Immunogenic outer-membrane proteins of *Haemophilus influenzae* type b in infection

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Outer-membrane proteins (OMPs) from *Haemophilus influenzae* type b (strain Eagan), grown both *in vitro* (broth) and *in vivo* (rat intra-peritoneal), were separated by SDS-PAGE. The major OMPs were present in both growth conditions although the amounts of OMP a and OMP d were reduced in rat-grown organisms. There were strong additional bands in *in-vivo*-grown organisms at 51 and 92 kDa. Antiserum was raised in rabbits against *in-vivo*-grown bacteria, and absorbed with lysates of *in-vitro*-grown bacteria. This serum was used in Western blot analysis of OMPs from *in-vitro*- and *in-vivo*-grown cells to identify immunogenic proteins present in infection. These infection-associated OMPs had apparent molecular masses of 43 kDa, 48 kDa, 81 kDa and > 200 kDa. Bands of reactivity, of the same molecular mass as some of these, were found on immunoblots when rat and human convalescent sera were used as the source of primary antibody. In particular, a band of 81 kDa was recognized by pooled rat and three human convalescent sera.

Introduction

Information concerning virulence factors and protective antigens of micro-organisms expressed *in vivo* is crucial in understanding disease processes and the design of antimicrobial and vaccine strategies. Bacteria can alter their metabolism and composition in response to environmental change (Brown & Williams, 1985). Examples of environmentally-controlled virulence factors include temperature regulation of fimbriae in *Escherichia coli* (Jacobs & de Graaf, 1985), osmotic regulation of cholera toxin (Miller *et al.*, 1987) and pilus production (Taylor *et al.*, 1987) in *Vibrio cholerae*, and iron regulation of *Corynebacterium diphtheriae* toxin (Murphy *et al.*, 1974). Many virulence factors are expressed only in environmental conditions similar to those found in the host (Moulder, 1985). In general, when studying bacterial virulence, investigators have used organisms grown in conventional broth media. Thus important antigens or virulence factors may have been overlooked.

Haemophilus influenzae type b (Hib) is one of the

commonest causes of meningitis in young children. The organism can colonize the nasopharynx (reviewed by Moxon, 1990) pass to the blood stream (possibly by epithelial/endothelial interactions), multiply in the vascular compartment (Rubin *et al.*, 1985) and/or macrophages (Williams *et al.*, 1991), cross the blood/CSF barrier by unknown mechanisms and multiply in the CSF. Adaptation and survival in these contrasting environments may require the expression of different surface components. The outer-membrane proteins (OMPs) of Hib have been extensively investigated to identify possible virulence factors and vaccine candidates. One approach has been to use acute and/or convalescent sera to investigate the immunological response of patients and experimental animals to OMPs by Western blot analysis (Hansen *et al.*, 1981; Gulig *et al.*, 1982; Loeb & Smith, 1982; Erwin *et al.*, 1988). However, without exception, outer membranes isolated from broth-grown organisms were used in these studies. In this study we have isolated outer-membrane material from Hib grown either in broth or harvested directly from infected rats, and used rabbit serum raised against whole cells of *in-vivo*-(rat)-grown bacteria to identify immunogenic proteins 'novel' to infection. Bands were observed on immunoblots of the same molecular mass as those present in infection when convalescent sera from rats and patients were used as the source of primary antibody.

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Abbreviations: ALP, alkaline phosphatase; BHI, brain heart infusion; Hib, *Haemophilus influenzae* type b; i.p., intra-peritoneally.

Methods

Bacterium and in vitro growth conditions. Hib strain Eagan (Anderson *et al.*, 1972) was grown to exponential phase ($OD_{490} = 0.27$) in Brain Heart Infusion (BHI) broth supplemented with $2 \mu\text{g ml}^{-1}$ protoporphyrin IX and $2 \mu\text{g ml}^{-1}$ NAD at 37°C on an orbital shaker (200 r.p.m.). Overnight Fe-restricted cultures were obtained by growth in supplemented BHI broth plus $100 \mu\text{M}$ -ethylenediaminedi-(*o*-hydroxy)phenylacetic acid (Morton & Williams, 1990), the latter compound having been freed from contaminating Fe by the method of Rogers (1973). Eagan was used because of the considerable data that has accumulated on this organism, leading to the suggestion that it should be adopted as a reference type b strain for investigations of Hib pathogenicity (Kroll & Moxon, 1989).

Growth of organisms in vivo (rat). Exponential-phase cells were harvested, washed once and resuspended in PBS at a concentration of 5×10^3 c.f.u. ml^{-1} and 0.1 ml of this was inoculated intra-peritoneally (i.p.) into 5-day-old Sprague Dawley rat pups. Twenty-four hours later, i.p. washings [1 ml per rat in MEM (Gibco)] were aseptically collected, pooled and centrifuged (150 g, 5 min, 4°C) to remove peritoneal cells. The supernatant was recentrifuged (5000 g, 20 min, 4°C), the pellet was resuspended in double distilled water to lyse remaining peritoneal cells and bacteria were collected by recentrifugation (5000 g, 20 min, 4°C). The pellet was resuspended in 1 ml 10 mM-HEPES (pH 7.4) and stored at -20°C until required. Pooled blood obtained by cardiac puncture from 3 animals, 4 weeks post i.p. infection with Eagan, was the source of convalescent rat serum (Hansen *et al.*, 1981). Sera were stored at -70°C as 0.1 ml samples until required.

Inoculation of rabbits. Rabbit antiserum was raised against *in-vivo*-grown bacteria (anti-*in-vivo*) as follows. Freshly-harvested *in-vivo*-grown bacteria were washed twice and resuspended in PBS to a concentration of 4×10^8 c.f.u. ml^{-1} and 0.4 ml was inoculated into the ear vein of New Zealand White rabbits (2 kg) at weekly intervals. After 9 weeks, rabbits were bled from the marginal ear vein of the opposite ear and serum was stored at -20°C in small aliquots until required.

Convalescent sera. Three patients with a positive diagnosis of Hib meningitis were the source of human convalescent sera. Age at diagnosis was 12 months (C), 3 months (D) and 14 months (E) and convalescent sera was collected at 3, 4 and 5 weeks post diagnosis, respectively.

Isolation of outer membranes and SDS-PAGE. Outer-membrane material was obtained by extraction of crude cell envelopes with Triton X-100/10 mM- Mg^{2+} (Schnaitman, 1971). Protein concentration was measured by a modification (Stoscheck, 1990) of the method of Bradford (1976) using bovine serum albumin as standard. Approximately 0.5–1.0 μg of membrane protein was obtained from each rat. SDS-PAGE was performed on 12.5% (w/v) acrylamide gels as described previously (Allan *et al.*, 1987) and OMPs were stained with silver using the Amersham kit (RPN.17).

Antibody absorption. Rabbit anti-*in-vivo* serum (1 ml) was absorbed twice with sonicates of 1×10^{11} c.f.u. of *in-vitro*-grown organisms in 0.5 ml PBS at 0°C for 30 min. Following centrifugation (13 500 g, 10 min, 0°C) absorbed antiserum was stored at -70°C until required. Any dilution of serum with PBS was taken into account, and a volume of absorbed serum equivalent to 0.1 ml of original serum was used in the immunoblots.

Immunoblotting. Proteins were transferred to 0.45 μm nitrocellulose sheets by the method of Towbin *et al.* (1979). Membranes were soaked in 10 mM-Tris/0.9% NaCl (pH 7.4)/0.3% Tween 20 (TN20) for 1 h to block non-specific binding sites. The nitrocellulose strips with transblotted proteins were incubated in primary antibody, consisting of

0.1 ml rabbit, rat or human sera in 20 ml TN20, for 2 h at room temperature, and washed extensively in TN20. This was followed by incubation (2 h at room temperature) in secondary antibody (1/1000 dilution in TN20) comprising affinity-isolated goat anti-rabbit IgG alkaline phosphatase (ALP) conjugate, goat anti-rat IgG ALP conjugate or goat anti-human IgG ALP conjugate (all from Sigma) as appropriate. After washing in buffer without Tween 20, blots were developed using the method of Blake *et al.* (1984). All washing and incubation procedures were performed on an orbital shaker (60 r.p.m.).

Results

OMP profiles

The OMP profiles of *in-vitro*- and *in-vivo*-grown organisms are shown in Fig. 1. The major OMPs were present in both growth conditions, although the amounts of OMP a and OMP d were reduced in rat grown organisms. There were strong additional bands in *in-vivo*-grown organisms at 51 and 92 kDa, and these were also prominent on Coomassie blue R-250 staining (data not shown). No detergent-insoluble material was found from i.p. washings of control animals or from inflammatory cell preparations obtained after the first centrifugation following direct harvesting (see Methods). The same result was found when sham-infected rats were used as controls (van Alphen *et al.*, 1990).

Identification of proteins present in infection and their immunogenicity to convalescent sera

OMPs present in infection were identified using rabbit serum raised against *in-vivo*-grown organisms and absorbed with whole cell lysates of *in-vitro*-grown bacteria. Four strong bands were present in membranes isolated from broth-grown organisms (Fig. 2, lane A1). These were bands equivalent to the major OMPs d (37 kDa), e (31 kDa), d* (29 kDa) and a minor protein at 79 kDa. d* is the non-heat modified form of d (van Alphen *et al.*, 1983). Exactly the same result was found if membrane preparations were used from Fe-restricted grown cells (data not shown). In the *in vivo* membrane preparation, a number of immunogenic proteins with molecular masses of 43 kDa, 48 kDa, 81 kDa and >200 kDa were also observed suggesting that these were present in infection (Fig. 2, lane A2). Rabbit pre-immune serum recognized bands at 37 kDa and 31 kDa in *in vivo* and *in vitro* membranes and in addition at 56 kDa in *in vitro* membranes (data not shown).

When convalescent sera were used as the source of antibodies, bands were seen that had the same molecular mass as the 'infection specific' proteins (Fig. 2, lanes B2, C2, D2 and E2). These were not present in membranes isolated from broth-grown organisms (Fig. 2, lanes B1,

Fig. 1. SDS-PAGE of OMPs (silver stained) of Hib strain Eagan from bacteria grown *in vitro* (lane 1) or *in vivo* (lane 2). Loading was 10 µg protein per lane. Molecular mass markers (kDa) are on the right and the nomenclature for the major outer-membrane proteins on the left.

C1, D1 and E1). In particular, a band of 81 kDa was recognized by pooled rat and all three human convalescent sera, although the serum from patient C also identified a faint band of this molecular mass in *in vitro*

isolated membranes. A 48 kDa band was found in patient D, and a 43 kDa protein in patient C and pooled rat sera. There were strong responses to OMPs d, d* and e in both membrane preparations by all sera. Serum from uninfected age-matched rats had a band of reactivity of the same molecular mass as OMP d (37 kDa) in both *in vitro* and *in vivo* membrane preparations (data not shown). No bands of reactivity were found with detergent-extracted inflammatory cell preparations obtained after the first centrifugation following direct harvesting (see Methods) with rabbit, rat or human sera (data not shown).

Discussion

The aim of this study was to detect immunogenic proteins 'novel' to infection. The approach taken was one similar to that used successfully in studies on *Vibrio cholerae* (Jonson *et al.*, 1989). A major problem with Hib is obtaining sufficient *in vivo* material. Practical and ethical considerations relating to humans, and the prohibitive numbers required for animal experiments preclude obtaining sufficient nasopharyngeal, blood or CSF material. One possible limitation of this study is that the *in vivo* material, isolated from i.p.-infected rats, may not reflect the membrane composition of bacteria from other anatomical sites in rat or man. However, given the above constraints, i.p.-grown bacteria were chosen as the source of *in vivo* derived organisms.

The results show that there were differences in OMP profile between *in-vivo*- and *in-vitro*-grown organisms. The major OMPs a and d may be down-regulated *in vivo*. van Alphen *et al.* (1990) also found that the amount of OMP d was reduced in similar circumstances. The role in virulence, if any, of the 51 and 92 kDa proteins which are relatively over-expressed *in vivo* remains to be determined.

Immunogenic proteins present in infection were identified using serum raised against *in-vivo*-grown organisms and absorbed with *in-vitro*-grown whole cell lysates. The most striking difference between *in-vivo*- and *in-vitro*-grown organisms in the immunoblots was the recognition of an 81 kDa protein by rabbit, rat and human sera in *in-vivo*-grown bacteria. Larger numbers of human sera need to be screened to determine the prevalence of immunological response to this protein. We cannot categorically say whether the proteins of the same molecular mass recognized by the different sera are the same. It is possible that co-migrating proteins of the same molecular mass were present, and were recognized by different sera. With silver staining there appeared to be 81 kDa proteins present in membranes of both *in-vitro*- and *in-vivo*-grown organisms. One possibility is

Fig. 2. Identification of immunogenic proteins in infection. Outer membranes of bacteria grown *in vitro* (lanes 1) and *in vivo* (lanes 2) (10 µg protein per lane) were immunoblotted to nitrocellulose and probed with different sera. Lane (A), rabbit anti-*in-vivo* absorbed with whole-cell lysates of *in-vitro*-grown organisms; lane (B), pooled convalescent rat sera. Lanes (C, D, E), human convalescent sera from patients C, D and E, respectively (see Methods). Molecular mass markers (kDa) are on the right. On the left, bands of reactivity present in infection (lane A 2) with comparable molecular mass bands in rat or human convalescent sera (see text for details). Their molecular masses were 43 kDa (▲), 48 kDa (■), 81 kDa (→) and >200 kDa (●).

that, assuming both proteins are the same in each preparation, the reactivity in immunoblots with *in vivo* membranes is due to a conformational change. Cross-reacting antibodies to other microbial species cannot be ruled out in the case of rabbit and human sera, although this is unlikely with the rat serum given the antibody response of the control rat serum. Rabbit serum raised against Hib was cross reactive with OMPs from *H. parainfluenzae* and *Bordetella pertussis*, although this was marginal with *B. pertussis* (Erwin *et al.*, 1988). Whether the proteins found in infection are surface-exposed is unknown.

Van Alphen *et al.* (1990) compared the OMP profiles of rat (i.p.), serum-, broth-, chocolate-agar- and Fe-restricted-grown Hib organisms. It was suggested that additional proteins in the 50–75 kDa range present in

serum-, rat- and chocolate-agar-grown organisms were Fe-regulated. In the present study, an identical immunoblot profile was found for broth- and Fe-restricted-grown cells, thus the immunogenic proteins present in infection did not appear to have counterparts expressed under Fe-restricted conditions. The response of Hib to Fe-restriction may, in part, be strain-specific. With Eagan, the same OMP profile in silver-stained gels was found in Fe-restricted and in Fe-replete conditions (Morton & Williams, 1990), although other strains have been shown to have inducible OMPs visible with Coomassie blue (Williams & Brown, 1986) or silver staining (Pidcock *et al.*, 1988) under conditions of Fe-restriction. Variations in strain, media source, growth phase and the Fe chelator used make comparison between studies difficult.

Ideally, an *in vitro* growth condition which results in bacteria having a composition or properties similar to that *in vivo* is optimal. In practice, this is difficult to achieve. Growth in broth plus serum filtrate, for example, produces serum-resistant cells resembling those found *in vivo* (Shaw *et al.* 1974). The rabbit and rat sera generated in this study can be used to determine if these, or other, *in vitro* conditions result in expression of immunogenic proteins present in infection. Once found, these will facilitate studies aimed at elucidating whether such proteins are important in the disease process. Studies are in progress to isolate enough of the 81 kDa protein for N-terminal sequencing. Subsequent genetic and virulence studies may elucidate the role, if any, of this protein in pathogenesis. Whether any of the other immunogenic proteins found in this study to be present in infection are important in the disease process, remains to be determined.

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References

- ALLAN, I., LOEB, M. R. & MOXON, E. R. (1987). Limited genetic diversity of *Haemophilus influenzae* (type b). *Microbial Pathogenesis* **2**, 139–145.
- ANDERSON, P., JOHNSTON, R. B. & SMITH, D. H. (1972). Human serum activities against *Haemophilus influenzae*, type b. *Journal of Clinical Investigation* **51**, 31–38.
- VAN ALPHEN, L., RIEMENS, T., POOLMAN, J. & ZANEN, H. C. (1983). Characteristics of major outer membrane proteins of *Haemophilus influenzae*. *Journal of Bacteriology* **155**, 878–885.
- VAN ALPHEN, L., GEELEN-VAN DEN BROEK, L. & VAN HAM, M. (1990). *In vivo* and *in vitro* expression of outer membrane components of *Haemophilus influenzae*. *Microbial Pathogenesis* **8**, 279–288.
- BLAKE, M. S., JOHNSTON, K. H., RUSSELL-JONES, G. J. & GOTSCHLICH, E. C. (1984). A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. *Analytical Biochemistry* **136**, 175–179.
- BRADFORD, M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of dye binding. *Analytical Biochemistry* **72**, 248–254.
- BROWN, M. R. W. & WILLIAMS, P. (1985). The influence of environment on envelope properties affecting survival of bacteria in infections. *Annual Review of Microbiology* **39**, 527–556.
- ERWIN, A. L., KENNY, G. E., SMITH, A. L. & STULL, T. L. (1988). Human antibody response to outer membrane proteins and fimbriae of *Haemophilus influenzae* type b. *Canadian Journal of Microbiology* **34**, 723–729.
- GULIG, P. A., MCCracken, G. H., JR., FRISCH, C. F., JOHNSTON, K. H. & HANSEN, E. J. (1982). Antibody response of infants to cell surface-exposed outer membrane proteins of *Haemophilus influenzae* type b after systemic disease. *Infection and Immunity* **37**, 82–88.
- HANSEN, E. J., FRISCH, C. F., MCDADE, R. L., JR & JOHNSTON, K. H. (1981). Identification of immunogenic outer membrane proteins of *Haemophilus influenzae* type b in the infant rat model system. *Infection and Immunity* **32**, 1084–1092.
- JACOBS, A. A. C. & DE GRAAF, F. K. (1985). Production of K88, K99, and F41 fibrillae in relation to growth phase, and a rapid procedure for adhesin purification. *FEMS Microbiology Letters* **26**, 15–19.
- JONSON, G., SVENNERHOLM, A.-M. & HOLMGREN, J. (1989). *Vibrio cholerae* expresses cell surface antigens during intestinal infection which are not expressed during *in vitro* culture. *Infection and Immunity* **57**, 1809–1815.
- KROLL, J. S. & MOXON, E. R. (1989). Genetic and phenotypic variation in the capsulation and virulence of culture collection strains of *Haemophilus influenzae* type b. *Microbial Pathogenesis* **7**, 449–457.
- LOEB, M. R. & SMITH, D. H. (1982). Human antibody response to individual outer membrane proteins of *Haemophilus influenzae* type b. *Infection and Immunity* **37**, 1032–1036.
- MILLER, V. L., TAYLOR, R. K. & MEKALANOS, J. J. (1987). Cholera toxin transcriptional regulator tox R is a transmembrane DNA binding protein. *Cell* **48**, 271–279.
- MORTON, D. J. & WILLIAMS, P. (1990). Siderophore-independent acquisition of transferrin-bound iron by *Haemophilus influenzae* type b. *Journal of General Microbiology* **136**, 927–934.
- MOULDER, J. W. (1985). Comparative biology of intracellular parasitism. *Microbiological Reviews* **49**, 298–337.
- MOXON, E. R. (1990). *Haemophilus influenzae*. In *Principles and Practice of Infectious Diseases*, pp. 1722–1730. Edited by G. L. Mandell, R. G. Douglas, Jr and J. E. Bennett. Churchill Livingstone: New York.
- MURPHY, J. R., PAPPENHEIMER, A. M., JR & TAYART DE BORMS, S. (1974). Synthesis of diphtheria toxin-gene products in *Escherichia coli* extracts. *Proceedings of the National Academy of Sciences of the United States of America* **71**, 11–15.
- PIDCOCK, K. A., WOOTEN, J. A., DALEY, B. A. & STULL, D. A. (1988). Iron acquisition by *Haemophilus influenzae*. *Infection and Immunity* **56**, 721–725.
- ROGERS, H. J. (1973). Iron-binding catechols and virulence in *Escherichia coli*. *Infection and Immunity* **7**, 445–456.
- RUBIN, L. G., ZWAHLEN, A. & MOXON, E. R. (1985). Role of intravascular replication in pathogenesis of experimental bacteremia due to *Haemophilus influenzae* type b. *Journal of Infectious Diseases* **152**, 307–314.
- SCHNAITMAN, C. A. (1971). Effect of ethylenediaminetetraacetic acid, Triton X-100, and lysozyme on the morphology and chemical composition of the isolated cell walls of *Escherichia coli*. *Journal of Bacteriology* **108**, 553–563.
- SHAW, S., SMITH, A. L., ANDERSON, P. & SMITH, D. H. (1974). The paradox of *Haemophilus influenzae* type b bacteremia in the presence of serum bactericidal activity. *Journal of Clinical Investigation* **58**, 1019–1029.
- STOSCHECK, C. M. (1990). Increased uniformity in the response of the Coomassie blue G protein assay to different proteins. *Analytical Biochemistry* **184**, 111–116.
- TAYLOR, R. K., MILLER, V. L., FURLONG, D. B. & MEKALANOS, J. J. (1987). Use of *phoA* gene fusions to identify a pilus colonisation factor coordinately regulated with cholera toxin. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 2833–2837.
- TOWBIN, H., STAHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4350–4354.
- WILLIAMS, A. E., MASKELL, D. J. & MOXON, E. R. (1991). Relationship between intracellular survival in macrophages and virulence of *Haemophilus influenzae* type b. *Journal of Infectious Diseases* **163**, 1366–1369.
- WILLIAMS, P. & BROWN, M. R. W. (1986). The influence of iron restriction on growth and the expression of outer membrane proteins by *Haemophilus influenzae* and *Haemophilus parainfluenzae*. *FEMS Microbiology Letters* **33**, 153–157.