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# Modulation of leukotriene generation by invasive bacteria

M. GRÖNE, J. SCHEFFER & W. KÖNIG *Lehrstuhl für Medizinische Mikrobiologie und Immunologie, Arbeitsgruppe für Infektabwehrmechanismen, Ruhr-Universität Bochum, Germany*

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## SUMMARY

The effect of invasive bacteria on the release of proinflammatory mediators (oxygen radicals, leukotriene release) from human polymorphonuclear neutrophils was studied. Bacterial stimuli were used including genetically cloned invasive *Yersinia enterocolitica* strains 108-P (bearing the phagocytosis-resistance plasmid) and 108-C (plasmidless variant), *Listeria monocytogenes* [SLCC 5779 (inv$^-$) and NCTC 7973 (inv$^+$)] as well as an *Escherichia coli* K 12 strain (pRI 203) in which the inv gene of *Y. pseudotuberculosis* was cloned. When human polymorphonuclear granulocytes were studied as target cells the inv$^+$ as well as the inv$^-$ strains were phagocytosed to a comparable amount with the exception of the *L. monocytogenes* strain (inv$^+$). Among the invasive strains *E. coli* HB 101 (pRI 203) was the most active to trigger polymorphonuclear leucocytes (PMN) for oxygen radical production. Preincubation of the cells with bacteria and subsequent stimulation with the Ca ionophore A23187 or opsonized zymosan suppressed the chemiluminescence response to a different degree. The various bacterial strains did not induce leukotriene release from endogenous arachidonic acid. Subsequent stimulation of the infected cells with Ca ionophore or opsonized zymosan led to an altered pattern of the combined amounts of leukotriene $B_4$ ($LTB_4$), 20-OH- and 20-COOH-$LTB_4$ as well as the ratio of $LTB_4$ versus 20-OH and 20-COOH-$LTB_4$. Infection of the cells also reduced strain dependently the number of $LTB_4$-receptor sites. Our data suggest that bacterial uptake modulates the inflammatory response of granulocytes (e.g. chemiluminescence response, leukotriene generation).

## INTRODUCTION

Adherence and uptake are prerequisites for the replication of intracellularly persisting bacteria. In this regard *Yersinia pseudotuberculosis* invades into the host cells by specific receptor molecules on the cell surface.[1,2] The process of invasion is regulated by the expression of different proteins which allow a specific binding of the bacteria to the target cells and factors which inhibit the phagocytosis of bacteria by phagocytes.[2–6]

We studied three isogenic pairs of bacterial strains which differ in their properties to adhere and penetrate into the cell: *Y. enterocolitica*,[7–12] *Listeria monocytogenes* and an *Escherichia coli* K 12 strain in which an invasion gene (inv gene) from *Y. pseudotuberculosis* was cloned.[1] The genera *Yersinia* all include species capable of causing an invasive diarrhoea in humans and often are associated with lymphadenitis.[13] The molecular biological prerequisites of invasiveness have been studied for *Y. enterocolitica*[3,13,14] and *Y. pseudotuberculosis*.[1] *Yersinia enterocolitica* species have two chromosomal loci, inv and ail.[15,16] The inv locus allows a high level of invasion and the ail locus (attachment invasion locus) confers a target specificity on the bacterial host different from that conferred by the inv genes. An important prerequisite for the infection with *Y. enterocolitica* is a specific plasmid of 42,000–44,000 MW.[8,14] The effect of the inv gene product is in part counteracted by plasmid-associated outer membrane proteins (YOP—*Yersinia* outer membrane proteins).[3,6,14,17–19] Plasmid-bearing *Y. enterocolitica* strains exhibit a decreased phagocytosis and inhibit oxygen radical production of polymorphonuclear granulocytes.[5] The genetically modified *E. coli* K 12 strain HPB 101 (pRI 203) contains the inv gene from *Y. pseudotuberculosis* which, in contrast to the plasmidless isogenic *E. coli* HB 101, permits invasiveness into HEp-2 cells.[2]

*Listeria monocytogenes* can cause severe infections in immunocompromised humans. Bacterial entry into cells is mediated by the expression of a 60,000 MW (p60) protein on the bacterial surface.[4,20] This protein is secreted in large amounts by all inv$^+$ strains. Inv$^+$ *L. monocytogenes* strains are therefore capable of penetrating HEp-2 cells in contrast to inv$^-$ strains which are only phagocytosed by granulocytes and macrophages. While major emphasis with regard to intracellularly persisting microorganisms was focused on the invasion into epithelial cells, few data exist on whether and to what extent they affect the release of proinflammatory mediators (oxygen radicals,[5] leukotrienes[21–23] and leukotriene $B_4$ receptor expression.[24,25] Human polymorphonuclear granulocytes synthesize leukotriene $B_4$ ($LTB_4$) which has potent chemotactic and immunomodulatory functions.[26–30] In addition, these cells inactivate leukotriene $B_4$ via $\omega$-

Correspondence: Professor W. König, Medizinische Mikrobiologie und Immunologie, AG Infektabwehr, Ruhr Universität Bochum, Universitätsstraße 150, 4630 Bochum, Germany.

oxidation into 20-OH- and 20-COOH-$LTB_4$. Stereospecific binding sites for $LTB_4$ were identified on human polymorphonuclear leucocytes (PMN) with high and low affinity which mediate chemotaxis as well as enzyme release and aggregation.[24,25,31,32]

Detailed information about the induction of proinflammatory mediators by extracellular bacteria has been recently obtained for *E. coli* (haemolysin±, adhesin±),[33-35] *P. aeruginosa*,[36] *Serratia* spp. and *Aeromonas hydrophila*.[35]

The purpose of this study was to elucidate the role of defined pathogenicity factors of intracellularly persisting bacteria (invasin of *Y. enterocolitica* and *Y. pseudotuberculosis*, *Yersinia* outer membrane proteins, p60 protein of *L. monocytogenes*) on the ability of neutrophils to generate oxygen radicals, leukotrienes and to express leukotriene $B_4$ receptors.

Thus, studies on the formation of newly generated mediators may allow a more precise analysis of the effect of intracellularly persisting bacteria on proinflammatory mediator release.

## MATERIALS AND METHODS

*Materials*

Ficoll 400 was obtained from Pharmacia (Uppsala, Sweden); Dextran-Macrodex (6%, w/v) was from Knoll (Ludwigshafen, Germany); sodium-metrizoate solution (75%, w/v) was purchased from Nyegaard (Oslo, Norway). Zymosan A, Ca ionophore A23187, cytochalasin B and heparin were obtained from Sigma (Munich, Germany); [$^3$H]$LTB_4$ (specific activity 1·2 TBq/mmol) was supplied by New England Nuclear (Dreieich, Germany). Brain–heart infusion broth was used as culture medium (Oxoid, Germany GmbH, Wesel, Germany). Acetonitrile and methanol [high-performance liquid chromatography (HPLC) grade] were purchased from Baker Chemicals (Gross-Gerau, Germany) and Riedel-de-Haën (Seelze, Germany). Synthetic leukotrienes $C_4$, $B_4$, 20-OH-$LTB_4$ and 20-COOH-$LTB_4$ were a generous gift from Merck Frosst (Pointe Claire, Québec, Canada). Gentamicin was from Sigma. Phosphate-buffered saline [(PBS), 120 mM NaCl, 10 mM $Na_2HPO_4$, 3 mM $KH_2PO_4$ and 3 mM KCl, pH 7·4] was used for all experiments. Tris-lysozyme buffer (TLB) contained 0·1 M Tris-HCl, 120 mM NaCl, 4 mM KCl, 40 mM EDTA and 100 μg/ml lysozyme, pH at 7·4. Fine chemicals were supplied from Merck (Darmstadt, Germany).

*Preparation of cells*

Human PMN were isolated from 200 ml of heparinized blood (15 U/ml) of healthy donors separated on a Ficoll-metrizoate gradient followed by dextran sedimentation as described previously.[37] This method led to more than 95% pure PMN. The cells were diluted to a final concentration of $2 \times 10^7$ cells/ml in PBS.

*Bacterial strains*

The following bacterial strains were analysed (Table 1): *E. coli* HB 101 (inv$^-$), *E. coli* HB 101 (pRI 203) (inv$^+$), *Y. enterocolitica* 108-C (plasmidless) and *Y. enterocolitica* 108-P (plasmid harbouring).

The *E. coli* HB 101 represents an *E. coli* K 12 strain, non-invasive and without MS fimbriae. The invasive *E. coli* HB 101 (pRI 203) (a gift from Prof. Dr S. Falkow, Stanford University, CA) is an *E. coli* HB 101 strain, containing the inv gene from *Y. pseudotuberculosis*.[2] The *Y. enterocolitica* strain 108-C, serotype 0:3, is plasmidless and cell adherent; the plasmid-bearing strain 108-P (containing a 42,000–44,000 MW plasmid) is cell adherent, however, resistant to phagocytosis for HEp-2 cells. Both strains were obtained from Prof. Dr J. Heesemann (Institute of Medical Microbiology, Würzburg, Germany).[3,13,14]

**Table 1.** Summary of strains under study

| Strain | Genetics | inv$^+$/$^-$ | Hly/List. |
|---|---|---|---|
| *E. coli* HB 101 | *E. coli* K 12, without MSH fimbriae | inv$^-$ | – |
| *E. coli* HB 101 (pRI 203) | *E. coli* K 12 HB 101 with an invasion gene from *Y. pseudotuberculosis* | inv$^+$ | – |
| *Y. enterocolitica* 108-C | Serotype 0:3, plasmidless, cell adherent, invasion capacity is chromosomal encoded | inv$^+$ | – |
| *Y. enterocolitica* 108-P | Isogenic to 108-C, but with a 42,000–44,000 MW plasmid, phagocytosis resistant | inv$^+$ | – |
| *L. monocytogenes* NCT 7973 | Invasion capacity is chromosomal encoded | p60$^+$ | (+) |
| *L. monocytogenes* SLCC 5779 | Rough variant | p60$^-$ | (+) |

The *L. monocytogenes* strains NCT 7973 (hly$^+$, inv$^+$) and SLCC 5779 (rough variant, hly$^+$, inv$^-$) were obtained from Prof. Dr W. Goebel (Institute of Genetics and Microbiology, University of Würzburg, Germany). Their characteristics are as described previously.[4,38]

*Bacterial growth*

*Escherichia coli* strain: BHI (10 ml) was inoculated with 100 μl of an overnight culture; bacterial growth proceeded for 4 hr at 37° on a shaker (4000 *g*).

*Yersinia enterocolitica*. The strains were cultured overnight at 30°. The overnight cultures were diluted 1:20 and grown for an additional 4 hr at 37° on a shaker (4000 *g*).

*Listeria monocytogenes*. 100 μl of an overnight culture was diluted in 10 ml BHI-medium and grown for 4 hr on a shaker at 37°. After culture the bacteria were centrifuged (4000 *g*/20 min), separated from the culture supernatant and washed in PBS buffer. The bacterial concentration was microscopically determined. For the actual experiments washed bacteria at concentrations of $2{\cdot}5 \times 10^8$ cells were studied.

*Analysis of adherence and uptake of bacteria*

Experiments were performed with neutrophil granulocytes; the uptake of radioactively labelled bacteria was studied.[39] Bacteria were grown in the presence of 55·5 kBq [$^3$H]thymidine for 2·5 hr and washed twice before use in PBS buffer (radioactivity incorporated ranged up to 40–60%). Human neutrophils ($1 \times 10^7$ cells/500 μl) were incubated for 5 and 30 min with the labelled bacteria. In phagocytosis inhibition experiments human neutrophils ($1 \times 10^7$/500 μl) were preincubated with

cytochalasin B (10 μg/ml) for 30 min/37° and then stimulated with the indicated bacterial strains.

After centrifugation the differences between total radioactivity added and the amount of radioactivity in the supernatant which reflects the combined amounts of adherent and ingested bacteria was determined by liquid scintillation (Rack beta 1209, LKB, Turku, Finland). The number of adherent bacteria was assessed after incubation of neutrophils at 4° in 500 μl TLB (30 min) and determination of the radioactivity within the supernatant. The percentage of ingested bacteria was evaluated by analysing the radioactivity from lysed cells.

*Killing of micro-organisms by phagocytes*

Human PMN ($1 \times 10^7$) were incubated with the different bacterial strains ($2{\cdot}5 \times 10^8$) over various times (5, 15, 30 min). During this time the bacteria were allowed to bind and to become phagocytosed by the PMN. Adherent bacteria were killed by gentamicin treatment (100 μg/ml) for 30 min. The cells were then washed twice with PBS and the intracellular bacteria were released after the addition of 2 ml 1% Triton X-100 in aqua dest. The diluted suspensions ($10^{-5}/10^{-3}$) were plated on BHI agar plates and the colony-forming units (CFU) were calculated after growth over 24 hr at 37°.

*Chemiluminescence*

Oxygen radical production was monitored by the luminol enhanced chemiluminescence as described elsewhere.[35] PMN ($1 \times 10^6$/270 μl) in the presence of luminol (0·25 mM) were incubated with the bacterial cell suspension ($2{\cdot}5 \times 10^8$ bacteria) and the production of oxygen radicals was monitored for 30 min. Chemiluminescence was analysed in a Lumacounter M 2080 (Lumac, Schaesberg, The Netherlands). In further experiments infected cells were subsequently stimulated with the Ca ionophore or opsonized zymosan and the chemiluminescence response was studied over various times.

*Stimulation of PMN*

PMN ($1 \times 10^7$/500 μl) were incubated in the presence of $Ca^{2+}$ (1 mM) and $Mg^{2+}$ (0·5 mM) with bacteria ($2{\cdot}5 \times 10^8$/50 μl) for 5 and 30 min at 37°. After preincubation with bacteria Ca ionophore A23187 (6·3 μM) was added to the cell suspension and the incubation proceeded for an additional 15 min. For stimulation with opsonized zymosan the PMN were preincubated with bacteria in the presence of $Ca^{2+}$ (1·25 mM) and $Mg^{2+}$ (3·12 mM) and freshly prepared opsonized zymosan was added (2 mg/final concentration). The reaction was terminated after 15 min by addition of 2 ml of methanol/acetonitrile (50/50, v/v).

*Analysis of leukotrienes*

Samples were processed and analysed as described previously.[31,32] In brief, the deproteinized samples were centrifuged (1200 *g*, 5 min), the supernatant evaporated and the residue resuspended in 600 μl methanol/water (30/70 v/v) and analysed by HPLC. The leukotrienes were identified and quantified by area integration of the absorption peaks at 280 nm and external standardization with synthetic standards.

*$LTB_4$ binding assay*

The quantitation of specific $LTB_4$-binding sites was performed as described previously.[24,25] PMN ($4 \times 10^6$) were incubated with bacteria for 5 and 30 min at 37°. The cells were washed twice with PBS and adjusted to $2 \times 10^7$/ml. The binding assays were performed in 96-well filtration plates with 5 μm pore size polyvinylidene fluoride membranes (Millipore, Eschborn, Germany). Each well contained 0·9 kBq [$^3$H]$LTB_4$ and 37·5 μg bovine serum albumin (BSA). Specific binding for $2 \times 10^6$ cells/well was determined in the presence of 200 nM unlabelled $LTB_4$. After 45 min of incubation at 4° the reaction was terminated by rapid filtration using a Millititre vacuum holder. The filters were transferred into scintillation vials; 0·5 ml methanol and 4·5 ml Rotiszint (Roth, Karlsruhe, Germany) were added and the radioactivity was measured by liquid scintillation counting (Rack beta 1209; LKB, Turku, Finland). Specific binding was expressed as total binding minus non-specific binding. All experiments were carried out in triplicate.

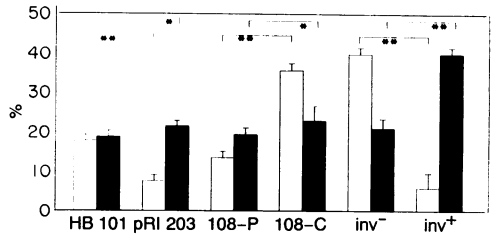


**Figure 1.** Adherence (□) and uptake (■) of the various bacterial strains into professional phagocytes. Human PMN ($1 \times 10^7$) were incubated with bacteria ($2{\cdot}5 \times 10^8$) at 37°. Data represent mean values of three experiments ($n = 3$); Values are expressed in per cent of total activity. * $P < 0{\cdot}05$; ** $P < 0{\cdot}01$.

*Enzyme release*

As a marker of potential cell damage the release of cytoplasmic lactate dehydrogenase[31] was studied. Under the conditions used the release of lactate dehydrogenase ranged up to 4·7–5·1% of the total cytoplasmic content. The release of β-glucuronidase (cytosolic and granular enzyme) was studied. β-glucuronidase was determined as described previously.[31] The release of β-glucuronidase ranged up to 8·9% of the total cellular content.

*Statistics*

All data were calculated as means ± standard deviation; the significance was evaluated by Student's *t*-test for independent means; $P < 0{\cdot}05$ was considered as significant.

## RESULTS

### Adherence and uptake of inv$^+$ and inv$^-$ bacteria into human granulocytes

Adherence and uptake of the bacterial strains were studied with human granulocytes as target cells. Since the granulocytes, unlike non-phagocyting cells, significantly killed the bacteria after phagocytosis within a time range of 30 min the CFU do not reflect the correct values for the combined amounts of adherence and phagocytosis. Bacteria ($2{\cdot}5 \times 10^8$) were therefore grown in the presence of 55·5 kBq [$^3$H]thymidine for 2·5 hr. Human neutrophils ($1 \times 10^7$) were incubated over various times (5, 15 and 30 min) with radiolabelled bacteria. The radioactivity incorporated ranged up to 40–60%. The percentage of adherence and of ingested bacteria was evaluated as described (see Materials and Methods). The adherence as is shown (Fig. 1) for

**Table 2.** Human PMN ($1 \times 10^7$) were incubated with the different bacterial strains ($2{\cdot}5 \times 10^8$) over various times (5, 15, 30 min). Adherent bacteria were killed by gentamicin treatment (100 μg/ml) for 90 min. After lysis of the cells with Triton X-100 the suspensions were plated on BHI agar plates and the CFU were calculated after growth over 24 hr.

| Gentamicin (final conc. μg/ml) | % growth (HB 101) |
|---|---|
| 500 | 0 |
| 250 | 0 |
| 125 | 0 |
| 100 | 0 |
| 62·5 | 0 |
| 31·2 | 0 |
| 15·6 | 0 |
| 7·8 | 22 |

| Strain | Gentamicin conc. | Incubation time (min) | % growth |
|---|---|---|---|
| HB101/pRI203 | 100 μg/ml | 5 | 15/0 |
| | | 10 | 0/0 |
| | | 15 | 0/0 |
| | | 30 | 0/0 |
| | | 60 | 0/0 |
| | | 90 | 0/0 |
| | | 120 | 0/0 |

the 15 min value ranged from 8% for *E. coli* HB 101 (pRI 203) to 40% for the non-invasive *L. monocytogenes* strain. The adherence of *Y. enterocolitica* 108-C amounted to 35% and of *Y. enterocolitica* 108-P to 12%. When phagocytosis was studied invasive and non-invasive bacteria were ingested in comparable amounts, phagocytosis ranged for all strains from 19 to 24%, with the exception of *L. monocytogenes* (inv$^+$). The uptake of *L. monocytogenes* (inv$^+$) was significantly increased up to 40%. These results emphasize that the interaction of invasive and non-invasive bacterial strains with the target cells is also dependent on cell-specific characteristics. Addition of cytochalasin B to granulocytes inhibited completely the phagocytosis at time-points of 5 min. Baseline values of ≈4·5% were obtained (data not shown).

### Killing of micro-organisms by phagocytes

Human PMN ($1 \times 10^7$) were incubated with the different bacterial strains ($2{\cdot}5 \times 10^8$) for 5, 15 and 30 min. Upon gentamicin treatment the CFU were calculated after 24 hr growth on BHI agar plates.

It is apparent (Table 2) that after 30 min of infection about 0–10% of bacteria remained viable.

### Modulation of the chemiluminescence response

In subsequent experiments the effect of the various bacterial strains on the chemiluminescence response of human granulocytes was studied. For this purpose human neutrophil granulocytes were incubated with the various bacterial strains (see Table 1) and the production of oxygen radicals was monitored up to 30 min. Subsequently, after 15 min Ca ionophore (6·3 μM) or

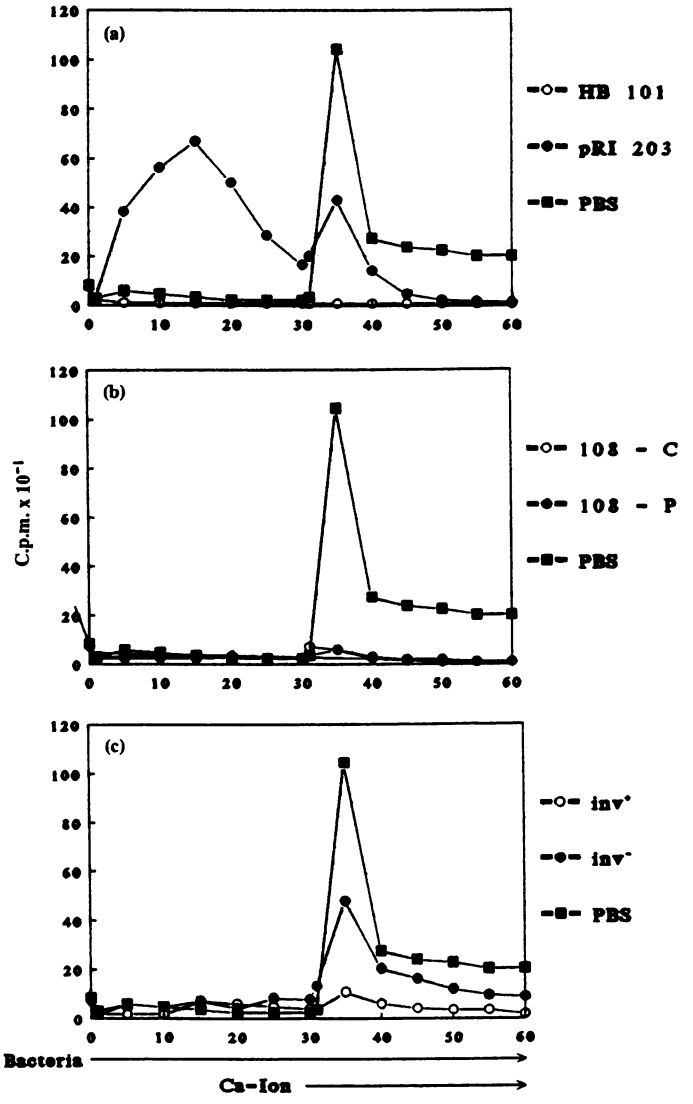


**Figure 2.** Induction of chemiluminescence by invasive and non-invasive bacterial strains—effect of subsequent Ca ionophore stimulation. Human PMN ($1 \times 10^6$) were stimulated with bacteria ($2{\cdot}5 \times 10^8$). (a) *E. coli*-, (b) *Y. enterocolitica*- and (c) *L. monocytogenes*-pretreated cells. The chemiluminescence response was monitored at different times (0–30 min), Ca ionophore (6·3 μM) was added and the chemiluminescence response was monitored (31–60 min). The results are expressed as c.p.m. One representative experiment out of $n = 6$ is shown.

opsonized zymosan (2 mg) were added and the production of oxygen radicals was studied for a further 30 min. Figure 2 represents a typical set of experiments out of six individual donors. In the absence of any bacterial stimulus the chemiluminescence signal did not exceed the amount of $4 \times 10^3$ c.p.m./$1 \times 10^6$ cells (i.e. control: cells and buffer). Incubation of the PMN with *E. coli* HB 101 (pRI 203) induced an increased chemiluminescence signal up to $60 \times 10^3$ c.p.m., *E. coli* HB 101 did not trigger the cells. The subsequent stimulation of these cells with the Ca ionophore (6·3 μM) led to the following results: in the presence of buffer the Ca ionophore induced a sharp increase up to $100 \times 10^3$ c.p.m. Preincubation of PMN with *E. coli* HB 101 (pRI 203) and subsequent stimulation with the Ca ionophore reduced the response ($40 \times 10^3$); no chemilumines-

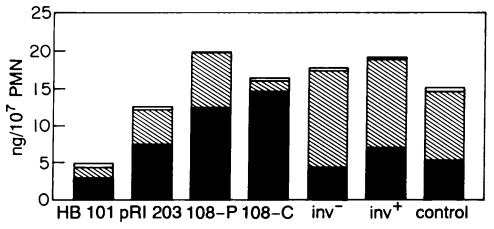


**Figure 3.** Effect of bacterial pretreatment on leukotriene release with the Ca ionophore: $LTB_4$ (■); 20-OH $LTB_4$; 20-COOH $LTB_4$ (□). Human PMN ($1 \times 10^7$) were incubated with bacteria ($2{\cdot}5 \times 10^8$) for 30 min at 37°. Ca ionophore (6·3 μM) was added and incubation proceeded for a further 15 min. Cells which were preincubated in the presence of PBS for 30 min at 37° and stimulated with the Ca ionophore served as the control. The results are mean values of three experiments. $P < 0{\cdot}01$ for *E. coli* strains HB 101 and pRI 203; $P < 0{\cdot}05$ for *Y. enterocolitica* 108-P.

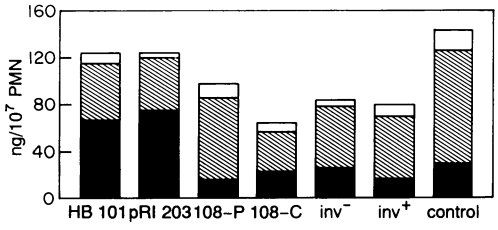


**Figure 4.** Effect of bacterial pretreatment on leukotriene release with opsonized zymosan: $LTB_4$ (■); 20-OH $LTB_4$ (▨); 20-COOH $LTB_4$ (□). Human PMN ($1 \times 10^7$) were preincubated with bacteria ($2{\cdot}5 \times 10^8$) for 30 min at 37°. Opsonized zymosan (2 mg) was added and incubation proceeded for a further 15 min. Cells which were preincubated in the presence of PBS for 30 min at 37° and stimulated with opsonized zymosan served as the control. The results are mean data of three experiments. $P \leq 0{\cdot}01$ for the *Y. enterocolitica* strains 108-P and 108-C; $P \leq 0{\cdot}05$ for *L. monocytogenes* inv⁺.

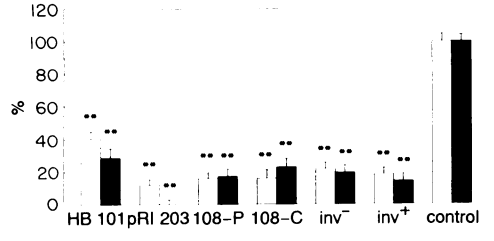


**Figure 5.** Modulation of $LTB_4$ receptor binding after bacterial pretreatment: 5 min (□); 30 min (■). Human PMN ($4 \times 10^6$) were prestimulated with bacteria ($2{\cdot}5 \times 10^8$) and then incubated with 2·3 nM [$^3$H]$LTB_4$ (and 220 nM unlabelled $LTB_4$ for the determination of non-specific binding) and 125 μg BSA for 45 min at 4°. Data of three experiments were represented as % binding compared to the buffer control (100% binding). ** $P < 0{\cdot}01$; * $P < 0{\cdot}05$.

cence was obtained in the presence of *E. coli* HB 101 after Ca ionophore stimulation. When opsonized zymosan was applied as a secondary stimulus using PMN from a different donor a similar pattern was obtained. For the primary incubation with bacteria the *E. coli* HB 101 (pRI 203) strain unlike *E. coli* HB 101 induced a chemiluminescence signal. A subsequent incubation with opsonized zymosan showed the following results. In the presence of buffer the cells revealed a prolonged increase of the chemiluminescence response ($900 \times 10^3$ c.p.m.) ranging from 30 to 60 min. Preincubation of the cells with *E. coli* HB 101 (pRI 203) revealed a low response up to $60 \times 10^3$ c.p.m. on stimulation with opsonized zymosan. In the presence of *E. coli* HB 101 no chemiluminescence signal was obtained on subsequent stimulation (data not shown). Incubation of the cells with *Y. enterocolitica* (108-C, 108-P) revealed a chemiluminescence signal below the level of the buffer control (Fig. 2b). The subsequent addition of the Ca ionophore reduced the chemiluminescence response and a marginal signal of $10 \times 10^3$ c.p.m. was obtained. Incubation of the cells (different donor as with Ca ionophore) with *Y. enterocolitica* 108-C and the subsequent stimulation with opsonized zymosan showed a chemiluminescence peak up to $130 \times 10^3$ c.p.m., while PMN treated with *Y. enterocolitica* 108-P showed a chemiluminescence response after stimulation with opsonized zymosan up to $30 \times 10^3$ c.p.m. (data not shown).

Incubation of neutrophils with the inv⁺ or the inv⁻ *L. monocytogenes* strains (Fig. 2c) showed a slight increase of the chemiluminescence signal ($18 \times 10^3$ c.p.m.) after 5 min of incubation.

Subsequent stimulation with the Ca ionophore reduced the chemiluminescence signal for the inv⁻ strain from $55 \times 10^3$ c.p.m. (buffer $100 \times 10^3$) to $12 \times 10^3$ c.p.m. (inv⁻) and to $8 \times 10^3$ c.p.m. for the inv⁺ strain. With opsonized zymosan as subsequent stimulus the inv⁺ strain as well as the inv⁻ strain inhibited the chemiluminescence signal from $850 \times 10^3$ c.p.m. (see buffer control) to $390 \times 10^3$ c.p.m. (data not shown); both, the inv⁻/inv⁺ strains changed the pattern of the chemiluminescence response significantly. These results indicate that preincubation of neutrophils with the various bacterial strains significantly suppressed the subsequent chemiluminescence response independently of whether the Ca ionophore or opsonized zymosan were used as secondary stimulus. The extracellular bacterial strain (*E. coli* HB 101) was even more inhibitory than the intracellular micro-organisms.

### Release of leukotrienes

Human granulocytes ($1 \times 10^7$) were incubated with the various bacterial strains ($2{\cdot}5 \times 10^8$) (see Table 1); no leukotrienes were generated after incubation of the cells for 5 (data not shown) and 30 min as was assessed by HPLC of the cell supernatant.

After preincubation with bacteria the cells were either stimulated with the Ca ionophore A23187 or with opsonized zymosan. The generation of $LTB_4$, 20-OH- and 20-COOH-$LTB_4$ was analysed. The combined amounts of leukotrienes ($LTB_4$ and ω-oxidized products) were studied after Ca ionophore stimulation.

Pronounced changes were observed when granulocytes were incubated with the bacteria for 30 min and subsequently stimulated with the Ca ionophore (Fig. 3) or opsonized zymosan (Fig. 4). The following results were obtained: The total amounts of $LTB_4$ and its metabolites (20-OOH- and 20-COOH-$LTB_4$) as

well as the ratio of $LTB_4$ versus the $\omega$-oxidized metabolites (20-OH- and 20-COOH-$LTB_4$ were studied.

Figures 3 and 4 summarize the data obtained for the total amounts of $LTB_4$, 20-OH-and 20-COOH-$LTB_4$. Ca ionophore-stimulated cells release $LTB_4$ and metabolites up to 143 ng/$1 \times 10^7$ cells. It is apparent that among the various strains *Y. enterocolitica*, 108-C-incubated cells produce less $LTB_4$ and metabolites (63·1 ng) as compared to 108-P (96·5 ng) and *L. monocytogenes* ($inv^+/inv^-$)-pretreated cells (82·9–87·3 ng). A significant reduction (10–15%) as compared to the control is also obtained when cells were preincubated with *E. coli* HB 101 and *E. coli* pRI 203 and subsequently stimulated.

We then analysed the ratio of $LTB_4$ to $\omega$-oxidized metabolites and observed significant differences. In the presence of both *E. coli* strains (HB 101, pRI 203) the amounts of $LTB_4$ exceeded by twofold the control value (29·0 ng) while the remaining strains with the exception of *Y. enterocolitica* 108-P (15·6 ng) did not significantly affect $LTB_4$ formation. However, all strains changed the metabolization of $LTB_4$ into 20-OH- and 20-COOH-$LTB_4$. While the PBS control showed 95·0 ng of 20-OH- and 20-COOH-$LTB_4$, *Y. entercoliticia* 108-C pretreated cells revealed a threefold reduced amount of 20-OH-$LTB_4$, the remainder strains a twofold reduction. A two- to threefold inhibition was also obtained for the generation of 20-COOH-$LTB_4$.

Figure 4 summarizes the data obtained after activation of bacteria-pretreated PMN with opsonized zymosan. Preincubation of the cells with *E. coli* HB 101 reduced the combined amounts of $LTB_4$ and metabolites by two- to threefold. A less pronounced inhibition ($\approx$17%) was obtained with *E. coli* HB 101 (pRI 203). The remaining strains enhanced the total amounts of leukotrienes by 10–30% as compared to the control (14·9 ng). When the generation of $LTB_4$ was considered *Yersinia*-pretreated cells showed a more than two- to threefold higher generation of $LTB_4$. While incubation of the cells with *E. coli* HB 101 suppressed $LTB_4$ formation to 2·8 ng, with *E. coli* HB 101 (pRI 203) a 50% enhanced $LTB_4$ formation (7·4 ng), as compared to the buffer control, was apparent. *Listeria monocytogenes*-pretreated cells also showed an enhanced formation of 20-OH-$LTB_4$. A significant reduction of 20-OH-$LTB_4$ formation was obtained with *E. coli* HB 101 and *Y. enterocolitica* 108-C; a less pronounced inhibition was obtained with *E. coli* HB 101 (pRI 203) ($\approx$50%) and *Y. enterocolitica* 108-P ($\approx$20%).

The data clearly indicate that the amounts obtained for the generation of $LTB_4$ and metabolites from bacteria-pretreated granulocytes vary depending on the subsequent secondary stimulus.

### $LTB_4$ receptor expression

Subsequent experiments were carried out to study the expression of $LTB_4$-binding sites after preincubation of granulocytes with the various bacterial strains (Table 1). It is apparent from Fig. 5 that a 5-min preincubation with bacteria reduced the binding sites for $LTB_4$ by $\approx$60% for *E. coli* HB 101 and up to $\approx$85% for *E. coli* HB 101 (pRI 203) and *Y. enterocolitica* 108-P. Preincubation of PMN with the *L. monocytogenes* strains suppressed $LTB_4$ receptor binding by 75–80%. After 30 min preincubation with *E. coli* HB 101 (pRI 203) no binding sites for $LTB_4$ were detectable, while with *E. coli* HB 101 the binding activity was $\approx$38%. For the remaining strains the prolonged preincubation did not significantly change the amounts of $LTB_4$ receptors.

## DISCUSSION

Major progress has been made in understanding the cell biological as well as bacterial requirements for the entry and adherence of enteroinvasive *E. coli, Shigella* ssp., *Salmonella* ssp. as well as *Yersinia* ssp.[10] Target cells studied were mostly epithelial cells (e.g. HeLa-, HEp-2-cells).[2,4,8-11] For *L. monocytogenes* the synthesis of an extracellular protein (p60) is apparently involved in the uptake by non-professional phagocytes.[4,20,38] Few data exist as to the effect of intracellular persisting bacteria on the activity of professional phagocytes which they also commonly use as host cells.[3,14] Our results emphasize that the interaction of bacteria (intracellular, extracellular) with inflammatory cells modulates their proinflammatory activities. Differences with regard to the cellular responses towards an extracellular micro-organism (*E. coli* HB 101) as compared to intracellular bacteria are obtained.

Defined surface components (e.g. inv, ail gene products, p60) have been described as mediating adherence and invasion.[1,15,16] Unlike epithelial cells (data not shown) the results obtained with granulocytes indicate that bacterial uptake primarily depends on the phagocytic properties of the target cells. Differences are apparent when bacterial adherence to phagocytes was studied. The time–course of chemiluminescence response induced by the various bacterial strains reflects additional differences. The interaction and phagocytosis of *E. coli* HB 101 (pRI 203) by PMN induces a significant chemiluminescence response unlike *E. coli* HB 101. Both *Y. enterocolitica* strains were not active. Indeed it has been shown that YOP suppress the chemiluminescence response.[5] The chemiluminescence induced by the $inv^+$ *L. monocytogenes* strain exceeds that of the *L. monocytogenes* $inv^-$ strain. The results suggest that although phagocytosis is most likely the predominant mechanism of bacterial uptake, defined surface properties (e.g. inv gene,[1,2] p60[4,20,38] and YOP[6,11]) are able to modulate the chemiluminescence signal.

PMN after phagocytosis of bacteria showed a completely suppressed (*Yersinia* ssp., *E. coli* HB 101) or partially suppressed [*E. coli* HB 101 (pRI 203), *L. monocytogenes* $inv^-$] secondary chemiluminescence signal induced by either the Ca ionophore or opsonized zymosan. *Listeria monocytogenes* $inv^-$ strains were less inhibitory compared to *L. monocytogenes* $inv^+$ strains. Recently it was shown with macrophages that intracellularly persisting pathogens enter the cell via the C3b and C3bi receptor.[40] The results suggest that independent of the intracellular pathogen, multiple factors may affect the chemiluminescence response of the pretreated granulocyte:[5] possible explanations are differences in the rate of killing, the activity of intracellular bacterial products, the compartmentalization of bacteria and possibly also differences in the activation of the cellular signal transduction cascade. In this regard it has been recently described that *Yersinia* outer membrane protein (YOP 2b) expresses tryosine phosphatase activity. It is believed that YOP induces dephosphorylation of the phagocyte membrane and may thus affect the signal transduction of the host cell.[41,42]

A number of observations recently suggested that extracellularly persisting bacteria by defined mechanisms (adhesins, haemolysins)[35] induce leukotriene formation from human granulocytes with chemotactic ($LTB_4$) and spasmogenic ($LTB_4$) properties. Walker & Hoover[23] have shown that typhus *Rickettsia* are able to induce leukotriene $B_4$ and prostaglandin $E_2$ release from mouse polymorphonuclear leucocytes. *Rickettsia*-induced stimulation of $LTB_4$ secretion was associated with its haemolytic activity; treatment of mouse PMN with non-haemolytic *Rickettsia* abolished the activity of *Rickettsia* to stimulate $LTB_4$ secretion from PMN. We demonstrated that leukotriene generation from endogenous arachidonic acid is significantly modulated as to the amount and pattern of metabolites and also depending on the subsequent stimulus. The intracellular bacteria (*Yersinia* ssp., *L. monocytogenes* $inv^+/inv^-$) suppressed the total amounts of leukotrienes. While the amount of $LTB_4$ was similar to the control (non-infected cells and Ca ionophore), the formation of 20-OH- and 20-COOH-$LTB_4$ was significantly reduced. These results suggest an impaired $LTB_4$ metabolization, which was more pronounced with the *E. coli* (HB 101, pRI 203) strains. An increased $LTB_4$ formation and impaired $\omega$-oxidation was obtained suggesting that intracellular and extracellular bacteria both affect similar enzymes of the leukotriene pathway. Obviously, the results observed after Ca ionophore stimulation are more likely affected by the *E. coli* background as compared to the expression of the inv gene. With opsonized zymosan as stimulus a different pattern was obtained. A pronounced suppression of the total leukotrienes is obtained for *E. coli* HB 101, which is less for *E. coli* HB 101 (pRI 203) pretreated cells. With both *E. coli* strains, the ratio of $LTB_4$ versus $\omega$-oxidized products is altered. The changes are even more pronounced with *Yersinia* ssp. pretreated cells. *Listeria monocytogenes* $inv^+/inv^-$ strains did not alter the granulocyte responsiveness towards opsonized zymosan for leukotriene generation. $LTB_4$ expresses its biological activity via distinct receptors (high affinity–chemotaxis and low affinity–degranulation). In this regard after 5 min of microbial interaction an impaired $LTB_4$ binding is obtained which is more pronounced for the intracellular bacteria as compared to *E. coli* HB 101. The data indicate that during microbial interaction apparently the surface properties of granulocytes are changed with a subsequent loss of $LTB_4$ receptors. Thus for defined proinflammatory properties (e.g. chemiluminescence) inflammatory cells react differently after interaction with intracellular and extracellular bacteria. The inv gene product apparently completely suppresses $LTB_4$ receptor sites. Not defined YOP affect the chemiluminescence and leukotriene metabolization and may possibly counteract the effect of the inv gene product.

However, there are important functions, e.g. arachidonic acid transformation, which are changed independently of intracellular and extracellular bacterial persistence. The phagocyte, although expressing reduced receptor sites for $LTB_4$, apparently counteracts the impaired chemotactic potential by a reduced $\omega$-oxidation and indirectly stabilizes the formation of the chemotactic factor $LTB_4$.

Thus, intracellular bacteria are able to modulate the pro-inflammatory mediator release of neutrophils. Future studies are directed to analyse the components of the signal transduction cascade of neutrophils modulated after bacterial interaction.

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