Porphyrin ring source can alter the outer membrane protein profile of non-typable *Haemophilus influenzae*

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Summary. Porphyrin ring source appears to alter the outer membrane protein (OMP) profile of some, but not all, non-typable (NT) *Haemophilus influenzae* strains isolated from sputum. When haemin was replaced with protoporphyrin IX, 41% of strains examined produced increased amounts of a polypeptide of 84 Kda and new OMPs of either 120 or 150 Kda. Immunoblotting with paired patient's sera revealed that antibodies reactive with these proteins were present, demonstrating OMP antigenicity and expression *in vivo* and indicating that these isolates of NT *H. influenzae* may display an altered OMP phenotype when growing in the human lung.

Introduction

Non-typable (NT) *Haemophilus influenzae* strains are frequently cultured from purulent lower respiratory tract secretions of patients suffering from chronic bronchitis, bronchiectasis and cystic fibrosis (May *et al.*, 1972; Smith *et al.*, 1976; Murphy and Apicella, 1987). Several authors have examined the outer membrane protein (OMP) profile of NT *H. influenzae* (see Murphy and Apicella, 1987). Despite an apparently constant OMP pattern during the growth cycle (Murphy *et al.*, 1983) and after repeated subculture of individual strains (Barenkamp, *et al.*, 1982; Murphy *et al.*, 1983), separate isolates of NT *H. influenzae* show great variation in OMP profiles (Loeb and Smith, 1980; Barenkamp *et al.*, 1982; Murphy *et al.*, 1983; Hansen *et al.*, 1985). Although *H. influenzae* type b demonstrates some measure of phenotypic OMP response to altered conditions of culture (Loeb and Smith, 1980; Williams and Brown, 1985), notably haem limitation (Coulton and Pang, 1983; Stull, 1987) and iron restriction by use of transferrin (Herrington and Sparling, 1985) or iron depletion (Pidcock *et al.*, 1988) *in vitro*, few data are available on alteration of OMP profiles amongst NT *H. influenzae* strains.

Although normal human sera usually contain antibodies that react with *H. influenzae* OMPs (Hansen *et al.*, 1985; Spinola *et al.*, 1986), probably

reflecting nasopharyngeal colonisation, convalescent sera from some patients with pneumonia caused by NT *H. influenzae* show quantitative increases in OMP-directed antibodies as well as the development of antibodies to OMPs not recognised by acute phase serum (Hansen *et al.*, 1985). Conversely, nasopharyngeal colonisation of children with NT *H. influenzae* involving sequential acquisition and loss of strains with distinct OMP profiles appears to be accompanied by few changes in serum antibody response (Spinola *et al.*, 1986); interestingly, this latter study demonstrated that OMPs not easily observed on sodium dodecyl sulphate—polyacrylamide gel electrophoresis (SDS-PAGE) could be detected by immunoblotting techniques, suggesting potent antigenicity of minor proteins. However, OMP antigens used in these studies (Hansen *et al.*, 1985; Spinola *et al.*, 1986) were of only one phenotype as expressed *in vitro*. Bacterial OMPs phenotypically expressed *in vivo* during growth in lungs have been shown to induce specific antibodies. These antibodies react with antigens present in bacteria grown *in vivo* and with antigens in bacteria grown *in vitro* under conditions that induce production of such OMPs (Anwar *et al.*, 1984; Cochrane *et al.*, 1988). Therefore, immunological recognition of novel OMP antigens phenotypically expressed *in vitro* may indicate production of these antigens *in vivo*. The purpose of this study was to examine the OMP phenotype response of NT *H. influenzae* resulting from substitution of porphyrin ring source and to determine expression of OMPs *in vivo* on the basis of the immunological responses detected in chronically infected patients.

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Materials and methods

Bacterial strains and culture conditions

The NT *H. influenzae* strains used in this study were isolated from purulent sputum samples from patients with bronchiectasis; samples of patients' serum were obtained at the same time. None of the patients was receiving antibiotics at the time. *H. influenzae* isolates were confirmed to be NT by failure to agglutinate with type-specific antisera. Bacteria were cultured in Brain Heart Infusion (BHI) Broth supplemented with growth yield excess NAD (5 mg/L) and with excess (5 mg/L) or growth yield limiting (0.25 mg/L) concentrations of either haemin or protoporphyrin IX (PPIX). To achieve iron-restricted conditions the iron chelator Desferal (Ciba-Geigy Ltd, Horsham Surrey) was added at a concentration of 30 μ M to cultures containing growth yield limiting concentrations of either haemin or PPIX. Growth was initiated by inoculating several colonies from chocolate-agar plates into 200 ml volumes of supplemented BHI contained in 1-L flasks fitted with polyurethane closures. Cultures were incubated at 37°C in an orbital shaking incubator (180 rpm) in air, i.e., without CO₂ enrichment. Growth was monitored by measurement of optical density at 470 nm.

Preparation of bacterial outer membranes

Outer membranes (OMs) were prepared from stationary phase bacteria (15-h cultures) by sarkosyl (sodium N-lauryl sarcosine; 2% w/v final concentration) as described previously (Kadurugamuwa *et al.*, 1987), except that centrifugation at 180 000 *g* was used to pellet the OMs. The protein content of each OM preparation was determined by the method of Lowry *et al.* (1951).

SDS-PAGE and immunoblotting

OMPs were separated by SDS-PAGE on 10% gels (5 cm in length) according to the method of Lugtenberg *et al.* (1975); 10 μ g of protein was loaded on to each well. Separated polypeptides were stained with Coomassie Blue and their molecular size was determined by comparison with standards of known molecular weight.

OMPs previously separated by SDS-PAGE were electrophoretically transferred on to nitrocellulose paper and antigenic sites were visualised by the method of Towbin *et al.* (1979). Although transfer of proteins was qualitatively complete, as judged by amido black staining of blotted nitrocellulose, transfer was quantitatively incomplete as judged by Coomassie Blue staining of blotted gels. Each *H. influenzae* isolate was probed with homologous patient's serum (1 in 25 dilution); protein A-peroxidase conjugate was used to visualise immune complexes.

Results

The effect of changing porphyrin source on the in-vitro growth of NT *H. influenzae* strain HM1 is

shown in fig. 1; the growth responses of other isolates were similar (data not presented). Although substitution of haemin 5 mg/L with PPIX did not alter the final cell yield or growth rate, reduction of the initial haemin or PPIX concentration from 5 mg/L to 0.25 mg/L resulted in 50% and 54% reductions in final cell yield, respectively, but had no effect on growth rate (fig. 1). Increasing the porphyrin source concentration from 5 mg/L to 10 mg/L changed neither the growth rate nor the final cell yield (data not presented).

Fig. 2a shows that strain HM1 grown in the presence of PPIX displayed an altered OMP profile compared with cultures grown with excess haemin; a band or bands of 84 Kda was (were) produced in increased amounts, and a novel 150-Kda polypeptide appeared. Interestingly, despite loading equal amounts of protein, major OMPs from strain HM1 grown in the presence of PPIX (or growth limiting concentrations of haemin plus Desferal) appeared to be present in increased amounts compared with those from bacteria grown with excess haemin (figs. 2a and 3); however, it should be noted that this may also reflect more intense staining of OMPs produced during the former growth condition. The reason for this is unclear, but it appears to be a constant feature of membranes prepared from strain HM1, although it was not observed with any other strain examined. Strain HM2 also expressed OMP profile changes with an additional protein of 120 Kda when grown in the presence of PPIX (fig. 2).

Supplementary experiments with strain HM1 (fig. 3) indicated that these OMP changes also occurred in bacteria grown in the presence of excess PPIX (5 mg/L) plus Desferal, and with growth yield limiting concentrations of PPIX (0.25 mg/L) in the presence or absence of Desferal, but not in haemin depleted (0.25 mg/L) media. Cultures in haemin depleted medium plus Desferal (fig. 3, lane 4) displayed increases in production of a band of 84 Kda compared with cultures grown in excess haemin. A doublet at 84 Kda appeared in membranes of cells grown with PPIX (with or without Desferal); however, this was inconsistently resolved (figs. 2a and 3). Additionally, a 150-Kda protein was faintly visible in membranes of cultures grown in haemin-depleted medium plus Desferal, but this was expressed too weakly to be photographed, in contrast to those membranes obtained from cultures grown with PPIX with or without Desferal (fig. 3). Furthermore, increases in a minor polypeptide below the 84-Kda protein (approximately 78 Kda) occurred during growth with PPIX or haemin in the presence of an iron chelator.

Fig. 1. The effect of initial haemin and PPIX concentration on the growth curve of *H. influenzae* strain HM1 at 37°C. Bacterial growth in BHI containing haemin (●, ■) or PPIX (○, □) 5 mg/L (●, ○) or 0.25 mg/L (■, □), was monitored turbidimetrically.

No apparent changes in OMP profile were seen with several other NT *H. influenzae* isolates when haemin was replaced by PPIX (data not shown). Out of a total of 24 strains examined, 10 (41%) displayed a phenotypic response to substitution of haemin with PPIX by alteration of the OMP profile; in the remaining 59%, the OMP profile remained constant irrespective of the source of porphyrin ring. Amongst those strains in which a phenotypic change was demonstrated, 10 showed increases in the 84-Kda polypeptide and four showed increases in the 78-Kda polypeptide, whereas three and seven expressed the novel 120-Kda or 150-Kda OMP, respectively.

Fig. 4 shows immunoblots of *H. influenzae* strains HM1 (fig. 4a) and HM2 (fig. 4b), demonstrating antibody activity directed against phenotypically expressed OMPs. Polypeptides of 150, 120 and 98 Kda present in membranes obtained from strain HM1 grown in media with PPIX were recognised by immune serum even though the 120-Kda polypeptide was barely visible on the corresponding SDS-PAGE gel (fig. 2a). Although with strain HM2 the 84-Kda OMP(s) increased by growth with PPIX or by growth limiting concentrations of haemin was strongly recognised by immune serum (fig. 4b), the novel PPIX-induced polypeptides of 120 and 150 Kda did not appear to be recognised. With the

Fig. 2. Alteration of the OMP profile of NT *H. influenzae* by porphyrin ring source. Sarkosyl-extracted OMs were obtained from bacteria grown in the presence of haemin (1) or PPIX (2) and analysed by SDS-PAGE. Panel **a** represents the OMP profile of strain HM1, and panel **b** the profile of strain HM2.

Fig. 4. Immunoblot of phenotypically altered OMPs of NT *H. influenzae*. OMPs from bacteria grown in the presence of excess (5 mg/L) haemin (1) or PPIX (2) were electrophoretically transferred to nitrocellulose and treated with homologous patient's serum; immune complexes were visualised with protein A peroxidase conjugate. Panel **a** represents the immunoblot obtained with OMPs from strain HM1 and panel **b** the immunoblot from strain HM2.

Fig. 3. Alteration of the OMP profile of *H. influenzae* strain HM1 by porphyrin ring source and iron restriction. Sarkosyl-extracted OMs were obtained from bacteria grown in the presence of excess haemin (lane 2), growth yield limiting haemin (lane 3), growth yield limiting haemin plus Desferal (lane 4), excess PPIX (lane 5), excess PPIX plus Desferal (lane 6), growth yield limiting PPIX (lane 7), and growth yield limiting PPIX plus Desferal. Mol. wt standards are shown in lane 1.

10 strains in which phenotypic OMP profile changes were demonstrated, all the paired patients' sera recognised the 84-Kda band and the reaction was consistently stronger when antigen was prepared

from bacteria grown with PPIX; 20% recognised the 120-Kda and 60% the 150-Kda OMP. The minor 78-Kda band did not appear to be strongly recognised in any case. Interestingly, homologous sera recognised a 98-Kda protein present in OMs prepared from strain HM1 grown with PPIX that was not visible in immunoblots from any other strain (fig. 4, lane 2).

Discussion

H. influenzae can acquire haem from a variety of substrates which are found *in vivo*, including haemoglobin, haemoglobin-haptoglobin, and haem-haemopexin (Stull, 1987; Pidcock *et al.*, 1988). Whereas these serum proteins may be utilised by bacteria growing in blood, their availability in sputum is not known. However, increased serum transudation into the lung appears to be a feature of infections overlying chronic obstructive pulmonary disease (Stockley and Burnett, 1980) and may account for the presence in sputum of haem sources

usable by *H. influenzae*. Both type b and NT *H. influenzae* strains can acquire iron from transferrin but not, generally, from lactoferrin (Herrington and Sparling, 1985; Pidcock *et al.*, 1988; Schryvers, 1988). The latter is found in mucosal secretions and, unlike transferrin, is present in increased concentrations in fluid obtained by lung lavage following experimental challenge by gram-negative bacteria (La Force *et al.*, 1986). The source of haem and iron for NT *H. influenzae* growing on mucosal surfaces or in the lung remains to be determined.

OMP changes have been shown to occur when *H. influenzae* type b strains are subject to haem or iron restriction. Different strains vary in response to haem limitation; they either produce 38-Kda OMP (Stull, 1987) or increase production of a 43-Kda OMP (Coulton and Pang, 1983). Iron restriction of type b strains results in the induction of a group of 90–95-Kda OMPs in BHI broth (Herrington and Sparling, 1985) or in defined medium (Pidcock *et al.*, 1988); production of additional OMPs appears to respond to the level of iron in the medium (Pidcock *et al.*, 1988). Interestingly, growth with PPIX has been a prerequisite for studies involving iron restriction, but the effect of substitution of porphyrin ring source on type b strains is not clear (e.g. Stull, 1987; cf. Pidcock *et al.*, 1988), although induction of a 73-Kda OMP in type b strains by PPIX has been reported (Williams and Brown, 1985). The changes described in these studies were not observed in the present study; this may be due to different experimental conditions, but may also be due to differences in the strains used. It should be noted that several strains isolated in the present study did not respond to the conditions tested; this was similar to the non-responsiveness reported previously (Williams and Brown, 1985).

The present study has shown that the OMP profiles of some NT *H. influenzae* can change when the bacteria are grown in the presence of PPIX. Although not all strains of *H. influenzae* responded phenotypically to substitution of porphyrin ring source, production of the 84-, 120- and 150-Kda OMPs appeared to respond to growth with PPIX. At least with strain HM1, the 84-Kda band was resolved inconsistently as a doublet, the lower band apparently produced in response to PPIX utilisation and production of the upper band increased with PPIX utilisation or during growth with haemin in the presence of an iron chelator. This latter growth condition also increased the production of a 78-Kda OMP which also occurred during PPIX utilisation.

Some of these proteins (especially the 84-, 120- and 150-Kda proteins) appear to be antigenic, they

were recognised by serum antibodies from chronically infected patients, and, therefore, are likely to be expressed by bacteria growing *in vivo*. However, it should be noted that the 120- and 150-Kda OMPs did not react consistently with homologous antisera and, although the reason for this is unclear, it may represent a failure of antibody induction, possibly due to a lack of antigen expression. The inconsistent resolution of the 84-Kda doublet prevents the use of the immunoblotting procedure to discern whether a single component of the doublet, or both components, are antigenic and produced *in vivo*. Interestingly, OMPs not readily detectable by SDS-PAGE were observed by immunoblotting when membranes prepared from bacteria grown with PPIX were used as antigen. However, these antibodies are probably not protective, at least in patients with bronchiectasis, as they are present in serum during the course of an infective exacerbation.

H. influenzae has the ability to utilise PPIX to form haem by the insertion of iron into the porphyrin ring by the enzyme ferrochelatase (White and Granick, 1963). However, it may be unlikely that an intracellular intermediate in mammalian haem synthesis such as PPIX (Israels *et al.*, 1975) is a significant physiological substrate, compared with haemin, when *H. influenzae* is growing *in vivo*, so that the reason for induction of OMP changes that produce membranes more like those found *in vivo* by PPIX is unclear. It is tempting to speculate that growth utilising PPIX as a porphyrin source may provide a signal for the induction of iron regulated OMPs. This is supported by the observation that the addition of an iron chelator to media containing PPIX apparently causes no further OMP profile changes, compared with PPIX alone (fig. 3). Furthermore, although haemin can apparently provide for the entire iron requirement of *H. influenzae* (Pidcock *et al.*, 1988), OMP profile changes similar to those induced by PPIX occur when Desferal is added to media containing growth yield limiting concentrations of haemin (fig. 3), conditions likely to reduce the concentration of iron available for the growth of *H. influenzae*. In this way, growth in media containing PPIX in place of haemin may mimic conditions of iron restriction, resulting in the expression of new OMPs. However, provision of PPIX as a porphyrin ring source may induce non-specifically a phenotypic change *in vitro* in some NT *H. influenzae* strains to produce OMP profiles more similar to those produced *in vivo*. The functions of these proteins, and the reasons for the apparent lack of their production by all strains, remain to be elucidated.

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