**Moraxella catarrhalis** stimulates the release of proinflammatory cytokines and prostaglandin E₂ from human respiratory epithelial cells and monocyte-derived macrophages

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**Abstract**

The outer membrane proteins of *Moraxella catarrhalis*, a bacterial pathogen which causes disease in both children and adults, play an important role in its phenotypic properties. However, their proinflammatory potential with regard to respiratory epithelium and macrophages is unclear. To this end, we examined the cytokine- and mediator-inducing capacity of a heat-killed wild-type *M. catarrhalis* strain and a nonautoagglutinating mutant as well as their outer membrane proteins and secretory/excretory products using the A549 respiratory epithelial cell line. The outer membrane proteins and secretory/excretory products from both isolates as well as the heat-killed bacteria all induced interleukin (IL)-6, IL-8 and prostaglandin E₂, but not IL-1β, from the A549 cell line in a dose- and time-dependent manner. Heat-killed bacteria and secretory/excretory products stimulated the release of IL-1β, IL-6, IL-8 and prostaglandin E₂ from human monocyte-derived macrophages. Both heat-killed isolates also stimulated nuclear translocation and transactivation of nuclear factor-κB. The heat-killed wild-type autoagglutinating isolate induced significantly greater amounts of IL-6 and IL-8 from A549 cells than the nonautoagglutinating mutant compared with the monocyte-derived macrophages but no significant differences in the amounts induced by the two strains were observed. These differences were also evident when the respiratory cell line was stimulated with outer membrane proteins as well as in the degree of nuclear factor-κB transactivation. There was little difference in the stimulatory activity of the secretory/excretory products. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis analyses revealed some differences in the outer membrane proteins and secretory excretory products between the two isolates. Combined, these data show that *M. catarrhalis* secretory excretory products and outer membrane proteins are associated with the induction of inflammatory responses in both respiratory epithelium and macrophages.

**Introduction**

*Moraxella catarrhalis* is an important cause of otitis media in children, and lower respiratory tract infections in adults with chronic obstructive pulmonary disease (Verduin et al., 2002; Murphy et al., 2005b). This association has stimulated much interest in determining its antigenic structure and in the host’s immune response during infection (Murphy, 1998; Murphy et al., 2005a). In this regard, particular attention has been directed toward the study of the outer membrane proteins (OMP) of *M. catarrhalis* and the immune responses they elicit, in the hope of finding a suitable vaccine candidate (Sethi et al., 1995; Murphy et al., 1998). However, such studies have been limited by the lack of a suitable animal model since, in contrast to the persistent nature of a human infection, *M. catarrhalis* is cleared from the lungs of model animals within 24 h (Unhanand et al., 1992; Murphy, 1996). Nevertheless, data from studies performed using mice and rats have consistently supported clinical observations showing that infection of the lower respiratory tract with *M. catarrhalis* results in a rapid influx of neutrophils, dendritic cells and other
inflammatory cells (McWilliam et al., 1994; Kyd et al., 1998; Jecker et al., 1999).

Moraxella catarrhalis, when cultured, has a tendency to clump, forming large aggregates, but the influence of this property on the pathogenicity or immunogenicity of the organism is unclear. However, a nonautoagglutinating mutant of M. catarrhalis lacking a 200 kDa OMP and demonstrating altered surface expression of the OMP-CD and lipooligosaccharide (LOS) as judged by flow cytometry using specific monoclonal antibodies has been shown to result in a reduced influx of neutrophils to, and reduced bacterial clearance from, the lung in a mouse model when compared to the wild-type (Kyd et al., 1998). The reasons for the reduced neutrophil influx are unclear. We hypothesized that it may be due to a decreased ability of the nonautoagglutinating variant to stimulate the release of proinflammatory cytokines, such as the neutrophil chemoattractant, IL-8, from the respiratory tract. We, therefore, determined the proinflammatory effects of a previously described wild-type M. catarrhalis isolate and its nonautoagglutinating variant (Kyd et al., 1998) on cells of the respiratory tract, namely, the respiratory epithelial cell and the macrophage.

Both cell types are known to respond to a range of bacterial components, such as LOS and cell wall proteins, by producing cytokines and mediators with diverse proinflammatory functions (Black et al., 1998). These include IL-6, which is involved in T-cell activation, growth and differentiation (Ionescu et al., 2000), IL-8, which is responsible for the recruitment of neutrophils (Black et al., 1998), and IL-1β and prostaglandin E2 (PGE2), both of which can also stimulate proinflammatory cytokine release from epithelium (Standiford et al., 1990; Tavakoli et al., 2001). Many of the above have also been shown to play an important role in the pathogenesis of M. catarrhalis-induced otitis media in an animal model of the disease (Sato et al., 1999) and can be detected in sputum from patients with chronic obstructive pulmonary disease (Chung, 2001). The data obtained from our studies indicate that culture supernatants and heat-killed autoagglutinating and nonautoagglutinating isolates of M. catarrhalis are potent proinflammatory agents for each cell type. The heat-killed nonautoagglutinating mutant, however, was significantly less potent than the autoagglutinating isolate, suggesting an important role for OMP in the induction of an inflammatory response during M. catarrhalis infection.

Materials and methods

Preparation of bacterial culture supernatants and heat-killed bacteria

Moraxella catarrhalis nonautoagglutinating mutant (Mc4223NC) and the autoagglutinating, wild-type isolate (Mc4223) were kindly provided by Professor T. F. Murphy, University of New York, Buffalo, New York, and grown on Mueller Hinton agar (MHA) plates overnight at 37 °C. Single colonies were removed from each plate and then grown overnight at 37 °C in 10 mL of Mueller Hinton broth (MHB) with shaking. The overnight cultures were then added to 100 mL of MHB in 1 L conical flasks and grown in an orbital shaker (Innova 4330; New Brunswick Scientific, Chatswood, New South Wales, Australia) for 24 h at 200 rpm at 37 °C to the same optical density. The cultures were centrifuged at 10 000 g for 25 min at 4 °C, and the culture supernatants filtered through 0.22 μm membrane filters (Gelman Sciences, Ann Arbor, MI) and stored at −20 °C. The culture supernatants were subsequently used at a dilution of 10% (v/v) in medium to stimulate the cells on the basis of prior optimization experiments. Bacteria were grown overnight in MHB, harvested by centrifugation and washed three times in sterile water. The concentrations were then adjusted to the same CFU mL⁻¹ (approximately 3 × 10⁸ mL⁻¹) in PBS and the bacteria were heat-killed by incubation at 60 °C for 1 h. Killing was assessed using MHA plates overnight at 37 °C prior to use. To obtain disaggregated wild-type (Mc4223) for experimental use, bacteria were disrupted by passing through a 26-gauge needle immediately prior to use. For consistency, the nonautoagglutinating (Mc4223NC) mutant was treated in the same manner. These preparations were then used over the range 1 × 10⁴ to 3 × 10⁸ CFU mL⁻¹ medium to stimulate the epithelial cells and human macrophages. Visual inspection of the colonies revealed characteristic differences in appearance and, in suspension, confirmed the autoagglutination and nonautoagglutination properties (Kyd et al., 1998). Escherichia coli LPS was obtained from Sigma Chemical Co. (St Louis, MO).

Isolation of OMP and SDS-PAGE analyses

Outer membrane proteins were isolated from two strains of M. catarrhalis, grown at 37 °C overnight in 10 mL of MHB as previously described (Murphy & Loeb, 1989). The bacteria were recovered by centrifugation at 10 000 g for 20 min and resuspended in 10 mL of phosphate buffer, 0.05 M (pH 7.4) containing NaCl (0.15 M) and EDTA (0.01 M). The suspension was incubated at 56 °C for 30 min and the bacteria were removed by centrifugation at 10 000 g for 40 min. The supernatants were then centrifuged at 70 000 g for 6 h at 4 °C and the pellet was resuspended in phosphate buffer for the determination of cytokine stimulatory activity or 500 μL sample buffer for SDS-PAGE. Protein concentrations were determined by absorption at 280 nm based on an absorbancy of 1 mg mL⁻¹ of 1. OMP samples were heated at 100 °C for 3 min and the proteins separated by electrophoresis at 150 V for 1 h on 10% (w/v) SDS-PAGE gels.
Supernatants from the overnight cultures were filtered through 0.2 μm syringe filters (Acrodisc, Pall Corporation, Ann Arbor, MI). Proteins were precipitated with an equal volume of TCA (20%, w/v), and the mixture incubated on ice for 30 min. After centrifugation at 2170 g for 15 min at 4 °C, the pellet was resuspended in 1 mL of acetone at −20 °C and the suspension centrifuged for 5 min at 4 °C. The wash process was repeated a further two times. The pellets were air-dried, resuspended in 300 μL of SDS-PAGE sample buffer and analyzed by 13% (w/v) SDS-PAGE electrophoresis. For total proteins, bacterial cells from 10 mL overnight cultures were resuspended in 500 μL and analyzed by 13% (w/v) SDS-PAGE electrophoresis. All gels were stained overnight with Coomassie blue R-250G (Gradipore Ltd, Frenchs Forest, NSW, Australia), destained with acetic acid (6%, v/v) and photographed using a Nikon Coolpix 990 digital camera (Nikon Corporation, Tokyo, Japan). The apparent molecular weights of the proteins of interest were determined by reference to low and high standards (Bio-Rad Laboratories, Sydney, Australia).

Culture and stimulation of A549 cells

The A549 cell line, a human pulmonary epithelial type II cell line (Lieber et al., 1976), was obtained from ATCC and maintained in F12 Kainh’s modification nutrient media (GibcoBRL, Melbourne, Vict., Australia) supplemented with fetal calf serum (FCS, 10%, v/v; Commonwealth Serum Laboratories, Parkville, Australia), penicillin (6 μg mL⁻¹), streptomycin sulfate (2.5 μg mL⁻¹) and Gungizone (1.5 μg mL⁻¹; GibcoBRL). Cells were passaged following harvesting with trypsin-EDTA (Sigma Chemical Co. Ltd) and grown until confluent (approximately 2.5 × 10⁵ cells per well) in 24-well tissue culture plates (Falcon, Becton Dickinson, NJ). Medium was then replaced as appropriate, and the cells incubated for a further 24 h. Cells were incubated with stimulants in media without FCS (basal medium) but in the absence or presence of polymyxin B (50 μg mL⁻¹; Sigma Chemical Co. Ltd) for the duration of the experiment and the supernatants harvested and stored at −20 °C for subsequent analyses.

Detection of IL-1β, IL-6, IL-8 and PGE₂

Tissue culture supernatants were assessed for the presence of IL-6 and IL-8 by ELISA as described previously (Asokananthan et al., 2002). IL-1β concentrations were determined by ELISA as per the manufacturer’s instructions (Pharmingen, San Diego, CA) and PGE₂ was measured by a competitive enzyme immunoassay according to the manufacturer’s instructions (Cayman Chemical, Ann Arbor, MI). Concentrations were expressed as pg mL⁻¹ or ng mL⁻¹ from 2.5 × 10⁵ epithelial cells or 1 × 10⁵ macrophages.

Inhibition of nuclear factor (NF)-κB and cyclooxygenase

The glucocorticoid dexamethasone and the serine protease inhibitor L-1-tosylamido-2-phenylalanine chloromethyl ketone (TPCK) are known to inhibit NF-κB activation (Dimango et al., 1998; Steer et al., 2000). On this basis, these inhibitors were used to evaluate the role of NF-κB in cytokine release. The cyclooxygenase inhibitor indomethacin was used to investigate the possibility that prostanooids per se were involved in cytokine release via an autocrine mechanism. Dexamethasone (DEX; 1 μM), TPCK (50 μM), indomethacin (Indo; 1 μM) and an antibody against IL-1β (anti-IL-1β; 0.1 μg mL⁻¹) were incubated with A549 cells for 1 h. Heat-killed M. catarrhalis wild-type (Mc) and nonautoagglutinating mutant (McNC; 10% v/v in medium) were then used to stimulate the A549 cells for 24 h. Production of IL-6 and IL-8 was then assayed and the data were expressed as a percentage reduction in cytokine detected following pre-treatment with inhibitor compared with detection in the absence of inhibitor.

Preparation of human macrophages from peripheral blood mononuclear cells (PBMC) and their stimulation

Buffy coat preparations from healthy donors were provided by the Australian Red Cross Blood Bank (Perth, WA, Australia), and PBMC prepared as described previously (de Almeida et al., 2000). The phenotype of the monocyte-derived macrophages used was established using phosphodiesterase content and CD68 expression (Gantner et al., 1997; de Almeida et al., 2000). The data indicated that 96% of cells were CD68⁺ and possessed the distinctive macrophage phenotype. Harvested macrophages were seeded in R10 with human AB serum (1%, v/v) in 96-well tissue culture plates at 1 × 10⁵ cells per well. The macrophages were then exposed to heat-killed M. catarrhalis wild-type (Mc) and nonautoagglutinating mutant (McNC; 6 × 10⁵ CFU mL⁻¹) or their culture supernatants (10%, v/v) for 24 h and cytokine production assayed.

Electrophoretic mobility shift assay (EMSA)

A549 cells were exposed to heat-killed wild-type and nonautoagglutinating bacteria (10% v/v in medium) or to PBS (10% v/v in medium) for 2 h, a time previously shown to result in maximum translocation of NF-κB (data not shown). Nuclear extracts were prepared from 2.5 × 10⁵ cells as previously described (Joyce et al., 1999). For the electrophoretic mobility shift assay, nuclear proteins (3 μg) were preincubated for 10 min at room temperature with 0.5 μg of poly(dI-dC) (Amersham Pharmacia Biotech, Uppsala, Sweden) in binding buffer containing the following components.
in a final reaction volume of 10 μL: HEPES buffer, 20 mM (pH 7.9); Ficoll (4%, v/v); EDTA (1 mM); dithiothreitol (1 mM); KCl (50 mM); IGEPAL CA-630 (0.05%, v/v). Appropriate specific antibodies (1 μg; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were included in the mixture for the supershift electrophoretic mobility shift assay. The appropriate oligonucleotide probe labeled with [α-32P]dCTP (Amersham Pharmacia Biotech) using Klenow fragment of E. coli DNA polymerase I (Promega, Madison, WI) was then added. Consensus binding sequences for NF-κB were designed as described previously (Steer et al., 2000). Ten minutes after the addition of probe, or 20 min after the addition of antibody, samples were loaded onto a polyacrylamide gel (4%, w/v) containing Tris-Borate-EDTA buffer (0.25 M/2 M), which had been pre-run for 2 h. Samples were then loaded onto the gel and electrophoresis performed at 150 V for 90 min. The gel was then dried and exposed to Cronex X-ray film (Agfa, Mortsel, Belgium), using a single intensifying screen (Amersham Pharmacia Biotech).

**NF-κB transactivation**

A549 cells were transiently transfected with the 3kB-luc reporter plasmid vector by the DEAE-dextran method as previously described (Steer et al., 2000). The 3kB reporter, which incorporates three consensus NF-κB binding sites in tandem, upstream of a minimal promoter and luciferase coding region, was kindly provided by Professor D. Baltimore (Caltech, Pasadena, CA). Cells were seeded in 24-well plates, left overnight and then starved in basal medium for 24 h before exposure to heat-killed wild-type and nonautoagglutinating mutant bacteria (10% v/v in medium) and to a control (PBS; 10% v/v in medium). Luciferase activity was measured in cell extracts 1, 3, 6, 12 and 24 h after stimulation using a commercial luciferase assay system according to the manufacturer’s instructions (Promega).

**Statistical analysis**

Unless stated otherwise, all data were expressed as mean ± standard error. Statistical significances were determined using a two-tailed Student’s t-test using Microsoft Excel for Macintosh with P < 0.05 regarded as significant. Bonferroni’s correction was used to adjust P-values when multiple comparisons were performed.

**Results**

**Culture supernatants from the wild-type and the nonautoagglutinating mutant induce the release of IL-6 and IL-8 but not IL-1β from A549 cells**

A549 cells were stimulated with wild-type or nonautoagglutinating mutant culture supernatants diluted to 10% (v/v) in medium. Broth alone at this dilution was inactive and culture supernatants at this dilution were previously shown to be optimal, and without observable adverse effects on cell viability. Significant concentrations of IL-6 and IL-8 are expressed as mean ± standard error of two experiments performed in triplicate. The IL-6 responses for both supernatants were significantly different from the PBS control (□) response at all time points (wild-type, •, P < 0.0001; nonautoagglutinating, ○, P < 0.0001). The IL-8 responses for both supernatants were significantly different from the control response at the 24 h time point (wild-type, P < 0.01; nonautoagglutinating, P < 0.0001).

[Fig. 1. Release of IL-6 and IL-8 from A549 cells following stimulation with Moraxella catarrhalis culture supernatants. Concentrations of IL-6 and IL-8 are expressed as mean ± standard error of two experiments performed in triplicate. The IL-6 responses for both supernatants were significantly different from the PBS control (□) response at all time points (wild-type, •, P < 0.0001; nonautoagglutinating, ○, P < 0.0001). The IL-8 responses for both supernatants were significantly different from the control response at the 24 h time point (wild-type, P < 0.01; nonautoagglutinating, P < 0.0001).]
Heat-killed wild-type and the nonautoagglutinating mutant bacteria induce the release of IL-6 and IL-8 but not IL-1β from A549 cells

A549 cells stimulated with heat-killed wild-type or nonautoagglutinating mutant (10% v/v in medium) released significant concentrations of IL-6 and IL-8 (Fig. 2), but not IL-1β (data not shown), in a time-dependent manner. Significantly greater concentrations of IL-6 were released at all time points when the A549 cells were stimulated with wild-type compared with the nonautoagglutinating mutant, but IL-8 concentrations were significantly different only at 6, 12 and 24 h (Fig. 2). As with the culture supernatants, the addition of polymyxin B had little significant effect on the concentrations of cytokine released (data not shown). The cytokine stimulatory activity of the OMP proteins from both isolates was also demonstrated, and a statistically significantly greater release was observed using OMP from the wild-type compared with the nonautoagglutinating mutant (Fig. 3).

Wild-type and the nonautoagglutinating mutant culture supernatants and heat-killed bacteria induce the release of PGE₂ from A549 cells

Stimulation of A549 cells with both wild-type and nonautoagglutinating mutant culture supernatants and heat-killed bacteria caused the release of significant concentrations of PGE₂ compared to the medium control (Table 1); however, no significant differences in PGE₂ release following stimulation with either culture supernatants or heat-killed bacteria were observed. Similarly, no significant differences in PGE₂ release from the wild-type and nonautoagglutinating mutant using the two types of stimuli were observed.
Role of IL-1β, PGE₂ and NF-κB in heat-killed wild-type- and nonautoagglutinating mutant-induced IL-6 and IL-8 release from A549 cells

An anti-IL-1β antibody (0.1 μg mL⁻¹) abolished IL-1β-induced IL-6 and IL-8 release from the A549 cell line (P < 0.05, n = 3; data not shown) but had little effect on heat-killed wild-type- or nonautoagglutinating mutant-induced cytokine release (Table 2). Similarly, indomethacin (1 μM) did not significantly inhibit heat-killed wild-type- or nonautoagglutinating mutant-induced IL-6 and IL-8 release from the A549 cell line (Table 2). However, dexamethasone (1 μM) and TPCK (50 μM) were shown to significantly inhibit heat-killed wild-type- and nonautoagglutinating mutant-induced IL-6 and IL-8 release (Table 2).

Heat-killed wild-type and the nonautoagglutinating mutant culture supernatants and heat-killed bacteria induce cytokine release from human monocyte-derived macrophages

Supernatants from wild-type and nonautoagglutinating mutant cultures and heat-killed bacteria stimulated the release of IL-1β, IL-6 and IL-8 from human monocyte-derived macrophages. In each instance (n = 3–11) the concentrations obtained were significantly greater than those obtained from medium alone (P < 0.05 for each cytokine except IL-8 responses obtained with culture supernatants) and cytokine release did not differ between the isolates. However, donors responded variably, with low cytokine concentrations compared to the A549 cell line responses. To overcome this, the bacteria-induced release was examined using a single donor for all extracts (Table 3). Again, all three cytokines were released but no significant differences were observed between the responses obtained using heat-killed wild-type and the nonagglutinating mutant (Table 3). The addition of polymyxin B had varying effects on induced cytokine release. For example, polymyxin B stimulated both heat-killed wild-type- and nonautoagglutinating mutant-induced IL-1β and IL-8 release, but not IL-6 release. Escherichia coli LPS at 1 μg mL⁻¹ also stimulated the release of significant concentrations of IL-1β, IL-6 and IL-8, all of which were inhibited by polymyxin B treatment (Table 3). Stimulation with culture supernatants and heat-killed wild-type and mutant also resulted in the release of significant concentrations of PGE₂ (Table 1) compared to medium control.

SDS-PAGE analyses

SDS-PAGE analyses showed no discernible differences between the total protein profiles of the wild-type autoagglutinating isolate and those of the nonautoagglutinating mutant (Fig. 4a). The OMPs from both isolates were similar, although there were some differences. For example, the OMP preparation from the wild-type contained 187, 166, 48 and 25 K bands that were absent in the OMP preparation from the mutant (Fig. 4b). The OMP from the nonautoagglutinating mutant contained a 49 and a 32 K band compared to the wild-type OMP preparation. Proteins were also found in culture supernatants from both isolates, although the spectrum varied (Fig. 4c). For example, a 74 K protein was found only in the wild-type, whereas 77, 45, 32 and 26 K proteins were evident in the supernatant from the nonautoagglutinating mutant but not from the wild-type isolate (Fig. 4c).

Exposure of A549 cells to heat-killed wild-type and the nonautoagglutinating mutant bacteria results in nuclear translocation of NF-κB binding proteins p50/p65, p65/c-Rel and c-Rel/c-Rel

Nuclear translocation of NF-κB proteins in the A549 cell line was detected after 2 h stimulation with either heat-killed

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Table 1. A549 cells and human monocyte-derived macrophages release PGE₂ following stimulation with heat-killed wild-type and nonautoagglutinating mutant (NC) of Moraxella catarrhalis and cell-free culture supernatants

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>PGE₂ (pg mL⁻¹) ± SEM</th>
<th>Stimulant</th>
<th>PGE₂ (pg mL⁻¹) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium control</td>
<td>262 ± 33</td>
<td>Moraxella catarrhalis</td>
<td>411 ± 30</td>
</tr>
<tr>
<td>Moraxella catarrhalis</td>
<td>398 ± 26</td>
<td>Moraxella catarrhalis NC</td>
<td>398 ± 26</td>
</tr>
<tr>
<td>Moraxella catarrhalis culture supernatant</td>
<td>496 ± 41</td>
<td>Moraxella catarrhalis culture supernatant</td>
<td>466 ± 11</td>
</tr>
<tr>
<td>Moraxella catarrhalis NC culture supernatant</td>
<td>1024 ± 8</td>
<td>Moraxella catarrhalis NC culture supernatant</td>
<td>1024 ± 5</td>
</tr>
</tbody>
</table>

*Indicates significantly greater mean release than medium control (P < 0.05).

Data are expressed as mean ± SEM, n = 2–6.

Table 2. Inhibition of heat-killed Moraxella catarrhalis induced IL-6 and IL-8 release from A549 cells by dexamethasone and TPCK but not indomethacin or an anti-IL1β antibody

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Inhibitor</th>
<th>IL-6</th>
<th>IL-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moraxella catarrhalis</td>
<td>DEX</td>
<td>90a</td>
<td>43a</td>
</tr>
<tr>
<td></td>
<td>TPCK</td>
<td>94a</td>
<td>58a</td>
</tr>
<tr>
<td>Moraxella catarrhalis NC</td>
<td>Indo</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Anti-IL1β</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>DEX</td>
<td>89a</td>
<td>50a</td>
</tr>
<tr>
<td></td>
<td>TPCK</td>
<td>98a</td>
<td>88a</td>
</tr>
<tr>
<td></td>
<td>Indo</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Anti-IL1β</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

*Indicates a significant reduction following treatment with the inhibitor (P < 0.05; n = 3).
wild-type or nonautoagglutinating mutant bacteria but not when the cells were incubated with PBS (all 10% v/v in medium) (Fig. 5). Each isolate induced a similar pattern of NF-κB dimers comprising p50/p65, p65/c-Rel and c-Rel/c-Rel, as shown by the ability of specific antibodies to shift appropriate bands (Fig. 5).

Heat-killed wild-type and the nonautoagglutinating mutant bacteria stimulation of A549 cells results in NF-κB transactivation

The A549 cells transiently transfected with a NF-κB-luciferase reporter construct and stimulated with heat-killed wild-

Discussion

Numerous studies have investigated the proinflammatory capabilities of *Moraxella catarrhalis* (McWilliam et al., 1994; Table 3. Human monocyte-derived macrophages release cytokines following stimulation with the heat-killed wild type and nonagglutinating mutant (NC) of *Moraxella catarrhalis* and their culture supernatants

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>IL-1β (p8− vs p8+)</th>
<th>IL-6 (p8− vs p8+)</th>
<th>IL-8 (p8− vs p8+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium control</td>
<td>4.4 ± 0.7</td>
<td>2.8 ± 1</td>
<td>92.8 ± 3</td>
</tr>
<tr>
<td><em>Moraxella catarrhalis</em></td>
<td>213.4 ± 20±7</td>
<td>98.6 ± 12±7</td>
<td>121.9 ± 2±7</td>
</tr>
<tr>
<td><em>Moraxella catarrhalis</em> NC</td>
<td>229.1 ± 13±7</td>
<td>95.8 ± 10±7</td>
<td>121.8 ± 2±7</td>
</tr>
<tr>
<td><em>Moraxella catarrhalis</em> culture supernatant</td>
<td>74.5 ± 6±7</td>
<td>20.2 ± 3±7</td>
<td>113.2 ± 2±7</td>
</tr>
<tr>
<td><em>Moraxella catarrhalis</em> NC culture supernatant</td>
<td>106.9 ± 30±7</td>
<td>31.7 ± 4±7</td>
<td>113.6 ± 3±7</td>
</tr>
<tr>
<td>Escherichia coli LPS</td>
<td>45.7 ± 4±7</td>
<td>36.6 ± 2±7</td>
<td>104.4 ± 0</td>
</tr>
</tbody>
</table>

aCytokine data expressed as pg mL−1 in the absence (−) or presence (+) of polymyxin B (p8, 50 μg mL−1).
bP < 0.05;
cP < 0.01;
dP < 0.001 comparing mean response obtained with stimulus versus that obtained with appropriate medium control;
eP < 0.01;
fP < 0.001 comparing mean responses obtained in the presence or absence of polymyxin B. Data obtained from a single experiment performed in triplicate.

culture supernatant 213.4 ± 20±7

**Table 3.** Human monocyte-derived macrophages release cytokines following stimulation with the heat-killed wild type and nonagglutinating mutant (NC) of *Moraxella catarrhalis* and their culture supernatants.
PGE2 was measured, perhaps due to specific signaling of infection. Such differences were not observed when the nonautoagglutinating mutant, at comparable multiplicity of infection, was significantly more potent in inducing cytokine release than the wild-type isolate. As the stimulatory capabilities of the culture supernatants were similar and adjusted for cell growth, the difference in responses obtained at 6 h was subsequently shown to be significant. We also investigated whether a paracrine/autoagglutinating isolate, although only at high concentrations. It is possible that the differential activity observed correlated with the previously reported differences in OMP (Kyd et al., 1998) as well as the differences described in our studies.

Fig. 6. Moraxella catarrhalis stimulation of A549 cells results in nuclear factor (NF)-κB transactivation. A549 cells were transiently transfected with an NF-κB-luciferase reporter construct and stimulated with heat-killed wild-type (●), nonautoagglutinating mutant (○) and a PBS control (□) for 1, 3, 6, 12 and 24 h. Relative light units were then measured and results are expressed as mean ± standard error of the mean, n = 3. The difference in responses obtained at 6 h was subsequently shown to be significant in two subsequent single time point experiments (P < 0.05).

Jecker et al., 1999; Gjorloff Wingren et al., 2002) and its structural components (Keller et al., 1992; Kyd et al., 1998), but relatively little is known about the proinflammatory properties of its secreted/excreted components or cell surface proteins, particularly with regard to cells derived from the respiratory tract. In this study, the availability of a nonagglutinating mutant of M. catarrhalis facilitated a comparison of the proinflammatory properties with a wild-type isolate.

The secretory/excretory products of both the wild-type and the nonautoagglutinating mutant induced the release of IL-6, IL-8 and PGE2 but not IL-1β from the A549 cell line. These results in accord with those from other studies examining the proinflammatory potential of other respiratory bacterial pathogens (Bresser et al., 1997; Clemans et al., 2000; Fink et al., 2003). Little difference in the stimulatory activity of the products from each isolate was observed. In contrast, the heat-killed wild-type bacterial suspension was significantly more potent in inducing cytokine release than the nonautoagglutinating mutant, at comparable multiplicity of infection. Such differences were not observed when PGE2 was measured, perhaps due to specific signaling followed by receptor/agonist interaction.

As the stimulatory capabilities of the culture supernatants were similar and adjusted for cell growth, the difference in proinflammatory potential observed with the heat-killed bacteria suggest the involvement of structural components of the bacteria, rather than any specifically secreted or excreted product. In this regard, the OMP from both isolates stimulated IL-6 and IL-8 release. Consistent with the heat-killed bacteria, OMP from the wild-type stimulated significantly more cytokine than the OMP from the nonagglutinating isolate, although only at high concentrations. It is possible that the differential activity observed correlated with the previously reported differences in OMP (Kyd et al., 1998) as well as the differences described in our studies.

Both the wild-type and nonautoagglutinating mutant stimulated the nuclear translocation of NF-κB proteins and transactivation following bacterial stimulation. As with cytokine release, greater NF-κB transactivation was observed with heat-killed wild-type compared to the mutant. The two isolates induced the same pattern of nuclear NF-κB proteins: namely, p50/p65, p65/c-Rel and c-Rel/c-Rel. Stimulation of respiratory epithelium with bacteria, such as Pseudomonas aeruginosa (Dimango et al., 1998) or with peptidases (Dr J. Fink, unpublished observations) results in nuclear translocation of the p50/p65 heterodimer, although the significance of these differences is unclear. The release of both IL-6 and IL-8 was sensitive to the glucocorticoid, dexamethasone, a finding consistent with the suppression of NF-κB activity by this class of drugs (Auphan et al., 1995; Steer et al., 2000).

Human monocyte-derived macrophages were also shown to respond to whole bacteria and secretory/excretory products, and released IL-1β, IL-6, IL-8 and PGE2. However, differences between the wild-type and mutant were not observed. Because of the possibility of LPS- or LOS-induced stimulation, experiments were performed in the presence or absence of polymyxin B. This treatment did not inhibit responses, suggesting that these bacterial products were not involved in cytokine induction. Interestingly, polymyxin treatment enhanced responses for IL-1β and IL-8, and these findings are consistent with those from recent studies where enhanced IL-1β production was observed in mouse macrophages primed with LPS and then treated with polymyxin (Shimomura et al., 2003).

Relatively few studies have documented the induction of PGE2 release from respiratory epithelium or macrophages and none, to our knowledge, has shown that M. catarrhalis induces its release. This is despite previous studies showing multiple proinflammatory effects of PGE2 such as the induction of IL-6 from respiratory epithelium (Tavakoli et al., 2001), stimulation of T cells to produce IL-4 and IL-5 (Snijewint et al., 1993), and a role in dendritic cell maturation (Kalinski et al., 1997; Smith et al., 2002). On the basis of our data, PGE2 induced by M. catarrhalis may be significant. We also investigated whether a paracrine/autoaggregative mechanism was involved in M. catarrhalis-induced cytokine and mediator release as both PGE2 and IL-1β have been shown to have proinflammatory properties. For
example, PGE₂ can induce the release of cytokines, such as IL-6, from respiratory epithelium (Tavakoli et al., 2001) while IL-8 can be similarly induced by IL-1β (Standiford et al., 1990). However, inhibitors of PGE₂ and IL-1β activity did not alter the release of IL-6 or IL-8 from M. catarrhalis-stimulated A549 cells, confirming the absence of a PGE₂- or IL-1β-mediated autocrine feedback mechanism. The absence of a PGE₂-mediated effect in the A549 cell line contrasts with observations reported for the BEAS 2B cell line where it was shown to induce IL-6 release (Tavakoli et al., 2001). It is likely that these findings reflect differential expression of prostancoid receptor subtypes.

The identity of components present in culture supernatants of M. catarrhalis involved in cytokine and mediator induction are unclear at this stage. In other systems, cytokine or mediator release may be induced from respiratory epithelium or macrophages by a variety of molecules including enzymes, LOS and LPS (Zughaier et al., 1999; Clemans et al., 2000; Smith et al., 2002). Unlike the responses seen with the monocyte-derived macrophages, polymyxin B treatment did not affect cytokine release from A549 cells, suggesting only a small role for M. catarrhalis LOS (Clemans et al., 2000). This finding was not unexpected (Fink et al., 2003) as these cells do not appear to express the CD14 receptor. In contrast, previous studies on Haemophilus influenzae demonstrated polymyxin B treatment reduced bacterial-induced cytokine responses by up to 70%, indicating a possible role for LOS in that system (Clemans et al., 2000). The stimulatory components present in the culture supernatants derived from M. catarrhalis were also found to be heat stable and similar characteristics have been reported for bacteria such as P. aeruginosa (Massion et al., 1994) and Burkholderia cepacia (Fink et al., 2003). Given previous studies showing the proinflammatory potential for peptidoglycan (Keller et al., 1992), and relative heat stability, it would be of interest to determine its involvement, as well as other constituents of the culture supernatants, on the responses observed in our studies.

Recently, both the Hag protein and OMP-CD were shown to be adhesins for the A549 cells (Pearson et al., 2002; Holm et al., 2004) and, on this basis, should be specifically examined for cytokine-inducing properties; however, other OMP differences between the wild-type and the mutant were observed, suggesting that components other than Hag and OMP-CD are involved in cytokine modulation in respiratory cells. Such differences have not been previously reported using the isolates examined here (Kyd et al., 1998). It is likely that differences in the methods used to prepare OMP (EDTA vs. zwittergent) account for our findings as it has been reported that some OMP, e.g. UspA2, may not be efficiently extracted by the EDTA method. It should be emphasized that despite the differences between wild-type and mutant, both M. catarrhalis isolates were potent inducers of proinflammatory agents.

In summary, we have demonstrated that M. catarrhalis and its secretory/excretory products are potent proinflammatory stimuli for respiratory epithelium and macrophages. Heat-killed M. catarrhalis wild-type induced greater concentrations of proinflammatory cytokine from the A549 cell line than did its nonautoagglutinating variant but not from macrophages, suggesting that different receptors and/or stimulatory molecules are involved with the latter. Our findings are consistent with those showing that the wild-type isolate used here induced enhanced recruitment of neutrophils to the lungs of a mouse model of infection compared with the nonautoagglutinating mutant (Kyd et al., 1998), and with those showing an association between reduced cytokine-releasing potential with persistent bacterial infections (Bresser et al., 1997).

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