+2% BSA at 2.5×10^6 cells/ml, and for enzyme release assays in HBSS+0.25% BSA at 6×10^6 cells/ml. This separation method yielded 95% pure PMNs; and trypan blue exclusion test showed greater than 96% viability.

**SOLUBLE STIMULI**

The synthetic chemotactic peptide FMLP (Sigma, St Louis, Mo, USA) was first dissolved in dimethyl-sulfoxide, diluted with PBS to 1×10^{-3} mol/l and stored at −20°C. The final concentration for daily use was diluted from this stock solution. The concentrations used were: 10^{-5} mol/l for chemotaxis and chemiluminescence and 2×10^{-9} mol/l for enzyme release assays.

Rabbit C5α was partially purified from yeast activated rabbit serum, as previously described,\textsuperscript{25} using CM-Sepharose and Sephadex G-100 columns (Pharmacia). The partially purified C5α had optimal chemotactic activity at a protein concentration of 10 µg/ml. The concentrations of C5α used were 0-1% for chemotaxis, 0.2% for enzyme release, and 0-7% or 1-4% for chemiluminescence assays.

Platelet activating factor (PAF; Sigma) was stored in chloroform, 2 mg/ml, at −20°C. For daily experiments chloroform was evaporated under nitrogen and PAF was dissolved in ethanol. This was diluted to the desired concentration with HBSS. In chemiluminescence assays 15 ng/ml of PAF was used.

Leucotriene (LTB₄) was stored in ethanol at −70°C. For each experiment, a fresh solution was prepared in HBSS. The concentrations of LTB₄ used were 5 ng/ml for chemotaxis, 20 ng/ml for enzyme release, and 15 ng/ml for chemiluminescence assays.

**MEASUREMENT OF PMN CHEMOTAXIS**

PMN migration was assayed using a modified Boyden chamber.\textsuperscript{26} The PMN suspension was placed in the upper compartment and the chemoattractants or buffer in the lower compartment separated by a nitrocellulose filter, pore diameter 3 µm (Sartorius, Göttingen, FRG). The chambers were incubated at 37°C for 35 minutes. The filters were then stained with Mayer's haematoxylin (Sigma), dehydrated with a rising alcohol concentration, and clarified with xylene. All experiments were performed in duplicate and five fields per filter were examined by the leading front method described by Zigmond and Hirsch.\textsuperscript{27} Unstimulated migration is the migration of PMNs towards the buffer alone, and chemotaxis is the difference between the unstimulated migration and the migration stimulated by a chemoattractant.

**RELEASE OF LYSOSOMAL ENZYMES**

Release of the azurophilic granule enzyme β-glucuronidase was measured with phenolphthalein glucuronic acid (Sigma) as substrate as previously described.\textsuperscript{22,28} Briefly, PMNs were first incubated with cytochalasin B (1 µg/ml; Sigma) at 37°C for 7-5 minutes. The soluble stimuli were added and the incubation continued at 37°C for 15 minutes. The
supernatants were collected and the enzyme activity in them measured after an 18 hour incubation with the substrate. Total enzyme activity was determined from lysates of PMNs exposed to 0-1% (v/v) Triton X-100. The non-specific release of enzyme was measured from supernatants of unstimulated PMNs. All the enzyme assays were performed in duplicate. The enzyme activities in the samples are expressed as a percentage of the total enzyme activity after subtracting the non-specific enzyme release. The spontaneous enzyme release during incubation served as a control of cell permeability and varied between 0% and 2.8%.

Release of myeloperoxidase was measured from cell free supernatants similar to those used in the β-glucuronidase experiments. The supernatants were allowed to react with 3.9 mM o-dianisidine (dimethoxybenzene; Sigma) in the presence of H₂O₂. The reaction was halted by addition of 1% NaN₃. The calculations were performed in a similar way to those in the β-glucuronidase experiments.

CHEMILUMINESCENCE ASSAY

The chemiluminescence assays were performed using a Luminometer 1250 (Wallac, Turku, Finland). The cell suspension (0.5 ml) was first incubated at 37°C. After 10 minutes 100 μl of 1 μM Luminol (Sigma) in HBSS was added and the mixture was further incubated for an additional 30 minutes. Soluble stimuli were added using the automated dispenser of the luminometer. The luminometer was programmed to detect responses at five second intervals for two minutes and to detect maximal responses. All chemiluminescence measurements were performed in duplicate.

In all experiments the cells were treated with cytochalasin B; 1 μg/ml was added before the addition of Luminol.

STATISTICS

Student’s t test was used to compare the chemiluminescence, chemotactic and secretory functions of PMNs, from normal and LPS treated rabbits.

Results

EFFECT OF LPS GIVEN IN VIVO ON PMN FUNCTIONS

Chemotaxis

When injected into rabbits 24 hours before the PMN harvest, 5 μg of Y enterocolitica LPS induced selective inhibition of PMN responses to endogenous mediators of inflammation. Chemotaxis towards FMLP was not altered in the LPS treated animals, whereas chemotaxis towards C5a and LTB₄ was significantly impaired (Fig. 1). Unstimulated migration of PMNs was increased in the LPS treated rabbits (80.5 (11.2) normal v 96.7 (9.9) LPS treated rabbits, mean (SD) in μm, p<0.001).

Enzyme release

Results for enzyme release closely paralleled those observed in chemotaxis. The release of β-glucuronidase and myeloperoxidase induced by C5a and LTB₄ was inhibited, whereas the responses to FMLP were not affected (Figs 2 and 3).

Chemiluminescence

The luminol induced chemiluminescence from PMNs in the presence of cytochalasin B was measured in response to one synthetic and three endogenous chemotactic stimuli. In the presence of cytochalasin B, which potentiates the chemiluminescence response, only the response to FMLP remained unaltered, and the responses to C5a, LTB₄, and PAF were inhibited in LPS treated animals (Table 1).

EFFECT OF IN VITRO LPS TREATMENT ON PMN CHEMILUMINESCENCE

To study the direct effects of Y enterocolitica LPS on PMN functions we first tested the ability of this LPS to induce chemiluminescence from PMNs. LPS concentrations up to 1 μg/ml were used, and the responses were followed for 20 minutes. Addition of LPS to the suspension of rabbit PMNs did not
Inhibition of polymorphonuclear leucocyte functions

Induce metabolic activation of the cells as measured by chemiluminescence (data not shown).

When PMNs were first incubated with either 0.01 μg/ml or 1 μg/ml of LPS simultaneously with Luminol in the presence of cytochalasin B and then

![Graph](image1)

**Fig. 2** Effect of in vivo administered lipopolysaccharide (LPS) on the release of β-glucuronidase from polymorphonuclear leucocytes (PMNs) in response to FMLP (2×10⁻⁹ M), partially purified rabbit C5a (0-2%), and leucotriene B₄ (LTB₄) (20 ng/ml). The results represent means (SEM) of the results for ten normal and eight LPS treated rabbits. □= Normal rabbit PMNs; △= PMNs from LPS treated rabbits.

![Graph](image2)

**Fig. 3** Effect of in vivo administered lipopolysaccharide (LPS) on the release of myeloperoxidase from polymorphonuclear leucocytes (PMNs). The concentrations of the stimuli are equal to those in Fig. 2. The results represent means (SEM) of the results for five normal and five LPS treated rabbits. □= Normal rabbit PMNs; △= PMNs from LPS treated rabbits.

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**Table 1** Effect of LPS† administered in vivo on PMN‡ chemiluminescence

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>PMNs from normal rabbit</th>
<th>PMNs from LPS treated rabbit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mV</td>
<td>n</td>
</tr>
<tr>
<td>FMLP† (10⁻⁹ M)</td>
<td>4282 (2265)‡</td>
<td>12</td>
</tr>
<tr>
<td>C5a (1-4%)</td>
<td>1848 (1384)</td>
<td>12</td>
</tr>
<tr>
<td>LTB₄† (15 ng/ml)</td>
<td>169 (126)</td>
<td>10</td>
</tr>
<tr>
<td>PAF† (15 ng/ml)</td>
<td>271 (165)</td>
<td>8</td>
</tr>
</tbody>
</table>

*p<0.05; **p<0.01.
†LPS=lipopolysaccharide; PMN=polymorphonuclear leucocyte; FMLP=n-formyl-methionyl-leucyl-phenylalamine; LTB₄=leucotriene; PAF=platelet activating factor.
‡The results are expressed as mean (SD).

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**Table 2** Effect of preincubation with LPS on PMN chemiluminescence

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Control PMNs</th>
<th>PMNs preincubated with LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 μg/ml</td>
<td>10 ng/ml</td>
</tr>
<tr>
<td>FMLP (10⁻⁹ M)</td>
<td>3782 (1313)*</td>
<td>4391 (951)</td>
</tr>
<tr>
<td>C5a (0.7%)</td>
<td>612 (353)</td>
<td>569 (220)</td>
</tr>
<tr>
<td>LTB₄ (15 ng/ml)</td>
<td>143 (37)</td>
<td>164 (90)</td>
</tr>
<tr>
<td>PAF (15 ng/ml)</td>
<td>203 (118)</td>
<td>195 (158)</td>
</tr>
</tbody>
</table>

*Values are expressed as means (SD) from at least three separate experiments.
No significant differences were detected between the responses of control and LPS treated PMNs.
For abbreviations see Table 1.

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stimulated with FMLP, C5a, LTB₄, or PAF, no modulation of their responses was detected (Table 2).

**Discussion**

In the present study we found that *Y enterocolitica* LPS, when administered in vivo, affects the responses of rabbit PMNs to endogenous PMN stimuli. A single intravenous injection of LPS induced clear inhibition of chemotactic and secretory responses of PMNs to the endogenous stimuli C5a and LTB₄, and chemiluminescence responses to C5a, LTB₄, and PAF. In contrast, responses to the synthetic chemotactic peptide FMLP in all functions tested were unaltered. *Y enterocolitica* LPS did not directly induce chemiluminescence responses from PMNs nor did it affect PMN chemiluminescence responses to soluble chemotactic stimuli. The present results basically parallel those obtained earlier with *E coli* LPS. There are differences, however,
that deserve attention. *E. coli* belongs to the normal intestinal flora and is not known to be arthritogenic. Because of the continuous contact of the body with *E. coli* there are natural antibodies that could modify the response to this LPS.\(^{10,24}\) Although inhibition of PMN function was found also with *E. coli* LPS,\(^ {23}\) *Y. enterocolitica* LPS was more potent in the induction of PMN inhibition. Five micrograms of *Y. enterocolitica* LPS induced an inhibition of PMN functions comparable with that induced by 100 µg of *E. coli* LPS in the previous study.\(^ {23}\) Also, the clinical illness in rabbits differed in regard to the LPS. Rabbits receiving *E. coli* LPS developed diarrhoea and were lethargic at the time of the collection of blood, whereas rabbits receiving *Y. enterocolitica* LPS had no clinical symptoms. The stimulus specific inhibition of PMN functions also differed in regard to the lipid stimuli LTB\(_4\) and PAF. *E. coli* LPS induced inhibition of chemotaxis only, whereas in the present study all the functions tested were clearly inhibited. This strongly suggests somewhat different mechanisms of action for these two LPSs.

The normal responses to FMLP, a stimulus not existing in the body, serve as a control of the condition of PMNs and strongly suggest that PMN functions are endogenously regulated. In vivo, LPS activates several biological mechanisms that also interfere with PMN function. Activation of the complement cascade leads to the production of several PMN stimuli, C5a being the most potent among them. In addition to its chemotactic and secretory activities, C5a has been suggested as the cause of the profound neutropenia following LPS injection.\(^ {30}\) Complement activation probably leads to further activation of the inflammatory process and the release of other inflammatory mediators—e.g. LTB\(_4\) and PAF, both known as strong PMN stimuli. Thus stimulus specific desensitisation of PMNs as a consequence of the production of these factors after LPS administration is one possible explanation for the defective PMN function we detected.

Macrophages have an important role in the regulation of the inflammatory reaction. When stimulated with LPS they actively secrete numerous immunologically potent factors.\(^ {32}\) Among these, interleukin 1 has been considered to affect PMN function, and it has also been characterised as a common endogenous mediator of inflammation.\(^ {21}\) Work is now in progress to characterise the possible modulation of PMN functions by interleukin 1. The in vitro effects of LPS on PMN functions have been extensively studied by several investigators. The results vary greatly depending on experimental conditions. The presence of serum or complement, adherence of PMNs, and good solubility of the LPS preparation favour the inhibition of PMN functions by LPS. As expected, absence of serum factors and inhibition of adherence lead to opposite findings.\(^ {33}\) In many in vitro studies very high concentrations of LPS have been used. For in vitro experiments we selected LPS concentrations based on the theoretical concentration reached in rabbit serum in the in vivo studies. In this study we could detect no effect on PMN function by direct exposure of the cells to LPS. This finding strongly indicates that the profound in vivo effects we observed are caused by the metabolic events following LPS administration, rather than by a direct, isolated effect of LPS itself.

During the arthritic disease an increased migratory function of PMNs in HLA-B27 positive subjects has been described.\(^ {34,35}\) In this study only the unstimulated motility was increased following LPS challenge; stimulated motility was selectively inhibited. This finding of PMN inhibition, however, may provide some insight into the initial pathogenesis of reactive arthritis. It can be postulated that during initial infection PMN functions are inhibited by LPS and the proper elimination of the pathogenic organism is disturbed. In genetically predisposed subjects the organism would then persist—for instance, within the intestinal lymphoid tissue—providing the source of LPS to maintain the high IgA response towards LPS.

Our present findings indicate that *Y. enterocolitica* LPS in vivo effectively induces a stimulus specific inhibition of PMN chemotaxis, enzyme release, and chemiluminescence. This inhibition is probably mediated by the activation of biochemical events in the host as the yersinia LPS itself in concentrations even higher than those theoretically reached during the in vivo challenge did not modulate PMN activation.

The authors thank Tom Stähberg, MSc, for preparation of the SDS-PAGE of the lipopolysaccharide and Dr Joshua Rokach (Merck Frosst, Quebec, Canada) for the gift of LTB\(_4\). This study was supported by grants from the Emil Aaltonen Foundation, Sigrid Jusélius Foundation, and the US Public Health Service (grant No 5 NO1 RO1 AM 33311 awarded by National Institute of Arthritis, Diabetes, and Kidney Diseases).

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doi: 10.1136/ard.48.1.42

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