Comparison of *Moraxella catarrhalis* isolates from children and adults for growth on modified New York City medium and potential virulence factors

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Initial studies found that *Moraxella catarrhalis* isolates from adults that grew on modified New York City medium (MNYC⁺) that contained antibiotics selective for pathogenic neisseriae differed from strains that did not grow on this medium (MNYC⁻) in their potential virulence properties. It was predicted that higher usage of antibiotics to treat respiratory illness in children might result in higher proportions of MNYC⁺ isolates if antibiotics were an important selective pressure for this phenotype. Two of 100 adult isolates (2 %) were MNYC⁺, compared to 88 of 88 isolates (100 %) from children (P = 0·000). MNYC⁺ strains were serum-resistant and bound in higher numbers to HEP-2 cells that were infected with respiratory syncytial virus (RSV). Endotoxin from an MNYC⁺ isolate induced significantly higher pro-inflammatory response levels than endotoxin from an MNYC⁻ strain. MNYC⁺ adult isolates expressed haemagglutinins and bound in lower numbers to RSV-infected cells, but serum resistance was variable. All isolates from children were MNYC⁺, serum-resistant and bound in greater numbers to RSV-infected cells. These results indicate that both RSV infection and antibiotic usage select for the MNYC⁺ phenotype.

INTRODUCTION

*Moraxella catarrhalis* has become an important human pathogen that causes otitis media, sinusitis, conjunctivitis, acute exacerbation of chronic bronchitis, pneumonia, endocarditis, septicaemia and meningitis (Catlin, 1990). Its virulence factors and pathogenesis mechanisms remain unclear. Resistance to complement-mediated killing is an important factor in Gram-negative bacterial infections (Hol et al., 1993; Verduin et al., 1994). Adherence factors contribute to pathogenicity in the respiratory tract; haemagglutinins of *M. catarrhalis* have been examined as markers for virulence (Murphy et al., 1997; Fitzgerald et al., 1999).

Colonization by *M. catarrhalis* is highest in young children and declines to < 5 % in adults (Ingvarsson et al., 1982; Ejlertsen, 1991; Aniansson et al., 1992). Sixty-eight per cent of Danish children in the 1–48-month age-range with upper or lower respiratory tract infections were colonized by *M. catarrhalis*, compared to 36 % of uninfected children (P < 0·001). After recovery, the isolation rate in the infected group fell to that of the uninfected group (Ejlertsen et al., 1994). Colonization by *M. catarrhalis* is associated with respiratory syncytial virus (RSV) and parainfluenza virus infections (Korppi et al., 1990, 1992; Nohynek et al., 1991).

Binding of several respiratory pathogens to HEP-2 cells was increased significantly by RSV infection (Raza et al., 1993; Saadi et al., 1993; El Ahmer et al., 1996). Initial studies of *M. catarrhalis* were carried out with two strains: MC1 grew on modified New York City medium (MNYC) and bound in greater numbers to RSV-infected cells, whereas MC2 was sensitive to antibiotics in the medium and bound in significantly lower numbers to RSV-infected cells (El Ahmer et al., 1996).

Endotoxins of Gram-negative bacteria are important virulence factors. *Neisseria meningitidis* isolates that express the L(3,7,9) lipo-oligosaccharide (LOS) immunotype are isolated from approximately 90 % of patients (Jones et al., 1992); L(3,7,9) LOS induced significantly higher levels of interleukin (IL)-6 and tumour necrosis factor (TNF) from the THP-1 cell-line than other immunotypes (Braun et al., 2002). Differences in LOS structure of *M. catarrhalis* were used to classify different strains (Vanechouette et al., 1990;
Rahman & Holme, 1996; Holme et al., 1999), but no studies were performed to determine if oligosaccharide structure influenced inflammatory responses.

Our objectives were: (1) to test the hypothesis that there would be a higher proportion of MNYC⁺ strains among those isolated from children than from adults; (2) to determine if MNYC⁺ clinical isolates had virulence factors similar to those of strain MC1; (3) to compare outer membrane protein (OMP) patterns of strains with the MNYC⁺ or MNYC⁻ phenotypes; and (4) to compare inflammatory responses to LOS obtained from strains MC1 and MC2.

 METHODS

 Isolates of M. catarrhalis. M. catarrhalis isolates from adult sputum samples (n = 100) and six from children were obtained from the Diagnostic Laboratory, Department of Medical Microbiology, University of Edinburgh, UK. Strains MC1 and MC2 were obtained from our culture collection: MC1 grew on MNYC (Young, 1978) that contained antibiotics selective for pathogenic neisseriae, but MC2 was sensitive to antibiotics. Strains MC1 and MC2 were obtained from our Diagnostic Laboratory, Department of Medical Microbiology, University of Edinburgh, UK. Strains MC1 and MC2 were obtained from our culture collection: MC1 grew on MNYC (Young, 1978) that contained antibiotics selective for pathogenic neisseriae, but MC2 was sensitive to antibiotics. Isolates from children (< 14 years of age) were obtained from three sources: Dr M. F. Hanson, Central Microbiology Laboratories, Edinburgh, Scotland (n = 15); Dr M. W. Raza, Newcastle, England (n = 16); and Dr A. Skoczynska, Sera and Vaccines Central Research Laboratory, Warsaw, Poland (n = 51).

 Culture. MC1 and other antibiotic-resistant isolates were grown on MNYC; antibiotic-sensitive isolates were grown on Columbia agar with 8 M NaCl. MC1 and other antibiotic-resistant isolates were grown on MNYC; antibiotic-sensitive isolates were grown on Columbia agar with 8 M NaCl. Culture media were prepared from serum of a donor with blood-group O. It was absorbed with sensitized sheep red blood cells as described previously (Zorgani et al., 1992). The complement source was C with a suspension of MC1 and MC2.

 Isolation of LOS. Isolation of LOS of M. catarrhalis OMP. OMP of each isolate was obtained from bacteria grown overnight at 37 °C on BA in a humidified atmosphere with 10 % CO₂. Bacteria were harvested, washed three times in Tris buffer (0·01 M, pH 7·4), reuspended in 50 ml 0·01 M Tris buffer and broken by sonication. Unbroken cells were removed by centrifugation at 3000 g for 20 min. Supernatant was then centrifuged at 100 000 g for 60 min at 4 °C. The pellet was mixed with 20 ml 1 % (v/v) sodium N-lauroyl sarcosinate (Sarkosyl; Sigma) for 60 min at 37 °C with shaking (Hancock & Poxton, 1988). The remaining outer membrane–peptidoglycan complex was sedimented by centrifugation at 60 000 g for 60 min at 4 °C, reconstituted in pyrogen-free (PF) water and stored at −20 °C. Protein concentration was estimated by the method described by Bradford (1976), before separation by SDSPAGE.

 SDS-PAGE for M. catarrhalis OMP. OMPs were separated by SDS-PAGE by using the SDS-discontinuous system of Laemmli (1970) on a Mini-PROTEAN II cell (Bio-Rad). Equal volumes of protein sample and sample buffer (pH 6·8, double-strength) that contained 0·125 M Tris, 4 % SDS (w/v), 2 % 2-mercaptoethanol (v/v), 20 % glycerol (v/v) and 0·002 % bromophenol blue (w/v) were mixed and heated to 100 °C for 5 min. Approximately 20 μl sample was applied to each lane and the separating gel was carried out at a constant voltage of 100 V through the stacking gel (10 % acrylamide) and a constant voltage of 60 V through the separating gel (10 % acrylamide). Proteins were visualized by staining overnight with 0·5 % (w/v) Coomassie brilliant blue in 25 % (v/v) propan-2-ol, 10 % (v/v) glacial acetic acid. Gels were destained four times with 10 % (v/v) glacial acetic acid for 1 h intervals. Molecular size markers (Sigma) in the range 29–205 kDa were run in parallel. M. catarrhalis OMPs were of the same molecular mass as those published previously (Murphy, 1990).

 Binding to RSV-infected cells. Assays for binding of bacteria to HEP-2 cells and HEP-2 cells infected with RSV were carried out as described previously (Raza et al., 1993). Two RSV strains were used: RSV-A (Edinburgh strain; Ogilvie et al., 1981) and RSV-B (strain 18573).

 Isolation of LOS. Four strains were used as sources of LOS: MC1, MC2 and L3 and L6 immunotype strains of N. meningitidis; 6275 (B : 2a : P1·2 : L3) and M992 (B : 5 : P1·7,1 : L6), obtained from Dr Wendall Zollinger, Walter Reed Army Institute of Research, Washington, DC, USA. Strains were grown for 18 h in 5 % (v/v) CO₂ on human blood agar (Oxoid) supplemented with 10 % (v/v) lysed human blood (Scottish Blood Transfusion Service, Edinburgh). Bacterial growth was harvested from plates, washed in sterile PF PBS, centrifuged at 1000 g and resuspended in PF distilled water. LOS was extracted by the hot phenol/water method described by Hancock & Poxton (1988).

 Inflammatory responses induced by LOS of M. catarrhalis isolates. The method was used that was published previously (Braun et al., 2002). Immature human monocyte cell-line THP-1 was incubated for 72 h with 10⁻⁷ M VD3 to induce expression of CD14 cell-surface antigen (Schwende et al., 1996; James et al., 1997). Triplicate samples of differentiated cells were challenged for 6 h with 100 ng ml⁻¹ of LOS from the Monaxella or Neisseria strains or purified Escherichia coli endotoxin, strain 026 : B6 (Sigma) (Braun et al., 2002). Samples were centrifuged at 300 g for 10 min; the supernatant was then centrifuged at 1000 g for 10 min and assessed for TNFα by a bioassay with L929 cells (Delahoucke et al., 1995). IL-6 was measured with an ELISA (Gordon et al., 1999).

 Statistical methods. Bacterial binding data were assessed by estimation of relative binding of bacteria to virus-infected HEP-2 cells
compared to uninfected cells, based on paired t-tests applied to logarithms of binding indices (Raza et al., 1993; El Ahmer et al., 1999). For cytokine levels, the mean and standard deviation (SD) were calculated and Student’s t-test was applied using MINITAB software for Apple Macintosh. Regression and analysis of variance showed that cytokine levels were distributed normally. Probability values were calculated with a confidence interval of 5% against the negative control (treated with PBS only). Differences between isolates from children and adults were assessed by the χ² method with the Mantel–Haensel correction.

RESULTS

Growth on MNYC
Isolates from adults and children were tested for growth on MNYC in parallel with strains MC1 and MC2 as positive and negative controls, respectively. Only two of 100 isolates from adults grew on MNYC, compared to 88 of 88 isolates from children (P = 0.000).

Assessment of complement-mediated killing assay
Among 100 adult isolates tested, the two with the MNYC+ phenotype were serum-resistant. Of 98 isolates that did not grow on MNYC, 93 (95%) were resistant and 5 (5%) were sensitive to serum killing. For each serum-sensitive isolate in the test sample, viable count was reduced by ≥90% compared to the controls. For serum-resistant strains, there was no difference between c.f.u. counts for test and control samples. All isolates from children were resistant to serum killing in the assay.

Haemagglutination
Of 98 MNYC− isolates from adults, all agglutinated the group O blood cells, but MNYC+ isolates (n = 2) did not. A similar pattern was observed with the antibiotic-resistant (MC1) and antibiotic-sensitive (MC2) isolates from the original study. Among the isolates from children, 81/88 (92%) expressed haemagglutinating activity.

Attachment of M. catarrhalis isolates to RSV-infected cells
In four experiments, a ratio of 400 bacteria per cell was used to assess the effect of infection with RSV-A or RSV-B on binding of M. catarrhalis isolates to HEp-2 cells. Compared to binding to uninfected cells, MNYC+ isolates from both children and adults bound in significantly greater numbers to cells infected with RSV-A (P < 0.01) or RSV-B (P < 0.01). Each of 48 MNYC− strains tested bound in significantly lower numbers to cells infected with RSV-A (P < 0.01) or RSV-B (P < 0.05). Results for two MNYC−, serum-sensitive and ten MNYC−, serum-resistant isolates are shown in Table 1. In contrast to the results obtained with isolates from adults, all isolates from children bound in significantly higher numbers to cells infected with RSV-A (P < 0.01) or RSV-B (P < 0.01).

Table 1. Binding of two antibiotic-sensitive, serum-sensitive strains (S5 and S6) and ten antibiotic-sensitive, serum-resistant strains (S7–S16) to HEp-2 cells infected or uninfected by RSV-A or RSV-B (mean of four experiments).

Results are expressed as mean binding index (BI) [= percentage of cells with fluorescence above control × mean fluorescence of the population (Raza et al., 1993)].

<table>
<thead>
<tr>
<th>Strain</th>
<th>Uninfected cells</th>
<th>RSV-A-infected cells</th>
<th>RSV-B-infected cells</th>
<th>Uninfected cells, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>S5</td>
<td>21167</td>
<td>16776</td>
<td>16402</td>
<td>55–87 (P = 0.05)</td>
</tr>
<tr>
<td>S6</td>
<td>19377</td>
<td>13732</td>
<td>16100</td>
<td>64–78 (P = 0.01)</td>
</tr>
<tr>
<td>S7</td>
<td>20634</td>
<td>16739</td>
<td>15481</td>
<td>76–87 (P = 0.01)</td>
</tr>
<tr>
<td>S8</td>
<td>26678</td>
<td>21096</td>
<td>19772</td>
<td>74–85 (P = 0.01)</td>
</tr>
<tr>
<td>S9</td>
<td>19993</td>
<td>15870</td>
<td>15875</td>
<td>71–89 (P = 0.01)</td>
</tr>
<tr>
<td>S10</td>
<td>15896</td>
<td>11791</td>
<td>11696</td>
<td>67–82 (P = 0.01)</td>
</tr>
<tr>
<td>S11</td>
<td>18690</td>
<td>13093</td>
<td>13144</td>
<td>61–79 (P = 0.10)</td>
</tr>
<tr>
<td>S12</td>
<td>16927</td>
<td>12596</td>
<td>12032</td>
<td>68–82 (P = 0.01)</td>
</tr>
<tr>
<td>S13</td>
<td>22769</td>
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<td>16799</td>
<td>59–93 (P = 0.05)</td>
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<tr>
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<td>21505</td>
<td>15420</td>
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</tr>
<tr>
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<td>11237</td>
<td>11542</td>
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<tr>
<td>S16</td>
<td>21398</td>
<td>15738</td>
<td>13917</td>
<td>63–84 (P = 0.01)</td>
</tr>
</tbody>
</table>
Comparison of OMPs among isolates

OMP profiles for four MNYC+ isolates [two from adults, one from a child and MC1 (strains R1–R4 in Fig. 1)] were compared to those of MNYC− isolates from adults (27 serum-sensitive and three serum-resistant isolates) (Fig. 1).

The two MNYC+, serum-resistant isolates from adults and strain MC1 lacked bands at 50 and 81 kDa, proteins which were present in MNYC− isolates. MNYC+ isolates of *M. catarrhalis* had a distinct band at 29 kDa that was not present in MNYC− isolates. OMP patterns for antibiotic-sensitive, serum-resistant and antibiotic-sensitive, serum-sensitive strains were similar.

All isolates from children expressed the 50 and 81 kDa OMPs. The OMP profiles for isolates from children all showed a similar pattern, except for those that did not agglutinate erythrocytes. These strains had a distinct band at 25 kDa that was not present in isolates that agglutinated erythrocytes or any isolate from adults.

Table 2 assesses the data with reference to source of the strain (adult or child) and Table 3 assesses the data with reference to growth of the strains on MNYC.

Comparison of inflammatory responses induced by LOS from different species

In six independent experiments with VD3-differentiated THP-1 cells, highest TNFα levels were obtained with LOS from meningococcal immunotype strains L3 (181·2 ± 8·31 IU ml⁻¹) and L6 (132·23 ± 9·02 IU ml⁻¹). TNFα levels for MC1 (125·0 ± 14·2 IU ml⁻¹), MC2 (90·2 ± 22·4 IU ml⁻¹) and *E. coli* (89·4 ± 5·34 IU ml⁻¹) were all significantly lower than those elicited by immunotype L3 LOS. TNF levels elicited by MC2 were similar to those elicited by *E. coli* endotoxin, but significantly lower than those for immunotype L6 and MC1. TNF levels elicited by MC1 LOS were similar to those elicited by LOS of immunotype L6.

DISCUSSION

The current study was initiated to examine further some differences noted between strains MC1 and MC2, which suggested that MC1 might express more virulence characteristics. The first objective was to test the hypothesis that the high incidence of antibiotic usage among children would result in a higher proportion of strains with the MNYC+ phenotype. Several proposed virulence factors were present in the majority of isolates from both children and adults.
serum resistance, haemagglutinins and 81 kDa OMP associated with serum resistance. The major differences were in growth on MNYC, binding of individual strains to RSV-infected cells and expression of the 29 kDa protein that appears to be associated with increased binding of M. catarrhalis to RSV-infected cells (Table 2).

When assessed for growth on MNYC, regardless of the source of the isolates (Table 3), the major differences were in relation to binding to RSV-infected cells and expression of the 29 kDa OMP.

Published reports that concern resistance of M. catarrhalis to killing by normal human serum indicate that many isolates are resistant to this complement-mediated lysis (Chapman et al., 1985; Jordan et al., 1990; Soto-Hernandez et al., 1989). The study by Jordan et al. (1990) attempted to correlate serum resistance and disease associated with M. catarrhalis; it suggested that strains isolated from infected sites are more likely to be serum-resistant than strains isolated from sputum of healthy persons. The majority of isolates from both children and adults were serum-resistant. There were three groups based on growth on MNYC and killing by pooled human serum: MNYC+, serum-resistant; MNYC−, serum-resistant; and MNYC−, serum-sensitive.

Studies of resistance of M. catarrhalis to complement-mediated killing found wide variation in the proportions of serum-resistant strains, ranging from 0 to 95% (Brorson et al., 1976; Chapman et al., 1985). A study by Verduin et al. (1994) found that only 10% of clinical isolates obtained from adults were serum-sensitive, a similar value to our finding of 5% serum-sensitive isolates in this study.

The 81 kDa protein (CopB) is associated with serum resistance. A mutant strain that lacked this protein was serum-sensitive (Helminen et al., 1993a). Reintroduction of CopB into this mutant abolished the serum-sensitive phenotype, confirming that CopB expression plays a direct or indirect role in serum resistance. A mAb to the 81 kDa protein bound to the majority (70%) of 23 M. catarrhalis strains tested (Helminen et al., 1993b), but there was no information about serum sensitivity or resistance of these strains. The majority (95%) of isolates from adults in our study expressed the 81 kDa protein and were serum-resistant. The protein was present in five serum-sensitive strains and MC2, but absent in MNYC+, serum-resistant isolates from adults. Each isolate from children expressed the 81 kDa protein and was serum-resistant.

The complexity of serum-resistance mechanisms operative in similar bacteria (e.g. Neisseria gonorrhoeae) (Britigan & Cohen, 1985; Parsons et al., 1989) suggests that serum resistance in M. catarrhalis is likely to be mediated by more than one mechanism. Differences in endotoxin of other Gram-negative species have been associated with serum sensitivity and antibiotic resistance (Nelson & Roantree, 1967). LOSs of strains MC1 and MC2 differed significantly in their ability to induce inflammatory responses from human monocyte cell-line THP-1. Haemagglutination activity associated with a 200 kDa protein does not appear to be associated with serum resistance, as five serum-sensitive strains from adults and MC2 agglutinated erythrocytes. For adult isolates, haemagglutinating activity was significantly associated with decreased binding to RSV-infected cells. In contrast, all isolates from children expressed haemagglutinins and bound in greater numbers to RSV-infected cells.

Decreased binding of strain MC2 to RSV-infected cells was the only exception we found to enhanced binding of a variety of pathogenic species to virus-infected cells. The same pattern of significantly decreased binding to RSV-infected cells compared to uninfected cells was observed for all 48 antibiotic-sensitive isolates from adults.

Patients with serological evidence of M. catarrhalis infection often have concomitant viral infection caused by RSV (Korppi et al., 1992). Although the majority of MNYC− strains from adults were serum-resistant, these strains might colonize RSV-infected patients less readily than MNYC+ strains that bind in greater numbers to RSV-infected cells. Current findings indicate that there is an association between increased binding of M. catarrhalis to RSV-infected cells, the 29 kDa protein and growth on MNYC.

Interactions observed for strains MC1 and MC2 and other MNYC− isolates are unique to RSV-infected cells. Both MC1 and MC2 bound in significantly greater numbers to HEp-2 cells infected with influenza A virus (El Ahmer et al., 1999).

Meningococcal LOS is the most potent inducer of inflammatory responses among Gram-negative endotoxins. LOS obtained from MC1 induced significantly higher levels of IL-6 and TNF than LOS from MC2, and the IL-6 responses were similar to those produced by LOS of two meningococcal immunotype strains. Further studies on LOS structures of M. catarrhalis strains are needed to determine if, as in meningococcal disease, inflammatory responses to different LOS structures contribute to severity of disease (Braun et al., 2002).

The present study found significant differences between M. catarrhalis isolates from children and adults. Based on these findings, we speculate that both RSV infection and antibiotic usage might select for the MNYC+ phenotype. The latter is supported by in vitro studies (data not shown) in which mutants of an MNYC−, serum-sensitive strain selected for resistance to penicillins, erythromycin, azithromycin, ciprofloxacin or cephalosporins grew on MNYC and were serum-resistant.

REFERENCES
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