The Expression of *Haemophilus influenzae* type b porin and porin deletions in the baculovirus expression vector system.

By

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December, 1993

A thesis submitted to the
Faculty of Graduate Studies and Research
in partial fulfilment of the requirements
of the degree of Master of Science.

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This thesis is dedicated to the memory of my father

W. Michael Chin

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I am indebted to many people who have provided guidance and assistance in the preparation of the material presented here. The most notable of these is my thesis supervisor, Dr. James W. Coulton, who provided the original ideas, suggestions, and goals for this work and who freely bestowed his time, guidance, and wisdom considerably beyond the call of duty.

Thanks to my fellow lab buddies, Ramakrishnan Srikumar, Gilles Carmel, Marie France Gras, David Dahan and Greg Moeck for their assistance, their friendship, and for the memorable afternoon teas, poutines and always, the laughter.

Thanks to Ken Ruffo who always managed to be there for me in spite of the 600 km. Thanks Ken for helping me manage my thoughts, time, and work.

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This work would not have been possible without the generous contribution of baculovirus transfer vectors by Dr. Chris Richardson and Jorge Viallard. Their comments and suggestions regarding this work are gratefully acknowledged.

Finally I thank my mother Caroline and my sisters for hanging in there!

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ABSTRACT

Haemophilus influenzae type b (Hib) is an important causative agent of endemic bacterial meningitis and other invasive diseases in infants and young children. The sole porin of Hib is a protein of molecular mass 40 kilodaltons (341 amino acids) which comprises almost 20% of the outer membrane proteins. The protein's location, abundance, and essential role in the outer membrane as a non-specific channel make it an ideal candidate for immune recognition. To extend our knowledge of the structure and function of porin and to study the role that porin may play in immunoprotection, it was desirable to synthesize recombinant Hib porin.

Preparing porin from Hib is limited by difficulties in purifying the protein of contaminating lipooligosaccharide (endotoxin) (LOS). We considered various expression systems for generating LOS free recombinant porin and chose to use the baculovirus expression vector system (BEVS). One of the major advantages of the BEVS over other expression systems is the abundant expression of some recombinant proteins which are in many cases antigenically, immunogenically, and functionally similar to their authentic counterpart.

The coding region of the gene for porin was isolated from an M13 library and was adapted and modified into intermediate plasmids and then into a baculovirus transfer vector. *Spodoptera frugiperda* (Sf9) cells were co-transfected with wild type DNA isolated from baculovirus and the recombinant transfer vector containing the full-length gene for porin. Pure recombinant virus was isolated and amplified in Sf9 cells. The recombinant porin was found to co-migrate with porin isolated from bacterial cells on SDS-PAGE. The recombinant porin also reacted with a monoclonal antibody directed against Hib porin.

In addition to the full-length recombinant porin, it was of interest to develop a family of carboxy-terminal deletion proteins in the BEVS. Four restriction endonuclease sites were chosen to linearize a plasmid containing the full-length porin gene. Truncated porin derivatives were generated by *in vitro* omega mutagenesis at these restriction sites. The insertions of the omega cassette at these four sites generated proteins that terminated after amino acids 139, 174, 182, and 264 of the primary sequence of mature porin.

SOMMAIRE

Haemophilus influenzae type b (Hib) est un agent pathogène important de la méningite endémique bactérienne et autres maladies envahissantes chez les nourrissons et les jeunes enfants. La seule porine de Hib est une protéine d'une masse moléculaire de 40 kilodaltons (341 aminoacides) comprenant presque 20 % des protéines de la membrane externe. Le site de la protéine, son abondance et son rôle essentiel dans la membrane externe à titre de voie aspécifique en font un candidat idéal pour la reconnaissance immunitaire. Dans le but d'approfondir nos connaissances de la structure et de la fonction de la porine, et d'étudier son rôle potentiel dans l'immunoprotection, la synthèse de la porine Hib recombinée s'imposait.

La préparation de la porine à partir de Hib est limitée par les difficultés que pose la purification de la protéine, soit l'élimination de la lipooligosaccharide contaminante (endotoxine) (LOS). Plusieurs méthodes d'expression visant la génération de porine recombinée sans LOS ont été considérées; le système vectoriel d'expression du baculovirus (SVEB) a finalement été choisi. Un des avantages importants du SVEB par rapport

aux autres méthodes d'expression est l'expression abondante de quelques protéines recombinées présentant des similarités antigéniques, immunologiques et fonctionnelles à leurs contreparties réelles.

La région de codage du gène pour la porine a été isolée à partir d'une archive M13 et a été adaptée et modifiée en plasmides intermédiaires, et ensuite en un vecteur de transfert de baculovirus. Des cellules *Spodoptera frugiperda* (Sf9) ont été co-transfectées avec du ADN de phénotype sauvage isolé du bacilovirus et le vecteur de transfert recombiné renfermant le gène intégral de la porine. Le virus recombiné pur a été isolé et amplifié en cellules Sf9. Nous avons constaté une co-migration de la porine recombinée avec la porine isolée des cellules bactériennes sur SDS-PAGE. Une réaction a également été constatée entre la porine recombinée et l'anticorps monoclonal dirigé contre la porine Hib.

En plus de la porine recombinée intégrale, la formation d'une famille de protéines à suppression de la terminaison carboxyle dans le SVEB. Quatre sites restrictifs d'endonucléase ont été choisis pour linéariser un plasmide renfermant le gène intégral de porine. Des dérivés tronqués de porine ont été générés par l'intermédiaire de mutagenèse oméga *in vitro* aux sites de restriction. L'insertion de la cassette oméga à ces quatre sites a généré des protéines terminant après les aminoacides 139, 174, 182 et 264 de la séquence primaire de la porine mûre.

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PREFACE TO THE THESIS

In accordance with the guidelines concerning thesis preparation, and with the approval of the Department of Microbiology and Immunology, I have opted to present the experimental portion of this thesis (chapters 2 and 3) in the form of original papers. A provision in the guidelines concerning thesis preparation reads as follows:

Candidates have the option, **subject to the approval of their Department**, of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis.

-If this option is chosen, **connecting texts, providing logical bridges between the different papers, are mandatory.**

-The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary form that is more than a collection of manuscripts published or to be published. **The thesis must include, as separate chapters or sections:** (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final overall conclusion and/or summary.

-Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (eg. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

-In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis of**

who contributed to such work and to what extent; supervisors must attest to the accuracy of such claims at the Ph.D Oral Defense. Since the task of Examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of different authors of co-authored papers.

With regard to the above conditions, I have included two original papers which have been published as chapters of this thesis. Chapters 2 and 3 contain their own Abstract, Introduction, Materials and Methods (chapter 2) or Experimental Procedures (chapter 3), as well as Results, and Discussion sections. A preface which serves as the connecting text to bridge the manuscripts is found prior to Chapter 3. A general introduction, thesis overview and summary chapter have also been included. References are collated alphabetically and found at the end of each chapter.

I conducted my experimental research in Dr. J.W. Coulton's laboratory, Department of Microbiology and Immunology, McGill University from September 1988 to May 1990. In May 1990, I requested a leave of absence from the Coulton lab for compassionate reasons.

The manuscripts, in order of their appearance in the thesis, are:

1. Coulton, J.W., Chin, A.C., and Vachon, V. 1992. Recombinant porin of ***Haemophilus influenzae*** type b. Journal of Infectious Diseases. 165(**suppl** 1):S188-91.

2. Srikumar, R., Chin, A.C., Vachon, V., Richardson, C.D., Ratcliffe, M.J.H., Saarinen, L., Kayhty, H., Makela, P.H., and Coulton, J.W. 1991. Monoclonal antibodies specific to porin of ***Haemophilus influenzae*** type b: localization of their cognate epitopes and tests of their biological activities. Molecular Microbiology. 6:665-676.

In manuscript 1 (chapter 2), I was responsible for all the research described with the following exceptions:

- Rino Stocco constructed the M13 mp18 library of chromosomal DNA of Hib strain ATCC9795.
- Dr. J.W. Coulton purified the recombinant Hib porin.
- Dr. V. Vachon performed the lipid bilayer experiments described in Figure 3.

In manuscript 2 (chapter 3), I created pACC33 by subcloning the full length porin gene (*ompP2*) in the baculovirus transfer vector pJV.P10Z. I generated the series of deletion mutants of *ompP2* by omega mutagenesis. These were made compatible to the BEVS by subcloning each into the baculovirus transfer vectors pJV.P10Z and pJVETLZ. I was also responsible for the co-transfections of these transfer vectors into Sf9 insect cells, the plaque assays, and the isolation and amplification of recombinant viruses. I identified the recombinant proteins from the insect cell extracts by SDS-PAGE (Figures 5-7).

The remainder of the work was distributed as follows:

- R. Srikumar was responsible for identification of the immunoreactive amino acid sequences recognized by the monoclonal antibodies against Hib porin.
- Dr. M.J.H. Ratcliffe assisted with the flow cytometry.
- Dr. V. Vachon developed a scheme for the purification of Hib porin.
- All the microbiological tests of the monoclonal antibodies were performed by Dr. H. Kayhty, Dr. P.H. Makela, and Dr. J.W. Coulton at the National Public Health Institute in Helsinki, Finland.
- Dr. C.D. Richardson provided the baculovirus transfer vectors pJVETLZ and pJV.P10Z. He also assisted me in resolving the proteins from insect cell extracts for Figure 5.

All of my research was conducted under the supervision of Dr. J.W. Coulton.

INTRODUCTION

Haemophilus influenzae: biology and role in disease

Haemophilus influenzae is a small gram-negative bacterium that is an apparently harmless component of the normal flora in the upper respiratory tract of humans. *Haemophilus influenzae* is often encapsulated. In 1931 Margaret Pittman identified six capsular serotypes, a-f, of *Haemophilus influenzae*. Strains of *Haemophilus influenzae* that produce a type b polysaccharide capsule have since been shown to cause almost all cases of invasive disease attributable to *Haemophilus influenzae* (Turk, 1984; Turk and May, 1987; van Alphen and Bijlmer, 1990). *Haemophilus influenzae* type b (Hib) is the only one of the six serotypes that is able to invade via the blood and thereby enter into the meninges tissues which surround the central nervous system. Until recently, Hib was the leading cause of endemic bacterial meningitis in infants and young children (Dajani *et al.*, 1979). In the United States alone, it is estimated that one in every two hundred children will contract Hib disease by the age of five. This translates to roughly 12,000 cases each year

(Granoff and Munson, 1986). Ten percent of these children will die from Hib disease, and anywhere from nine to twenty nine percent of surviving children will experience permanent neurological sequelae. In addition to bacterial meningitis, Hib can also cause septicemia, epiglottitis, cellulitis, arthritis, osteomyelitis, pericarditis, and pneumonia. The use of antimicrobial therapy has profoundly altered the outcome of infection by lowering the fatality rate to ten percent. The emergence of Hib organisms in the past twenty years which are resistant to antibiotics such as ampicillin, chloramphenicol and the cephalosporins threatens the ability to treat infections successfully (Wilfret, 1990; MMWR Bulletin, 1984). This threat, coupled with the severity of Hib induced disease and the strain imposed on the health care system by such illnesses, has spurred researchers to focus studies on the prevention of Hib disease by immunization. Universal vaccination has been projected as the most cost effective solution (Hay and Daum, 1987).

Normally maternal antibodies present at birth protect the infant against any harmful pathogens, including Hib. As the levels of these antibodies decline the potential of disease increases until the child reaches the age of two to three years. At this age the child's own protective antibodies have developed. Even though all children younger than five years of age are at great risk of Hib disease, it is no surprise then that almost seventy five percent of all systemic Hib infections occur in children under the age of eighteen months, with peak susceptibility at six to seven months of age. Children of susceptible age are more likely to have meningitis than any other form of invasive Hib disease. For an immunization program to be successful in preventing Hib invasive disease it must be effective in infants younger than six months of age. Ideally, the vaccine should be safe and should generate a protective and boostable immune response in infants by the age of six months (Madore *et al.*, 1989).

Capsular polysaccharide: important for virulence, PRP vaccine, and PRP-D conjugate vaccine

The type b capsular polysaccharide comprises linear polymers of the pentoses, ribose, and ribitol linked by phosphodiester bonds, and is referred to as polyribitolribosyl phosphate (PRP). PRP is an important virulence factor of Hib. In fact, loss of the type b PRP capsule by the organism is associated with the loss of pathogenicity (Zwahlen *et al.*, 1983). In addition, serum antibodies directed against the type b capsule activate complement-mediated bactericidal and opsonic activity *in vitro* (Schneerson *et al.*, 1971; Anderson *et al.*, 1972), confer protection against Hib bacteraemia in experimental animals, and protect against disease in humans (Peltola *et al.*, 1984).

In April of 1985 a vaccine (PRP) composed of the Hib polysaccharide was licensed for use in the U.S. This was a result of an earlier Finnish trial in which the vaccine was observed to be 90% effective in children older than 18 to 24 months of age (Peltola *et al.*, 1984). Since the vaccine did not produce protective levels of antibodies in infants younger than 18 to 24 months of age it was recommended for use with infants older than 24 months. Its use in the U.S. has been of varied success. Reports of vaccine efficacy has been as high as 88% in a multicentre case control study conducted in Connecticut, Dallas County and greater Pittsburgh; yet a similar study performed in Minnesota reported a lack of efficacy (Sharpiro and Berg, 1990). As well, Granoff and colleagues (1986) reported several cases in which apparently normal children developed Hib disease after receiving the Hib polysaccharide vaccine. They concluded that some apparently normal children who were at least two years old lacked the ability to respond immunologically to the capsular polysaccharide of Hib and perhaps other polysaccharide antigens. In addition, the vaccinated population (24 months of age and older) accounts statistically for only twenty percent of all invasive Hib infections in the U.S. That is, the PRP vaccine was ineffective for the population at greatest risk (Shapiro and Berg, 1990). The PRP vaccine consists of purified Hib capsular polysaccharide. The repeating nature of such an antigen requires thymus (T) cell independent processing which appears late in ontogeny; hence the unresponsiveness of this younger aged group to the PRP vaccine. Based on the above it would appear that this vaccine has limited use in North America where the incidence of disease is more prevalent in young infants.

A second generation of Hib vaccines, the PRP-conjugate vaccines, were initially licensed for use in the U.S. in 1987. In conjugate vaccines the carbohydrate antigen is conjugated to a protein carrier in order to enhance the antibody response (Granoff and Munson, 1986). The first such vaccine, PRP-D, consists of the Hib capsular polysaccharide conjugated to the diphtheria toxoid protein. The PRP-D conjugate vaccine, unlike the PRP vaccine, can recruit helper T cells yielding a strong T-dependent response which characteristically has an enhanced IgG memory response upon re-injection (Garner and Pier, 1989). This is advantageous as infants rely on T-dependent processing for their immune responses and are often not responsive to the polysaccharide alone. Although the PRP-D vaccine is a superior vaccine to the earlier PRP vaccine it is still not ideal as shown by the following.

Two studies were conducted to determine whether or not the PRP-D vaccine provided protective efficacy in children immunized prior to six months of age. In the first, a large randomized trial was conducted in Finland. Infants received three primary PRP-D immunizations at 3, 4, and 6 months of age. The levels of antibody induced after the series of three immunizations was observed to be minimal or nonexistent (Eskola *et al.*, 1985; Ward, 1990).

The second study involved 2113 Native Alaskan infants, a population considered to be at extreme risk to Hib invasive diseases. It was a randomized, double blind, and placebo controlled experiment. Infants were immunized at 2, 4, and 6 months (Ward *et al.*, 1988). As with the Finnish study, immune response to the PRP-D vaccine was minimal. In fact, after the three doses of the vaccine, detectable responses were found in only one half of the infants. In these infants the mean antibody levels were only 0.2 ug/mL, corresponding to approximately ten percent of the levels observed in older children after one dose of PRP-D. In the Alaskan trial there were 32 episodes of Hib disease with 20 occurring after the third immunization (8 episodes in the vaccine group and 12 in the control group). The protective efficacy of the vaccine after three doses was determined to be 35%, a level which is not clinically significant. Due to the minimal efficacy of this vaccine in younger infants it was not recommended for routine use.

In order to overcome the inadequate immune response of the Hib capsular polysaccharide vaccine in infants, two strategies evolved to identify alternative vaccine candidates. The first is to increase the immunogenicity of the capsular polysaccharide by conjugating it to alternate protein carriers. This has already met some success with the PRP-D conjugate vaccine. Additional work is currently in progress to determine if other proteins, such as the tetanus toxoid protein (Ahonkhai *et al.*, 1990), CRM_{197}, a nontoxic form of diphtheria toxin (Anderson, 1983), and outer membrane proteins of group B *Neisseria meningtidis*

(Vella *et al.*, 1990) may prove more immunogenic.

The second strategy is to identify surface exposed outer membrane proteins (OMP) of Hib which have potential for vaccine development. There are many reasons to consider the OMPs as vaccine candidates. The human antibody response develops earlier for proteins than for polysaccharide antigens. This is particularly important with Hib as younger children and infants are at greatest risk of disease. It has been observed that sera obtained from animals and humans of all ages, including infants convalescing from Hib induced meningitis, make antibodies to a variety of OMPs found on the organism (Loeb and Smith, 1982). There also appears to be considerable cross reactivity among homologous proteins from different isolates (Loeb and Woodin, 1987). But more importantly, the use of OMPs of Hib that might themselves be immunogenic would avoid carrier-induced suppression of anti-PRP antibodies. Conjugate vaccines have traditionally employed carriers such as the tetanus toxoid or the diphtheria toxoid because of their long usage in human vaccination programs without undue adverse effects. As a result most infants who now receive a PRP-conjugate vaccine may already have been primed to the carrier protein through previous immunization programs. Immunization with a carrier protein can induce the delayed onset of carrier-specific suppressor T cells (CT_s) (Herzenberg *et al.*, 1980). These CT_s persist and will continue to suppress antibody responses to any new epitopes found with the carrier. This suppression is specific to the individual epitope regardless whether subsequently conjugated to a new carrier (Herzenberg *et al.*, 1982). Potential suppression of anti-PRP antibody could be circumvented by use of Hib OMPs which would be of the same source as PRP and perhaps immunogenic themselves.

The outer membrane protein profile of Haemophilus influenzae

Outer membrane proteins are a source of vaccine candidates against Hib disease. The outer membrane of Hib is typical of gram-negative bacteria. It consists of phospholipids, lipooligosaccharide (LOS), and approximately 24 proteins. Six of these proteins account for about 80% of the total proteins and are hence called the major outer membrane proteins. Two nomenclatures exist for the six major OMPs: protein a or P1, protein b/c or P2, protein d/f or P5, protein e or P4, and protein g or

P6 (Granoff and Munson, 1986; Loeb and Smith, 1982).

Porin: a nonspecific channel

An essential role of the outer membrane is to act as a permeability barrier to the diffusion of deleterious substances across the cell envelope. Much attention has been focused on OMPs of Hib which may serve as molecular sieves to regulate the access of hydrophilic molecules to the cytoplasmic membrane. The OMP P2 was previously purified (Vachon et al., 1985, 1986) and shown to have porin activity when reconstituted in liposomes and in planar black lipid membranes. Hence the P2 protein of Hib was given the porin designation. As with porins of other gram-negative bacteria, the Hib porin forms trans-membrane water filled channels that permit the diffusion of hydrophilic solutes across the membrane. When Hib porin was reconstituted into vesicles of lipopolysaccharides and phospholipids, it was found to have a molecular mass exclusion limit of 1400 dalton (Vachon *et al.*, 1985). Solutes smaller than this pass through the outer membrane. Porin is estimated as having a cross sectional diameter of 0.9 nm from the reconstitution of the porin into planar black lipid membranes (Vachon *et al.*, 1986). In addition, porin was observed to have a trimeric organization in the outer membrane (Vachon *et al.*, 1988).

Hib porin accounts for almost 20% of the outer membrane proteins. Of the major OMPs only Hib porin has been shown to act as a pore-forming protein. Hib porin was shown to be surface exposed (Hamel *et al.*, 1987a and 1987b). A monoclonal antibody, Hb-2, recognized native porin at the surface of intact cells (Hamel *et al.*, 1987b) and reacted with 453 of 455 type b strains which were collected from diverse sources. In addition, Munson and colleagues (1989) observed that the gene for porin was highly conserved. They looked at the porin gene from prototype strains representing 3 major clones of Hib which cause invasive type b disease. They found that each contained only one porin gene and that the sequences had very few variations. More importantly, when purified porin was injected into mice the sera was found, by Western blot analysis, to contain high levels of Ab reacting with porin. In addition, the mice all survived when challenged with a lethal dose of Hib. Munsen *et al.* concluded that porin had potential as a vaccine candidate against Hib infection. Alternatively, porin may also be considered as a protein carrier in a new conjugate vaccine with the Hib capsular polysaccharide.

Expression of porin in baculovirus expression vector system

The goal of this research is to produce substantial amounts of porin protein for the purpose of studying its role in immuno-protection. The availability of large amounts of this protein is also essential for structural and functional studies. Several expression systems have been used successfully for the production of high levels of desired proteins. One system is the baculovirus expression vector system (BEVS).

Baculoviruses are useful vectors for the high level expression of foreign genes in insect cells. A major advantage of the BEVS over other expression systems, including bacterial, yeast, and mammalian systems, is that the recombinant proteins are often antigenically, immunogenically, and functionally similar to their authentic counterparts (Luckow and Summers, 1988b). Baculovirus are naturally occurring viruses that are limited to arthropods hosts. None have been shown to infect or replicate in other invertebrates, microorganisms, plants, or vertebrates. Thus baculoviruses are very attractive from the standpoint of safety.

In addition, the expression of porin in the baculovirus system has the inherent advantage of producing purified porin protein without any contaminating LOS, which is intimately associated with porin when isolated from bacterial cells. In our experience, porin purified from the bacterial cell surface by fast protein liquid chromatography (FPLC) contains LOS contaminant at about 100 ng of LOS per ug of purified porin. This LOS could not be removed in spite of repeated passage through a MonoQ column. A major limitation on the use of bacterial porin in the formulation of novel vaccinogenic materials is this problem of purifying porin free of LOS (an endotoxin). The production of a recombinant porin protein in a LOS free system such as the BEVS will also allow for the study of the role of LOS in the structure and function of the porin protein.

Baculovirus: Biology

Autographa californica nuclear polyhedrosis virus (AcNPV) is the prototype virus of the family Baculoviridae, which causes lethal infections in invertebrates. During baculovirus infection two forms of the virus are produced: extracellular virus particles (ECV) and occluded virus particles (OV). The OV are embedded in a crystalline protein matrix composed primarily of a single 33 kilodalton (kDa) polypeptide called polyhedrin. This polyhedral inclusion body protects the virus as it moves from one host to another. The virus can persist for years, even in soil or most other environments, until it is ingested by a host.

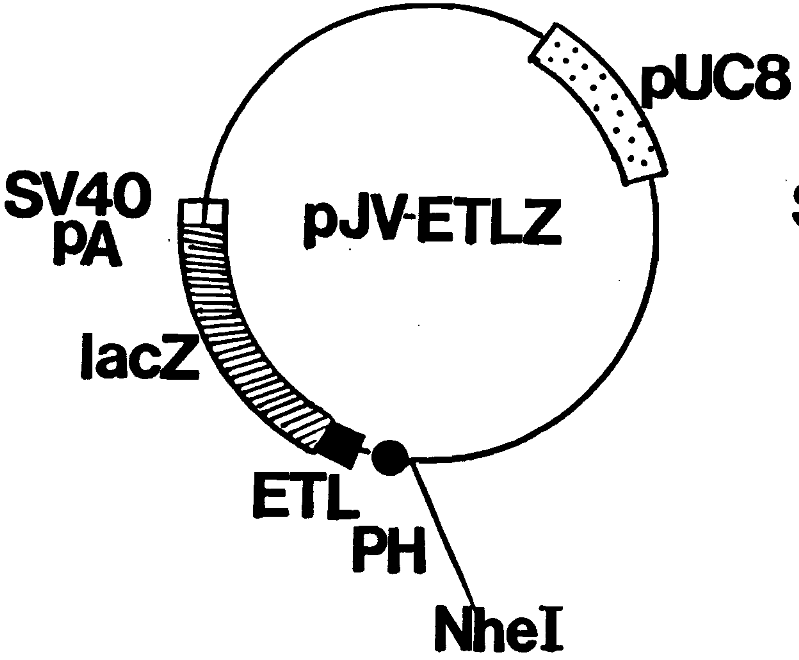
Insects become infected with baculovirus when they ingest OV in contaminated food. During the passage of the OV through the foregut of the insect the viruses are protected by the polyhedrin matrix. Once in the midgut, the alkaline pH environment causes the polyhedrin matrix to dissolve resulting in the release of numerous nucleocapsids. These virions absorb to the midgut epithelial cells and the infection process commences. ECV are formed by the budding of viral nucleocapsids through the plasma membrane of infected cells. These ECV cause secondary infection via the hemolymph of the insect. The formation of ECVs and OVs is biphasic with the production of ECV occurring between 12 and 24 hours post infection (hpi). The production of polyhedrin, which is necessary for the OV, begins 20 hpi reaching a maximum level at 48-72 hpi. About fifty polyhedra are produced from each cell such that in infected *Spodoptera* *frugiperda* cell cultures, polyhedrin accounts for 50-75% of the total protein produced (Summers and Smith, 1988). In 1983 Summers *et al.* showed that the polyhedrin gene is not essential for the replication of the infectious ECV. Deletion or inactivation of the polyhedrin gene by insertion results in mutants which do not produce occlusions in infected cells. Another very late gene product is the p10 protein, which, like polyhedrin, is also produced in large quantities. The function of the p10 protein is unclear, but it has been postulated that it plays a role in the assembly of occlusion bodies (Smith *et al.*, 1983). The p10 protein is known to be nonessential for the formation of virions.

Replacement of the coding sequences for polyhedrin or the p10 protein by foreign genes forms the basis of most baculovirus vectors. This exchange results in the ability of the strong late polyhedrin or p10 promoters to drive the expression of the foreign gene. In most studies, the use of baculovirus expression vectors in the production of foreign gene products has been for structure-function analysis of these proteins.

Baculovirus expression vectors

The baculovirus transfer vectors utilized were pJVETLZ and pJVP10Z, which were generously provided for our use by Dr. C. Richardson (Vialard *et al.*, 1990) (Fig. 1). They are improved baculovirus vectors developed in order to expedite the screening and purification of recombinants, and are examples of co-insertion vectors. In each the gene for β-galactosidase is expressed along with the gene of interest. β-galactosidase is an enzyme which hydrolyses the substrate halogenated indoyl-β-D-galactoside (Bluo-Gal) to produce a dark blue product. The expression of the β-galactosidase gene in pJVETLZ and pJVP10Z is driven by the ETL and P10 promoters respectively. In both vectors the expression of the gene of interest is driven by the polyhedrin promoter. Baculovirus sequences found just upstream and downstream of the polyhedrin gene flank the insertion site as well as the gene for β-galactosidase.

Integration of the foreign genes into the wild-type virus genome depends on a process of homologous recombination between the flanking sequences of the vector and the wild-type DNA. Thus the co-transfection of any one of these two vectors with infectious wild type viral DNA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pJV-ETLZ
lacZ
ETL
PH
NheI

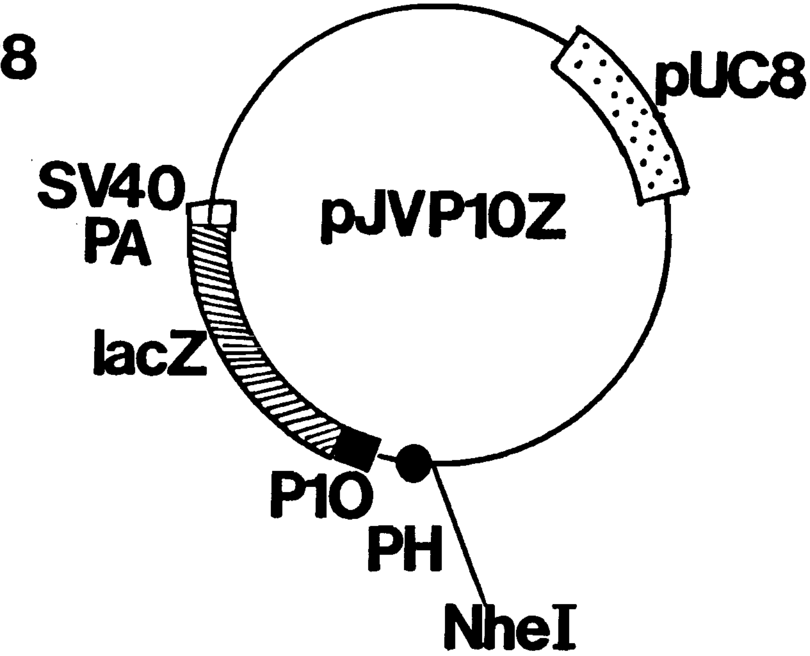

pUC8
SV40
PA
pJVP10Z
lacZ
P10
PH
NheI

Figure 1. Baculovirus transfer vectors. The two baculovirus transplacement vectors, pJVETLZ and pJVP10Z, are shown. Important features include: the strong late polyhedrin promoter (shown as a black circle) to drive the expression of the gene of interest; a unique restriction site for insertion of the foreign gene (NheI site); a second promoter either the late ETL promoter in pJVETLZ or the p10 promoter in the pJVP10Z construct (both are shown as black squares); the gene for β-galactosidase (striped area labelled lacZ); the polyadenylation signal from Simian virus 40 (clear rectangle); AcNPV sequences flanking the polyhedrin gene including the polyadenylation signal of polyhedrin (shown as thin lines); and sequences from pUC8, a bacterial plasmid for cloning and amplification in *E. coli* (stippled rectangle).

results in recombination such that the gene sequence for β-galactosidase and its promoter, as well as the sequences for the foreign gene and the polyhedrin promoter, are inserted into the viral genome as a single unit (Fig. 2). This event occurs at a low frequency. A successful recombinant virus will have acquired both gene products, thereby allowing for visual detection of the recombinants. In plaque assays, recombinant viruses produce blue plaques as a result of hydrolysis of the substrate Bluo-Gal, included in the agar overlay, by β-galactosidase.

Recombinant porin deletions using omega mutagenesis

Aside from the full length porin protein it was also desirable to generate a series of porin derivatives. These protein derivatives would differ in the amount of porin that was removed from the carboxy-terminus. That is, we wanted to generate a series of proteins which would all have the same amino terminus as the original porin protein, but which would vary in length. These recombinant proteins, along with the full length recombinant porin protein, would be valuable for immunological studies (including the mapping of epitopes recognized by monoclonal

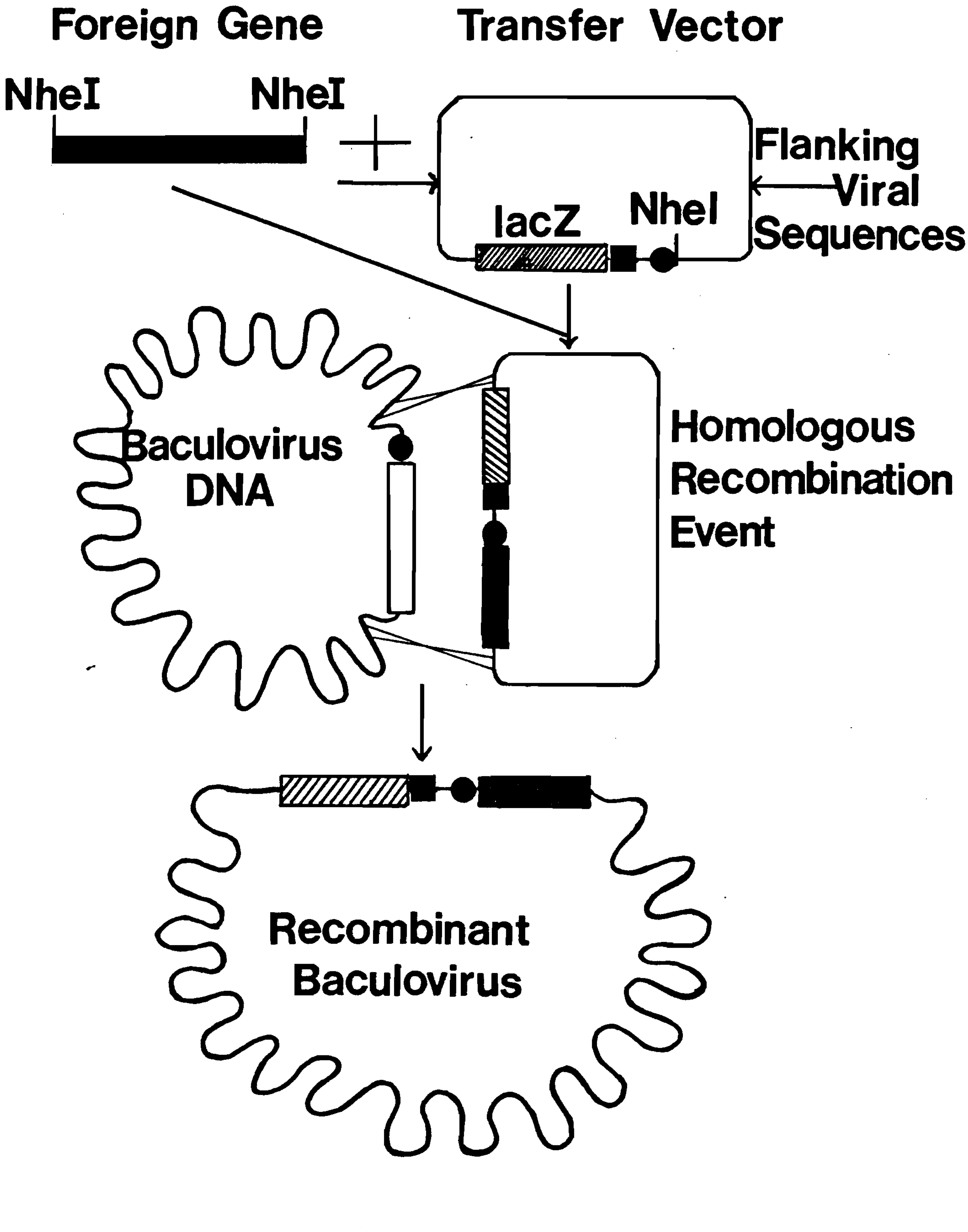

Foreign Gene
Transfer Vector
NheI
NheI
+
Flanking Viral Sequences
lacZ
NheI
Baculovirus DNA
Homologous Recombination Event
Recombinant Baculovirus

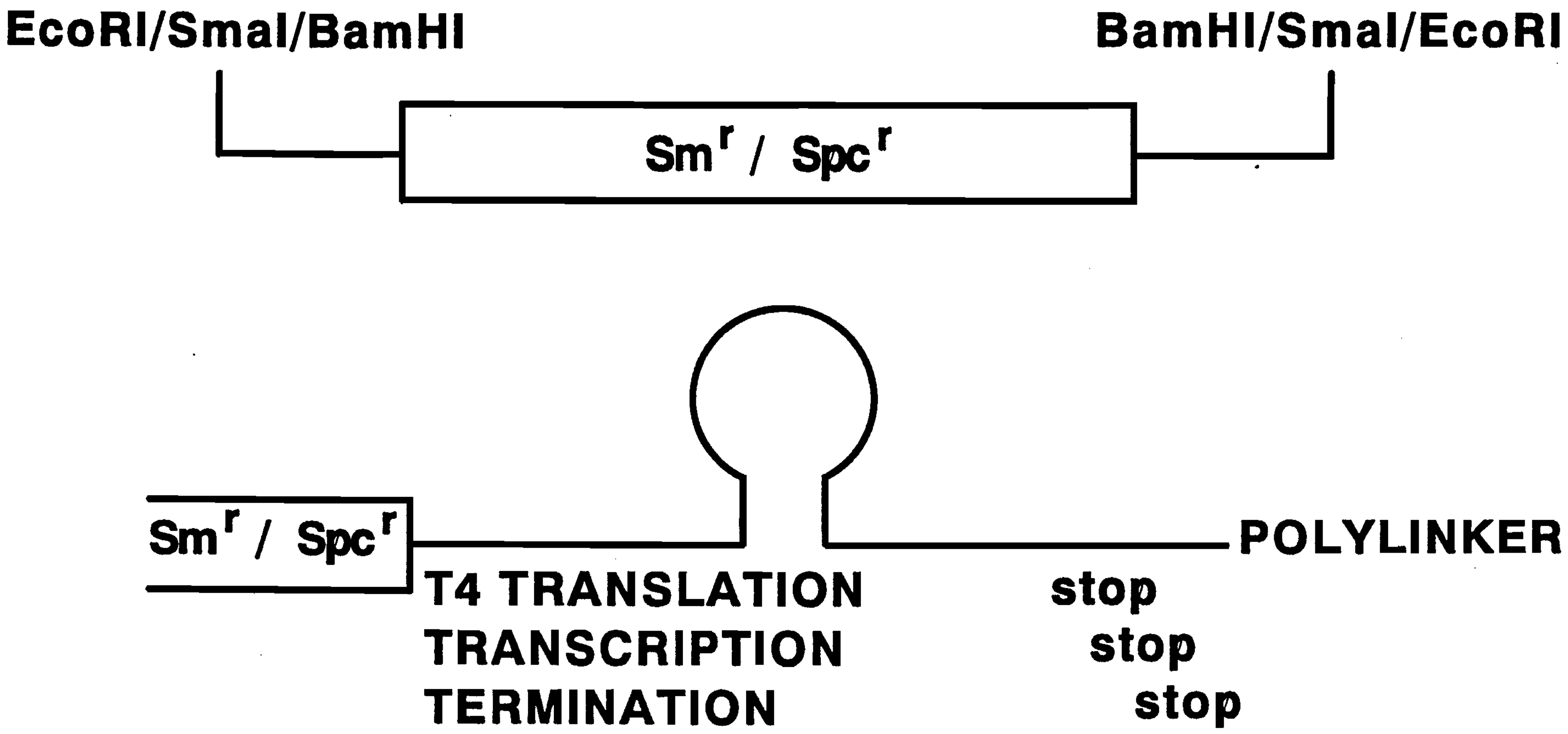
Figure 2. Integration of foreign gene in baculovirus genome by homologous recombination. The gene of interest is inserted in a baculovirus transfer vector. The gene is transferred to the viral genome as a result of a homologous recombination event between flanking viral sequences (found on either side of the gene of interest) and similar sequences on the viral genome.

antibodies against porin), as well as for structural and functional studies of the protein.

The family of truncated porin proteins was created by the premature termination of both transcription and translation at specific sites in the porin sequence. This was accomplished by introducing an omega cassette into selected sites in the porin gene using genetic engineering techniques. The omega cassette is approximately 2.0 kb long. It consists of the antibiotic resistance genes for streptomycin and spectinomycin (Sm^r/Spc^r), a 1.7 kb PvuII-HindIII fragment from the R100.1 plasmid flanked by short inverted repeats carrying transcription and translation terminal signals, and synthetic polylinkers (Prentki and Krisch, 1984). The transcription-termination fragment included the last eight codons of gene 32, and a characteristic stem-loop structure for transcription termination (Krisch and Allet, 1982). This sequence is known to be active as a rho-independent termination site for RNA polymerase *in vivo* and *in vitro* (Prentki, and studies (including the mapping of epitopes recognized by monoclonal antibodies against porin),Krisch, 1984). The transcription and translational termination signals are found at both ends of the cassette in both orientations. Thus the cassette can be inserted in either orientation to cause premature termination (Fig. 3).

The cassette was inserted into a plasmid linearized at various sites in the porin sequence. The sites chosen were the unique DraI, EcoRI, SnaBI, and AseI endonuclease restriction sites, found within the coding sequence of porin. The recombinant DNA molecules were then selected for resistance to the antibiotics ampicillin, spectinomycin, and streptomycin. The presence of the omega cassette terminated RNA synthesis prematurely at these sites, resulting in truncated porin proteins.

OMEGA MUTAGENESIS



EcoRI/SmaI/BamHI

BamHI/SmaI/EcoRI

Sm^r / Spc^r

Sm^r / Spc^r

POLYLINKER

T4 TRANSLATION
TRANSCRIPTION
TERMINATION

stop
stop
stop

Figure 3. Omega Mutagenesis. Upper panel. The diagram depicts the omega cassette used for premature termination of translation and transcription. Lower panel. Closeup of one end of the cassette. The Sm^r/Spc^r segment from R100.1 (open rectangle) is flanked by the short inverted repeats carrying the T4 transcription-termination signals (shown as a stem loop structure), translation stop signals in all three reading frames (shown as "stop"s), and the polylinker region consisting of the BamHI, SmaI, and EcoRI sites seen in the upper panel (Prentki, and Krisch, 1984).

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PREFACE TO CHAPTER 2

The goal of my studies has been to express porin of *Haemophilus influenzae* type b in an LPS free environment to aid the study of its role at the surface of the organism.

Chapter 2 reports the subcloning of the gene for porin of Hib into baculovirus expression vectors and the subsequent expression of the recombinant protein in the baculovirus expression vector system. The biophysical properties of the recombinant protein were also assessed in a reconstitution system.

Recombinant Porin of *Haemophilus influenzae* Type b

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A protein of ~40 kDa in the outer membrane of *Haemophilus influenzae* type b (Hib) behaves as a porin and permits transmembrane diffusion of low-molecular-weight solutes. The gene for Hib porin was cloned from an M13 library of chromosomal DNA of Hib strain ATCC 9795. The gene was subcloned into a new transfer vector as a prerequisite for its use in the baculovirus expression vector system. Pure recombinant virus (AcPORIN) was isolated. On infection of a cultivated insect cell line Sf9 with AcPORIN, a novel protein was detected in cell lysates, and this novel protein reacted with an anti–Hib porin monoclonal antibody. The purified recombinant Hib porin was tested for its pore-forming properties in a synthetic black lipid membrane. The biophysical activity of purified recombinant Hib porin was identical to porin isolated from the bacterial outer membrane.

The outer membrane of *Haemophilus influenzae* type b (Hib) contains a number of abundant proteins that are designated P1, P2, P4, P5, and P6 in order of decreasing molecular mass as estimated by SDS-PAGE: 46, 40, 30, 27, and 16 kDa, respectively [1, 2]. The cloning and sequencing of the genes for some of these proteins [3–7] represents considerable progress toward our understanding their structure at the bacterial cell surface. In spite of these advances, the specific functions most of these proteins provide for the bacterial cell are unclear.

Because the outer membrane acts as a permeability barrier to the diffusion of deleterious substances across the cell envelope, we have directed attention to proteins that permit the transmembrane passage of solutes across the outer membrane. One protein species of ~40 kDa was purified from the outer membrane of Hib and reconstituted into vesicles of lipopolysaccharide (LPS) and phospholipid. A value of 1400 Da was determined as the molecular mass exclusion limit for this protein [8]. Water-soluble solutes smaller than this encounter little barrier to their free diffusion across the aqueous channels formed by assembly of this protein. No other protein was found to have channel-forming properties.

Confirmation that this protein acted as a porin was provided by two complementary experimental systems. The 40-kDa protein spontaneously incorporated into a synthetic black lipid membrane and caused a stepwise increase in conductivity across the membrane [9]. An estimate was made of the cross-sectional diameter of Hib porin (0.9 nm) on the basis of assumptions of the cylindrical nature of the pore and the thickness of the outer membrane. The liposome swelling assay was used to assess the pore-forming properties of the 40-kDa protein [10]. A value of 1.8 nm for the cross-sectional diameter was calculated using the Renkin equation. The apparent variance of this estimate with that obtained from black lipid membranes remains unexplained. Finally, we have shown that Hib porin exists as a trimer in the outer membrane [10].

Not only does porin play a central role in the physiology of Hib, but it has also been reported to be a target for immunoprotection [11, 12]. Rabbit antibodies were prepared against Hib porin from one outer membrane protein subtype and were found to be cross-reactive by Western blotting with Hib porin from two other subtypes. The antibodies passively protected infant rats against infection caused by the homologous organism but not against that caused by challenge with a heterologous strain. These experiments suggested that determinants on the cell surface interacted with protective antibodies and that they were strain- or subtype-specific.

Such results served to heighten the interest in using Hib porin as a vaccine component or as a carrier in new formulations of a conjugate vaccine. Indeed, Hib porin has been used as a protein carrier to prepare conjugate vaccines with Hib capsular polysaccharide [13]. A major limitation in preparing Hib porin is that it is difficult to purify free from LPS (endotoxin). LPS binds strongly but noncovalently to Hib porin [14]. If such materials are to be used for administration to humans, the Hib porin should be LPS-free.

We considered various expression vector systems for generating LPS-free recombinant porin. *Escherichia coli* was excluded because of the unavoidable problems of purification of the recombinant Hib porin from another gram-negative bacterium that produces LPS. We report here the expression of the Hib porin gene in the baculovirus expression vector system (BEVS). The expression of a foreign gene under the control of the strong polyhedrin promoter of *Autographa californica* nuclear polyhedrosis virus (baculovirus) is an attractive system because it offers abundant expression of recombinant proteins in an LPS-free environment. In many cases,

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the expressed proteins have been shown to be antigenically and functionally similar to their authentic counterparts [15].

Materials and Methods

Molecular cloning of the Hib porin gene. An M13 mp18 library of chromosomal DNA of Hib strain ATCC 9795 was constructed by digestion of the chromosome with *Pvu*II. Size-classed fragments were ligated into the M13 vector cut with *Sma*I. The Hib porin gene was cloned by a strategy similar to that recently reported [4]. From the double-stranded restriction fragment form of the virus, the Hib porin gene was excised by double digestion with *Kpn*I and *Bam*HI; a fragment of 1.1 kb was isolated by agarose gel electrophoresis. This fragment was then modified at both the 5′ and 3′ ends to make it compatible with the BEVS transfer vector that contains a unique *Nhe*I cloning site [16]. A synthetic adaptor was added at the 5′ end of the porin gene: Its sequences contained an *Nhe*I site, the TATAAAT motif found in the −1 to −7 position upstream from the polyhedrin translation initiation codon, and an ATG start codon to initiate the translation of recombinant Hib porin. The design of the gene for recombinant Hib porin excluded codons for the normal signal sequence of 20 amino acids. Met-Gly-Thr-Pro were placed at the amino terminus instead of Ala1. At the 3′ end of the Hib porin gene and beyond the TAA stop codon were 102 nucleotides of chromosomal DNA from Hib that included sequences of dyad symmetry capable of forming a factor-independent transcription termination signal. An *Nhe*I linker was added to the 3′ end.

BEVS. This *Nhe*I-tailored Hib porin gene was cloned into the BEVS transfer vector pJV P10Z, one of a new family of transfer vectors that incorporates the gene for β-galactosidase (*lacZ*) to facilitate screening of recombinants [16]. The cultivated insect cell line Sf9 from *Spodoptera frugiperda* was cotransfected with wild-type DNA from *A. californica* plus purified transfer vector DNA containing the cloned Hib porin gene. By recombination through homologous viral sequences, a small number of progeny contained the Hib porin gene incorporated into the wild-type genome. The gene for polyhedrin was lost through this recombination event. Purification of recombinant viruses was simplified by incorporation of the *lacZ* sequence into the transfer vector. A chromogenic indicator identified blue plaques. Three rounds of plaque purification were necessary to separate Hib porin–containing recombinant virus free from wild-type virus. A high-titer virus stock (AcPORIN) was grown to 7.5×10^7 pfu/ml.

Purification of recombinant Hib porin. Recombinant Hib porin was purified by using the same extraction steps and detergents as were used to purify the protein from the bacterial cell surface [8]. Sf9 cells infected by AcPORIN were treated with 1% cetyltrimethylammonium bromide followed by detergent exchange into 0.1% zwitterionic detergent (Zwittergent Z-3, 14; Calbiochem, La Jolla, CA), yielding a preparation that was suitable for immunoaffinity column chromatography. Anti–Hib porin antibodies were raised in rabbits and the immunoglobulins were isolated from the sera by repeated ammonium sulfate fractionation and then by passage over a protein A–Sepharose (Pharmacia, Piscataway, NJ) column. Immunoglobulins were conjugated to Affi-Gel Hz (Bio-Rad, Richmond, CA), thereby generating a column for purification of detergent-solubilized Hib porin. Extracts of recombinant Hib porin were passed over the anti-Hib porin antibody column. The bound recombinant Hib porin was eluted with 0.10 *M* glycine, 0.5 *M* NaCl, and 0.05% Zwittergent Z-3, 14 (pH 3.0) and immediately neutralized with 0.1 vol of 1.0 *M* TRIS-HCl, pH 8.0.

Lipid bilayer experiments. The apparatus for determining the biological activities of Hib porin and recombinant Hib porin was a Teflon chamber in which two compartments were separated by a hole of 0.1 mm^2 [9]. Lipid bilayers were formed across the hole with a solution of 2.5% glyceryl monooleate in *n*-decane. Bilayer formation was monitored by the membrane's turning optically black to incident light. The purified porin was added to the aqueous phase either before membrane formation or after the membrane had turned black. Conductance across the membrane was measured by applying a fixed transmembrane potential. A pair of Ag-AgCl electrodes was inserted into symmetrical solutions on both sides of the membrane. An operational amplifier (type AD 40K; Analog Devices, Norwood, MA) was used in a current amplifier configuration such that the current could be monitored on a storage oscilloscope and recorded on a strip chart recorder.

Results

Expression of recombinant Hib porin by BEVS. When wild-type *A. californica* nuclear polyhedrosis virus infects insect cells, the polyhedrin gene is temporally regulated such that expression of polyhedrin protein is activated late in infection. The polyhedrin gene is dispensable: It is required neither for infection in cultivated insect cells nor for virus replication. Expression of the Hib porin gene under control of the polyhedrin promoter would be expected to follow kinetics similar to those of the temporal expression of polyhedrin. Sf9 cells were infected with AcPORIN and samples were harvested at varying times postinfection. The infected cells were lysed in electrophoresis sample buffer, loaded onto a 12% SDS-polyacrylamide gel, electrophoresed, and identified by staining with Coomassie brilliant blue. The proteins identified by SDS-PAGE (figure 1) 12 and 24 h postinfection were similar to those before infection; no additional proteins were apparent. At 48, 72, and 96 h postinfection, a viral glycoprotein (62 kDa) appeared in infected cell extracts; this protein is an index of successful infection. In addition, two novel protein species of ~38–40 kDa appeared. The intensities of all these new proteins were equivalent for each of the three time points (48, 72, and 96 h) as judged by the intensity of staining with Coomassie blue.

Confirmation that one of the proteins of 38–40 kDa was recombinant Hib porin was obtained by electrophoretic transfer onto nitrocellulose of a duplicate gel of the extracts collected at all time points between 0 and 96 h postinfection. The transferred proteins were reacted with a mouse monoclonal anti-porin antibody POR.1 and detected with a second-

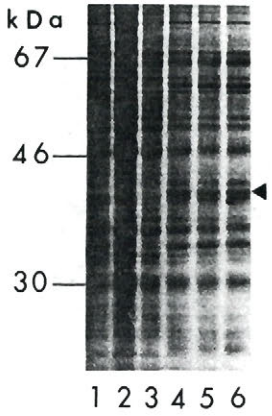

Figure 1. Total proteins produced in Sf9 cells infected with recombinant virus AcPORIN. Cells were lysed at 0, 12, 24, 48, 72, and 96 h postinfection (lanes 1–6, respectively). Arrowhead indicates novel protein of ~38 kDa that appears at 48 h.

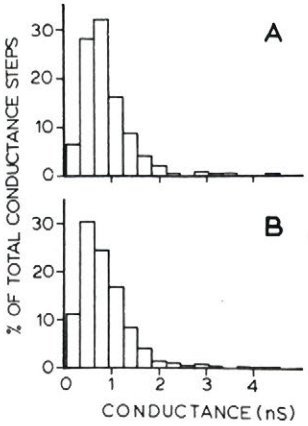

Figure 3. Conductance steps induced by purified-Hib porin (**A**) or purified recombinant Hib porin (**B**). Most conductance steps were insertion events that incorporated open pores.

ary antibody conjugated to alkaline phosphatase. A single species of recombinant Hib porin was detected (figure 2). It was identical in mobility to porin isolated from the outer membrane of Hib.

Biophysical properties of recombinant Hib porin. The recombinant Hib porin from BEVS was purified from Sf9 cells that had been infected with AcPORIN. We used the same detergents (cetyltrimethylammonium bromide and Zwittergent Z-3, 14) to extract recombinant Hib porin because they are compatible with the porin that was purified from the Hib outer membrane. Immunoaffinity chromatography was used to purify recombinant Hib porin from other membrane proteins of the insect cells.

Reconstitution experiments were done to measure the conductance of single channels induced by the incorporation of Hib porin or recombinant Hib porin into black lipid membranes. To obtain well-resolved conductance steps, small amounts of porin (1.0 ng/ml) were added to the aqueous phase (1 *M* KCl) bathing an artificial membrane. Porin isolated from the outer membrane of Hib was measured for its single-channel conductance at 50 mV. For 249 steps, each representing the insertion of a functional pore into the membrane, a mean value for single-channel conductance of 0.89 nS was determined (figure 3A). For recombinant Hib porin, 398 conductance steps were measured and they gave an average single-channel conductance of 0.88 nS (figure 3B).

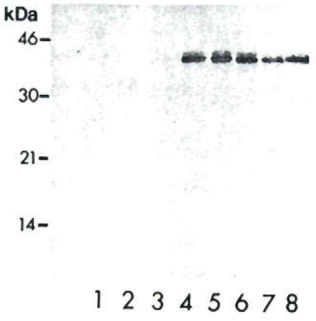

Figure 2. Immunoblotting of extracts of Sf9 cells infected with AcPORIN. Lanes 1–6, samples taken at 0, 12, 24, 48, 72, and 96 h postinfection; lanes 7 and 8, 100 and 200 ng of purified porin.

Discussion

Our objectives were to generate recombinant Hib porin in an expression vector system that is devoid of LPS and to test the recombinant protein for its biophysical properties in a reconstitution system. We used the BEVS to express high levels of the porin gene of Hib. The recombinant Hib porin appeared to be stable and not subject to endogenous proteolysis. No breakdown products were detected by immunoblotting with mouse monoclonal antibody POR.1. Some estimate of the yield of recombinant Hib porin was made by preparing samples for Western blotting. The staining of Hib porin standards was compared with the staining of recombinant Hib porin. Using such criteria, 1 μg of recombinant Hib porin was generated by 4.8×10^6 Sf9 cells that were grown as a monolayer in 4 ml of medium. Although the level of expression of recombinant Hib porin seems modest, this number of Sf9 cells is also small. Cultivation on a larger scale could augment the amount of recombinant Hib porin that can be produced by the BEVS.

The black lipid membrane system is considered one of the most sensitive assays to evaluate the properties of pore-forming proteins. Such proteins need to be purified preparations. Contaminating proteins that insert into the membrane without forming pores generate electronic noise that precludes the observation of isolated insertion events. For this reason, we used immunoaffinity chromatography to purify recombinant Hib porin. This sample was functionally identical to porin that was isolated from the outer membrane of Hib: The average values for single-channel conductance were 0.89 nS and 0.88 nS for Hib porin and recombinant Hib porin, respectively. We conclude that the conformation into which the recombinant Hib porin folded is similar or identical to the conformation of the Hib porin. Furthermore, because the BEVS contains no LPS, we propose that LPS plays no role in modulating the conformation of recombinant Hib porin.

The suitability of recombinant Hib porin as a vaccinogenic candidate or as an alternative carrier for capsular polysaccharide in the design of new conjugate vaccines merits continuing investigation.

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PREFACE TO CHAPTER 3

The potential of using recombinant porin as a vaccinogenic candidate or as an alternative carrier for capsular polysaccharide in a new generation of vaccines against Hib disease merits an investigation of porin's antigenic structure. The successful expression of a stable recombinant porin protein in the baculovirus expression vector system, as identified in chapter 2, suggested the development of a family of deletion proteins to further these studies. In chapter 3 the recombinant porin is tested for reactivity against anti-Hib porin monoclonal antibodies. In this chapter I also report the generation of a series of mutant *ompP2* genes by the insertion of an omega cassette in order to prematurely terminate transcription at four unique sites within the porin coding sequence. These were then subcloned into baculovirus transfer vectors and their subsequent expression in the BEVS is reported here. The recombinant truncated proteins were then tested for their reactivities to a panel of monoclonal antibodies made against porin of Hib.

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Monoclonal antibodies specific to porin of *Haemophilus influenzae* type b: localization of their cognate epitopes and tests of their biological activities

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Summary

The major outer membrane protein of *Haemophilus influenzae* type b (Hib) is porin (M_r 38 000, 341 amino acids). To identify antigenic determinants on Hib porin that might be exposed at the bacterial cell surface, seven mouse monoclonal anti-Hib porin antibodies were generated. The monoclonal antibodies were tested for their binding to intact cells by flow cytometry; all but one bound to the cell surface. Digestions of Hib porin with cyanogen bromide, hydroxylamine or trypsin generated fragments, the identities of which were confirmed by microsequencing of the amino termini. Following electrophoresis and immunoblotting of the fragments, the specificities of the monoclonal antibodies for their cognate sequences were determined. The porin gene *ompP2* was expressed in the baculovirus expression vector system; the recombinant porin was recognized by all of the monoclonal antibodies. Deletions were created by omega mutagenesis of *ompP2*, generating proteins truncated after amino acids 139, 174, 182, and 264. These deletion proteins were tested for reactivities with the monoclonal antibodies, thereby establishing the boundaries of three antigenic determinants that were recognized by the monoclonals: domain (i), amino acids 104–139; domain (ii) amino acids 162–174; and domain (iii), amino acids 267–341. The biological activities of monoclonal antibodies that were representative of these three classes were tested for their bactericidal activity in complement-mediated lysis of whole cells. The monoclonal antibodies were also tested for their immunoprotective properties in the infant rat model of bacteraemia. Although the monoclonal antibodies were surface-binding, they were neither bactericidal nor protective.

Introduction

Haemophilus influenzae type b (Hib) is the most common cause of bacterial meningitis in infants under 18 months. To elicit protective antibodies against this disease, much attention has been directed to the capsular polysaccharide polyribosyl ribitol phosphate (PRP) of this bacterium and its use as a vaccine (Granoff *et al.*, 1986). Because PRP alone was poorly antigenic in young infants most susceptible to disease, a new generation of vaccines was developed based on conjugation of PRP to carrier proteins. These polysaccharide–protein conjugates are of recognised value in preventing disease (Eskola *et al.*, 1990; Mäkelä *et al.*, 1990). Other surface components of Hib have also been considered as vaccine candidates (Granoff and Munson, 1986). Studies performed with an experimental infant rat model demonstrated that antibodies specific for non-capsular surface components might play a role in humoral defence mechanisms. A key observation was that antibodies directed against non-capsular antigens were able to protect against the experimental infection (Munson *et al.*, 1983). One of the non-capsular antigens is porin (M_r 38 000), the most abundant protein in the outer membrane of this bacterium.

We have studied the Hib porin extensively and have shown that it has a molecular mass exclusion limit to solutes greater than 1400 Da (Vachon *et al.*, 1985), a cross-sectional diameter of 0.9 nm (as determined by reconstitution into black lipid membranes; Vachon *et al.*, 1986), and a trimeric organization in the outer membrane (Vachon *et al.*, 1988). Furthermore, Hib porin is surface-exposed and conserved; monoclonal antibody Hb-2 recognized native porin at the surface of intact cells (Hamel *et al.*, 1987b) and reacted with 453/455 type b strains collected from a wide variety of sources (Hamel *et al.*, 1987a). Information on the structural and antigenic conservation of Hib porin (Hansen *et al.*, 1989b) has been extended to the cloning and sequencing of the porin gene

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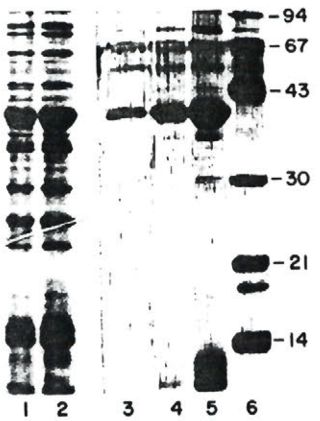

Fig. 1. Purification of porin from Hib strain ATCC9795. Samples were run on 13% SDS–PAGE and silver stained. Lanes 1 and 2: 1500 and 3000ng of porin prepared by the procedures of Vachon *et al.* (1985) and previously appearing as a single band after staining with Coomassie Brilliant Blue staining. Lanes 3, 4 and 5: 250, 500, 1000ng of FPLC-purified porin prepared as described in the *Experimental procedures.* Lane 6: molecular mass markers in kDa.

(Hansen *et al.*, 1989a; Munson and Tolan, 1989). Additional DNA sequence data have been recently generated for Hib porin genes from prototype strains of three major clone families of different outer membrane protein (OMP) subtypes: 2L, 3L, and 6U. These gene sequences showed variations of 1, 0, and 13 nucleotides respectively, over the entire sequence (Munson *et al.*, 1989) relative to the first reported porin sequence. The variations correspond to 1, 0, and 10 amino acid changes in primary sequence relative to porin from OMP subtype 1H.

To extend the information on porin of Hib beyond its biophysical behaviour and its gene structure, we wished to identify regions of porin that might be surface-exposed and accessible to antibodies. Our approach was to generate a panel of anti-Hib porin monoclonal antibodies (mAbs), to map the epitopes on porin that were recognized by them, and to identify those epitopes that are at the bacterial cell surface. Because polyclonal antibodies against Hib porin have been reported to be bactericidal and protective, the mAbs were also tested for their biological activities in complement-mediated bactericidal tests and immunoprotection in the infant rat model of infection.

Results

Monoclonal antibodies against Hib porin

The purification scheme for porin of Hib strain ATCC9795 as described by Vachon *et al.* (1985) yielded a single species of protein when stained with Coomassie Brilliant Blue. Upon silver staining of the preparations, several contaminating bands appeared (Fig. 1). The use of fast-protein liquid chromatography (FPLC) improved the purification to produce one major species by silver staining, provided high yields (10mg per run), and showed some contamination with lipopolysaccharide (LPS) of less than 100ng per μg of FPLC-purified protein. LPS could not be removed even by repeated passage of the sample over the MonoQ column.

BALB/c mice were immunized with FPLC-purified Hib porin suspended in Freund's incomplete adjuvant. Polyclonal sera were collected from the mice and analysed for the presence of Hib porin-specific antibodies by immunoblotting. These sera reacted strongly with the 38kDa porin and weakly with other OMPs. To generate stable hybridomas from the cells, the porin-challenged splenocytes were fused with the SP2/0 myeloma cell line. Tissue-culture supernatants from the hybridomas were screened for antibodies by means of the enzyme-linked immunosorbent assay (ELISA) using outer membrane preparations from Hib and FPLC-purified Hib porin as coating antigens, and the positive ones were further analysed for their capacity to recognize denatured porin by immunoblotting. OMPs were solubilized in electrophoresis sample buffer by boiling, electrophoresed on a 12% acrylamide gel, and transferred to nitrocellulose. Immobilized proteins were reacted first with the culture supernatants and then with a secondary anti-mouse immunoglobulin conjugated to peroxidase. If a band of 38kDa was observed in this second screen, then the hybridoma was cloned by limiting dilution and retested in the above two assays. Using these strategies, seven anti-Hib porin monoclonal antibodies, mAb POR.1 to mAb POR.7, were generated, each specifically reacting with a band in the preparation of the OMPs that co-migrated with purified Hib porin. The mAbs differed according to the reciprocal titres of their reactivities against FPLC-purified Hib porin and were of different IgG isotypes (Table 1).

Surface binding of monoclonal antibodies

While the above results demonstrated that mAbs POR.1 to POR.7 reacted with Hib porin in outer membrane vesicles (ELISA) and with denatured porin (immunoblotting), it was important to assess whether the seven monoclonals bound to native Hib porin at the bacterial cell surface. Flow cytometry is a sensitive technique for assessing the surface location of cellular macromolecules by their reaction with specific antibodies. Intact bacteria were mixed with a mAb, washed free of unbound mAb, and the surface-bound mAb was detected with anti-mouse kappa light-chain mAb 187.1 (Yelton *et al.*, 1981) conjugated to fluorescein or anti-mouse IgG heavy chain conjugated to fluorescein. Surface-bound fluorescence on 20 000 cells

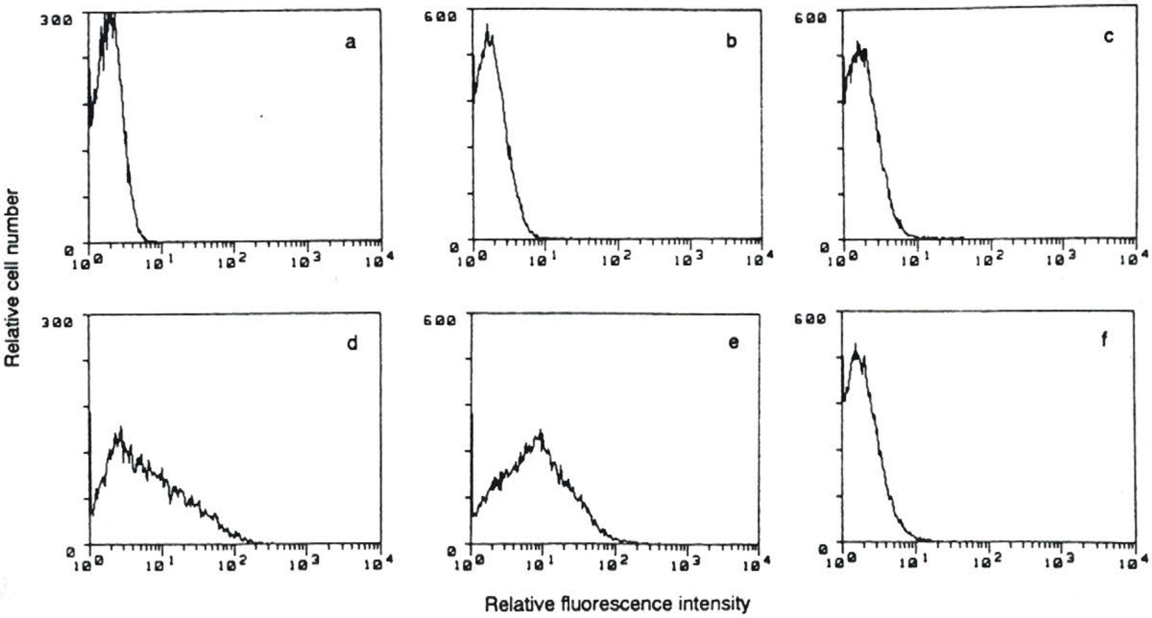

Fig. 2. Flow cytometry of *Haemophilus influenzae* type b with anti-Hib porin monoclonal antibodies. Bacteria were prepared and stained as described in the *Experimental procedures*. Panel a. Hib strain ATCC9795 stained only with secondary antibody conjugated to FITC; panels b and c, Hib strain DL42/2F4⁻ stained with mAb POR.4 and mAb POR.1, respectively; panels d, e and f: Hib strain ATCC9795 stained with mAb Hb-2 and mAb POR.4 and mAb POR.1, respectively.

per sample was assessed by flow cytometry. As a control for non-specific binding of antibody, we used Hib strain DL42/2F4⁻, a porin-deletion mutant. No staining of strain DL42/2F4⁻ was observed with any of mAbs POR.1 to POR.7 or with mAb Hb-2 (Fig. 2). When Hib strains ATCC9795 or RH3527 were assayed, there was no detectable staining with mAb POR.1. However, all other POR.2 to POR.7 mAbs and mAb Hb-2 stained these cells, as shown by the increase in relative fluorescence intensity. *H. influenzae* strain RH3528 is a non-capsulated variant derived from RH3527 and was stained by mAbs POR.2 to POR.7 and mAb Hb-2 but not by mAb POR.1.

Identification of immunoreactive amino acid sequences of Hib porin

To identify the primary amino acid sequences with which the seven mAbs reacted, several chemical and enzymatic digestions were conducted using FPLC-purified protein. The digestion products were separated by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS–PAGE), blotted onto nitrocellulose, and probed with each mAb. Cyanogen bromide digested the Hib porin at a unique Met-266 to two fragments of 30 kDa and 8 kDa. Different patterns of mAb reactivity were distinguished: (i) mAbs POR.1, POR.2, POR.3, POR.4, and POR.5 reacted with the amino-terminal 30 kDa fragment; (ii) mAbs POR.6 and POR.7 reacted with the carboxy-terminal 8 kDa fragment.

Cleavage with hydroxylamine occurs primarily between Asn–Gly bonds. In Hib porin there are three potential cleavage sites for this reagent: between amino acids 74/75, 181/182, and 218/219. Digestion conditions were selected to favour partial cleavages and so a family of five fragments was observed upon staining of the electropherograms. These fragments were 30, 24, 20, 18, and 14 kDa

Table 1. Monoclonal antibodies against porin of *H. influenzae* type b.

	Reciprocal Titre of Antibody Reactivity		
mAb designation	bacterial porin[a]	recombinant porin BEVS[b]	Immunoglobulin subclass
POR.1	10 000	25 000	IgG1, κ
POR.2	5000	5000	IgG1, κ
POR.3	5000	5000	IgG1, κ
POR.4	1000	5000	IgG1, κ
POR.5	1000	1000	IgG3, κ
POR.6	1000	500	IgG2a, κ
POR.7	100	500	IgG2a, κ
Hb-2	500	500	IgG2a, κ

a. FPLC-purified bacterial porin (10 μg ml⁻¹) from strain ATCC9795 in carbonate buffer was adsorbed to the ELISA plate, overnight, at room temperature; dilutions of mAbs were reacted with antigen, followed by a secondary antibody conjugated to alkaline phosphatase. The reciprocal titre is expressed as the dilution of mAb which gave an absorbance reading of at least 0.2 over background for the colorimetric assay of enzymatic activity.

b. Lysate from Sf9 insect cells infected with AcPOR virus (1.5 μg ml⁻¹) was adsorbed to the ELISA plate. Conditions for primary and secondary reactions with antibodies and with substrate were the same as for the reaction with FPLC-purified porin.

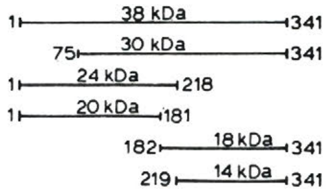

Fig. 3. Cleavage of Hib porin by hydroxylamine and identification of the cleavage products by microsequencing. Five polypeptides were generated by partial digestion with hydroxylamine: 30, 24, 20, 18, and 14 kDa. Each polypeptide was sequenced from its amino terminus and was oriented relative to the others by sequence and by molecular size. The numerical values at the ends of each line represent the confirmed amino-terminal amino acids and the assigned carboxy-terminal amino acids, based upon the location of Asn–Gly linkages in the primary sequence of porin.

(Fig. 3). When these five porin-derived peptides were immunoblotted and probed with mAbs, the following reactivities were detected: mAbs POR.1, POR.2, POR.3, POR.4, POR.5, POR.6 and POR.7 reacted with the 30 kDa fragment; mAbs POR.1, POR.2, POR.3, POR.4, and POR.5 reacted with the 24 and the 20 kDa fragments; mAbs POR.6 and POR.7 reacted with the 18 and 14 kDa fragments. To confirm the sites of cleavage, microsequencing of the first five amino acids of each fragment was conducted. These sequences were then matched with the primary amino acid sequence of Hib porin as follows: Gly-75–Ser–Asp–Asn–Phe, 30 kDa fragment; Ala-1–Val–Val–Tyr–Asn, 24 and 20 kDa fragments; Gly-182–Ile–Gln–Val–Gly, 18 kDa fragment; Gly-219–Val–Leu–Ala–Thr, 14 kDa fragment. These data indicate that the boundaries of epitopes recognized by mAbs POR.1, POR.2, POR.3, POR.4, and POR.5 are between amino acids 75 and 181 and that the boundaries of epitopes recognized by mAbs POR.6 and POR.7 are between amino acids 219 and 341.

Although porins from other bacteria are generally resistant to enzymatic cleavage (Rosenbusch, 1990), porin of Hib was cleaved by trypsin when the digestion was performed overnight in detergent. Trypsin was predicted to cleave this protein more than 40 times at the Arg and Lys residues. After overnight incubation and with a high amount of trypsin, seven cleavage products (and sometimes eight) were resolved by SDS–PAGE on a 20% gel followed by silver staining. Although these fragments were not always of the same intensity, their relative mobilities and therefore molecular sizes could be accurately estimated. The trypsin cleavage products were 19.5, 19.1, 18.9, 18.4, 17.1, (15.5), 15.0, and 14.0 kDa (Fig. 4A). Monoclonal antibodies POR.6 and POR.7 reacted with both the 19.5 and 19.1 kDa fragments (Fig. 4B). Monoclonal antibodies POR.2, POR.3, POR.4, and POR.5 reacted only with the 18.9 and 15.5 kDa fragments and mAb POR.1 reacted with all six trypsin fragments between 18.9 and 14.0 kDa. Microsequencing of the peptides showed that the amino termini were Ala-171–Gly–Glu–Val–Arg, 19.5 kDa fragment; Ile-176–Gly–Glu–Ile–Asn, 19.1 kDa fragment; Ala-1–Val–Val–Tyr–Asn, 18.9, 18.4, and 17.1 kDa fragments; Gln-33–Gln–His–Gly–Ala, 15.5, 15.0, 14.0 kDa fragments. Considering the locations and the relative susceptibilities of trypsin-sensitive cleavage sites between amino acids 156 and 175 and using the sizes of trypsin cleavage products calculated from the primary sequence, tentative assignments were made for the carboxy-termini of the fragments. The proposed *C*-terminal residues are: Phe-341 for the 19.5 and 19.1 kDa fragments; Arg-175 for the 18.9 and 15.5 kDa fragments; Lys-170 for the 18.4 and 15.0 kDa fragments; Lys-161 for the 17.1 and 14.0 kDa fragments. A summary of these assignments is found in Fig. 4C. From these data it is proposed that mAb POR.1 reacts with some amino acid sequences up to Lys-161, that mAbs POR.2, POR.3, POR.4, and POR.5 react with sequences between Gly-162 and Arg-175, and that mAbs POR.6 and POR.7 recognize some amino acids in the carboxy-terminal half of Hib porin. Instead of digesting Hib porin in solution, experiments were conducted to digest Hib porin by trypsin that was immobilized in a polyacrylamide gel. One of the many

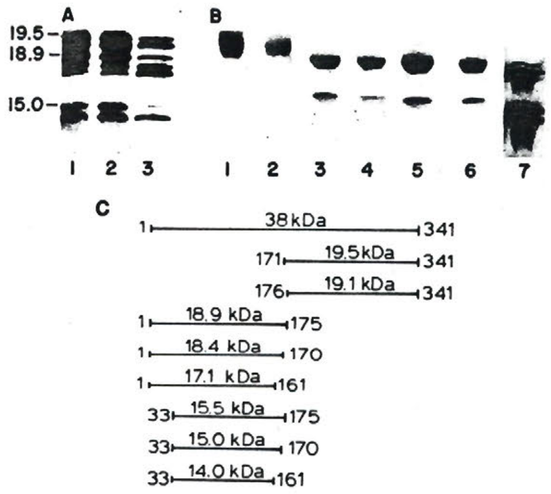

Fig. 4. Cleavage of Hib porin by trypsin and identification of monoclonal antibody reactivities against the cleavage products.
Panel A. Hib porin in solution was digested with trypsin, aliquots were removed at 1, 2, and 16 h of incubation (lanes 1, 2 and 3), electrophoresed on a 20% SDS–PAGE, and silver stained to identify the fragments. The molecular masses (kDa) of three of the fragments are indicated for reference in the left margin.
Panel B. Reaction of anti-Hib porin mAbs against trypsin digestion fragments. Lanes 1 to 7: mAbs POR.6, POR.7, POR.2, POR.3, POR.4, POR.5, and POR.1, respectively.
Panel C. Orientation of fragments from trypsin cleavage of Hib porin. Fragments were sequenced from their amino-termini; the first amino acid from the sequence data is the numerical value on the left of each line. Carboxy-termini were assigned, based upon sizes of the fragments; the numerical value on the right of each line indicates the carboxy-terminus of the cleavage fragments.

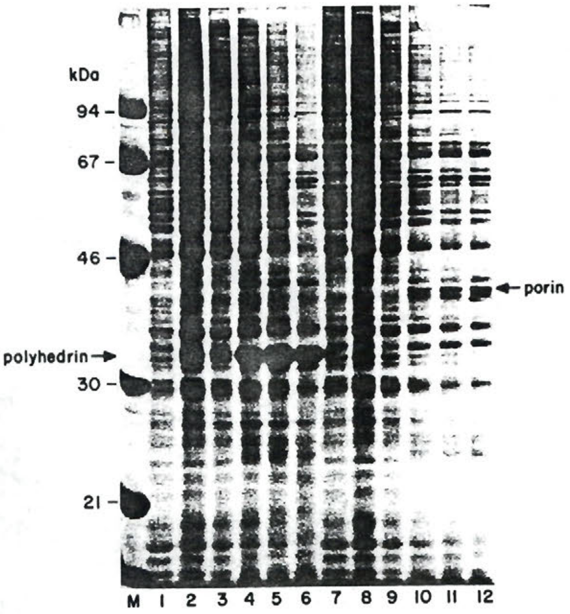

Fig. 5. Identification of proteins in extracts of Sf9 insect cells infected with wild-type baculovirus AcNPV (lanes 1–6) or with recombinant virus AcPOR (lanes 7–12). Replicate Sf9 cultures were infected with virus at a multiplicity of infection of 10:1 and samples were taken at the following hours, post-infection: 0 (lanes 1 and 7); 12 (lanes 2 and 8); 24 (lanes 3 and 9); 48 (lanes 4 and 10); 72 (lanes 5 and 11); and 96 (lanes 6 and 12). Samples were lysed in electrophoresis sample buffer, sheared by passage through a fine-guage needle, and electrophoresed on a 10% acrylamide slab gel. Proteins were stained with Coomassie Brilliant Blue. Molecular mass standards (in kDa) are indicated in the column labelled 'M'. The arrow in the left-hand margin indicates the position of polyhedrin; the arrow in the right-hand margin indicates the position of recombinant porin.

fragments of this partial proteolysis had a M_r of 27 000 and an amino terminus of Ala-104–Lys–Thr–Ile–Ala; such a fragment would extend to Phe-341. This 27 000 porin fragment reacted with mAb POR.1 as well as with mAbs POR.2, POR.3, POR.4, POR.5, POR.6 and POR.7. Such a result served to narrow further the domain of reactivity for mAb POR.1 to between Ala-104 and Lys-161.

Recombinant porin from baculovirus expression vector system

An independent verification that the mAbs were reacting against the Hib porin protein and not against contaminating materials such as LPS was obtained by expressing the porin gene *ompP2* in a non-bacterial expression vector system. The baculovirus expression vector system (BEVS) is an attractive system because no LPS is synthesized by Sf9 insect cells. The *ompP2* gene was cloned into pJV.P10Z, one of a new generation of baculovirus transfer vectors which possess the following features: the polyhedrin promoter drives the expression of the foreign gene; the gene for β-galactosidase is divergently transcribed from the P10 promoter and in the presence of a chromogenic indicator serves to identify recombinant plaques on a lawn of Sf9 cells. Blue plaques were purified by three cycles of plaque purification and a single isolated plaque was amplified to give a high-titre lysate designated AcPOR. Because of the cloning strategy that was used, the nucleotide sequence of recombinant porin was extended to create four amino acids at the amino terminus Met–Gly–Thr–Pro followed by Val-2-Val-3–Tyr-4–Asn-5– – –Phe-341 of the mature porin protein.

The time course of synthesis of recombinant porin from BEVS was monitored by infecting Sf9 cells with a stock of recombinant virus AcPOR. For comparison, wild-type baculovirus AcNPV was used to infect a parallel culture of Sf9 cells. Samples were extracted at various times post-infection and the total proteins of the infection mixture were identified by SDS–PAGE (Fig. 5). Up to 24 hours post-infection (hpi), the spectrum of proteins in cells infected with AcNPV or with AcPOR was indistinguishable. At 48 hpi, a major protein corresponding to polyhedrin (33 kDa) appeared in the lysate of cells infected with AcNPV. The relative amounts of this protein were equivalent in the samples taken up to 96 hpi. For Sf9 cells infected with AcPOR, a novel species of protein appeared at 48 hpi and was of the M_r anticipated for recombinant porin (38 000). No polyhedrin protein was detectable in these same samples, thereby confirming the purity of the AcPOR virus stocks. Again the amounts of this recombinant protein were comparable in all samples up to 96 hpi.

Two assays were used to identify the presence of recombinant porin and its reactivities with mAbs. First, samples of the above extracts were blotted onto nitrocellulose, probed with each of the mAbs POR.1 to POR.7 and detected with a secondary antibody conjugated to alkaline phosphatase. Extracts from mock-infected cells and from cells infected with AcNPV served as controls. For the latter two extracts, no bands were detected by immunoblotting. All mAbs reacted with recombinant porin by Western blotting. The kinetics of synthesis of recombinant porin (Fig. 6) matched the appearance of the novel protein species of M_r 38 000 that was identified by staining with Coomassie Brilliant Blue staining. Recombinant porin first appeared at 48 hpi and was also observed in samples taken at 72 and 96 hpi. Recombinant porin co-migrated with FPLC-purified Hib porin from the bacterial outer membrane. The absence of lower molecular-weight species on Western blotting suggested that there was little or no degradation of recombinant porin.

A second assay showed that recombinant porin reacted in a solid-phase system in a manner similar to porin isolated from Hib. Extracts of Sf9 insect cells infected with AcPOR were applied to ELISA plates and then tested with increasing dilutions of mAbs POR.1 to POR.7. The

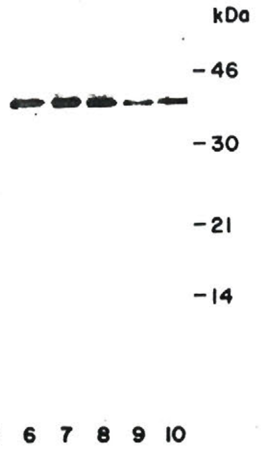

Fig. 6. Kinetics of synthesis of recombinant porin in BEVS. At different times post-infection of Sf9 insect cells by AcPOR, the samples were collected and lysed. After separation by SDS–PAGE, proteins were transferred to nitrocellulose, probed with mAb POR.1, and detected with a secondary anti-mouse mAb conjugated to alkaline phosphatase. Mock-infected cells (lane 1) and cells infected with AcNPV (lane 2) were used as negative controls. Porin isolated from Hib was the positive control for immunoreactivity: 100ng (lane 9) and 250ng (lane 10). Recombinant porin was not detected in samples at 0 (lane 3), 12 (lane 4), or 24 (lane 5) hpi. It appeared first in the sample taken at 48hpi (lane 6) and was also detected in samples at 72 and 96hpi (lanes 7 and 8 respectively). Molecular mass (in kDa) markers are indicated in the right margin.

reciprocal titres of mAb reactivities are shown in Table 1 and these values are comparable with those obtained for the reactivities of mAbs against Hib porin.

Truncated porin proteins from BEVS

Because we were able to synthesize stable, full-length recombinant porin without apparent endogenous proteolysis by Sf9 insect cells, it was of interest to generate stable carboxy-terminal deletion proteins in the BEVS and to use these deletion proteins to confirm the domains of reactivities of the panel of seven mAbs. By omega mutagenesis, the transcription and translation termination signals from gene *32* of T4 phage were inserted as a spectinomycin/streptomycin-resistance cassette at four hexanucleotide recognition sites *Dra*I, *Sna*BI, *Eco*RI, and *Ase*I within the porin gene. The insertions of the omega cassette at these positions within *ompP2* were expected to generate proteins that terminated after amino acids 139, 174, 182 and 264 of the primary sequence of mature porin. These four deletion proteins were designated POR139, POR174, POR182, and POR264 and they were used to clarify the domains of reactivities of the mAbs with particular reference to the carboxy-terminal boundaries.

Mouse polyclonal anti-porin IgG and mAb POR.1 recognized all four deletion proteins (POR139, POR174, POR182, POR264) as well as full-length recombinant porin from BEVS and bacterial porin (Fig. 7). Monoclonal antibodies POR.2, POR.3, POR.4 and POR.5 did not recognize the truncated protein POR139 but they showed a positive reaction on immunoblotting against proteins POR174, POR182, and POR264. Monoclonal antibodies POR.6 and POR.7 did not react with the truncated protein POR264 but were positive against full-length recombinant porin. These data served to establish the following carboxy-terminal boundaries of reactivity: mAb POR.1, amino acid 139; mAbs POR.2, POR.3, POR.4, and POR.5, amino acid 174; mAbs POR.6 and POR.7, amino acid 341.

Biological activities of mAbs

Because six mAbs were demonstrated to bind to the surface of Hib, we tested the mAbs for their biological activities in two different experimental systems. Bactericidal assays provided an indication of the ability of the mAb to bind complement and to activate complement-mediated killing of intact cells. The immunoprotection of the mAbs was assessed in the infant rat model of bacteraemia; passive transfer of protective antibodies, prior to intraperitoneal challenge with a dose of Hib, results in clearance of the infecting bacteria. Antibodies that are not protective are unable to abrogate bacteraemia, the index of infection.

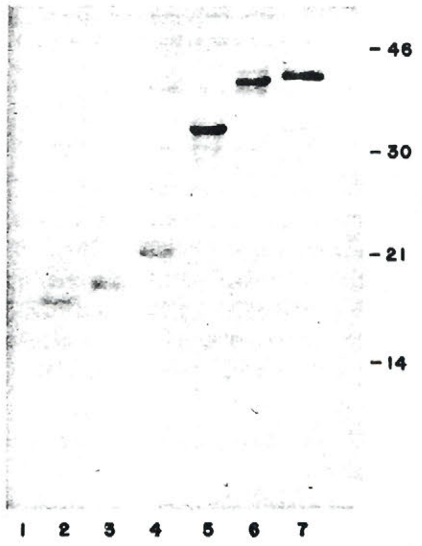

Fig. 7. Identification of recombinant carboxy-terminal deletion proteins and their immunoreactivities with monoclonal antibodies. Four truncated genes were created by omega mutagenesis of *ompP2* and the deletion proteins were expressed in BEVS. The following samples were subjected to the SDS–PAGE: lane 1: mock-infected Sf9 cells; lanes 2, 3, 4 and 5: Sf9 cells infected with recombinant virus that expressed POR139, POR174, POR182, and POR264, respectively; lane 6: Sf9 cells infected with AcPOR; lane 7, FPLC-purified porin from Hib. Proteins were electrophoresed, transferred to nitrocellulose, probed with mAb POR.1, and detected with an enzyme-linked secondary antibody. Molecular mass (in kDa) markers are indicated in the right margin.

Table 2. Passive protection of infant rats by antibodies against PRP or against porin.

Antibody	Bacteraemia[a]	High-level bacteraemia[b]	Geometric mean titre (c.f.u. × 10^3 ml^{-1} blood)
None	11/11 (100%)	11/11 (100%)	60.2
Anti-PRP	0/11 (0%)	0/11 (0%)	0.05
POR.1	8/8 (100%)	8/8 (100%)	38.7
POR.2	9/9 (100%)	8/9 (89%)	28.2
POR.5	9/9 (100%)	9/9 (100%)	60.0
POR.6	9/9 (100%)	9/9 (100%)	47.0
Hb-2	9/9 (100%)	9/9 (100%)	33.6

a, b. Values listed are numbers of rats showing viable c.f.u. of Hib in blood samples at 18 hours post-challenge; high-level bacteraemia is defined as greater than 2.5 × 10^3 c.f.u. ml^{-1} of blood.

The Hib strain ATCC9795 was avirulent for the infant rat since intraperitoneal challenge of 40 or 400 or 4000 colony-forming units (c.f.u.) did not result in bacteraemia. Upon injection of the same numbers of c.f.u. of Hib strain RH3527, bacteraemia was routinely established. This strain was therefore chosen for both biological assays. In addition, a spontaneously derived non-capsulated derivative (RH3528) was tested as a second target strain for bactericidal activities. Some mAbs were selected for assay according to the primary porin sequences to which the antibodies bound: POR.1, POR.2 and POR.5, and POR.6. As controls, mouse anti-Hib porin mAb Hb-2 (Hamel *et al.*, 1987b), a human pool of anti-PRP antibodies, and mouse anti-LPS mAb 3DA11 were used.

Hib strain RH3527 was resistant to 25% human complement and was not lysed by any of the monoclonal or polyclonal antibodies alone. In the presence of complement plus anti-PRP antibodies up to a dilution of 1/128, 50% killing of the input number of Hib strain RH3527 was observed. There was no bactericidal effect if complement had been heat-inactivated. In the presence of complement plus each of the mAbs POR.1, POR.2, POR.5, POR.6, or Hb-2, no reduction in c.f.u. was detected, even with the lowest dilution (1/100) which corresponded to 0.5 μg of affinity-purified mAb. This lack of bactericidal activity by the mAbs was not a strain-dependent phenomenon since the same results were obtained with Hib strain Eagan. Nor was the phenomenon related to the inhibition of complement action by the presence of capsule. *H. influenzae* strain RH3528, resistant to only 5% complement, was not lysed by any of the antibodies alone, but was efficiently killed by 5% complement plus anti-LPS mAb 3DA11 (IgG3) up to 1/3000 dilution. Again, 5% complement plus either mAb POR.1, POR.2, POR.5, POR.6 or Hb-2 was tested and shown not to be bactericidal against strain RH3528.

Infant rats in seven groups (with a minimum of eight rats per group) were injected with saline or anti-PRP antibodies (400 ng per animal) or mAbs POR.1, POR.2, POR.5, POR.6 or Hb-2 (all affinity-purified, up to 8 μg per rat). Upon administering a challenge dose of 4000 c.f.u. of Hib strain RH3527 followed by an interval of 18 hours, the geometric mean titre for the control group indicated high-level bacteraemia; no rats were bacteraemic when anti-PRP antibodies were passively transferred before challenge. The geometric mean titres for rats receiving anti-porin mAbs indicated that none of these antibodies conferred protection against bacteraemia (Table 2).

Discussion

The major pore-forming protein in the outer membrane of *H. influenzae* type b plays an essential role in permitting the transmembrane passage of water-soluble solutes into the periplasm. Our initial characterization of the biophysical behaviour of porin (Vachon *et al.*, 1986) has recently been extended by the cloning and sequencing of the *ompP2* gene (Hansen *et al.*, 1989a; Munson and Tolan, 1989). Although the primary sequences for the porin gene from four OMP subytpes of Hib are now available (Munson *et al.*, 1990), there is little information on the relationship between these variations of sequence and the immunobiological response that they elicit. There is also a paucity of information about the native topological organization of porin of Hib as it is oriented in the outer membrane. An obvious goal in the characterization of porin of Hib is to confirm the role of this protein in pathogenesis and immunity to infection. The studies in this paper were prompted by the often-cited report that polyclonal antibodies against porin of Hib have protective activity in the infant rat model of bacteraemia (Munson *et al.*, 1983). We wish to provide some answers to the following questions: what antigenic determinants on porin of Hib elicit an immune response; can the epitopes recognized by antibodies be assigned to the outer or inner surface of the outer membrane; what amino acid sequences on Hib porin are recognized by antibodies; do the antibodies against bacterial porin also recognize recombinant porin; and what is the role of such antibodies in conferring immunoprotection against bacteraemia?

Generating a panel of seven monoclonal antibodies against porin of Hib (also designated OmpP2) has provided experimental tools with which to answer some of these questions. Our mAbs reacted with both native and denatured OmpP2, and all but one was shown by flow cytometry to react to porin of Hib and to porin on a non-capsulated strain of *H. influenzae*. Taken together, these results implied that linear amino acid sequences at the cell surface were recognized by the mAbs and that strategies of protein fragmentation could be used to identify the cognate sequences. Such an analysis would have been more difficult if conformational epitopes had been recognized by the mAbs (Hansen *et al.*, 1989b). A combination of chemical and enzymatic digestions of OmpP2 gave information about the primary sequences to which the mAbs bound. The mAbs were tentatively assigned to three groups based upon their patterns of reactivities. While protein fragmentation gave clearly different patterns of recognition by mAbs, some caution was appropriate because of the report of the isolation of anti-Hib porin mAbs (Gulig *et al.*, 1983) which were later confirmed to be directed against LPS (Gulig and Hansen, 1985). The cloning and expression of *ompP2* in the BEVS, an LPS-free system, provided unequivocal evidence that the mAbs were reactive against Hib porin and not against any other biopolymer of *Haemophilus*. In the BEVS, four stable carboxy-terminal deletion proteins were synthesized: POR139, POR174, POR182 and POR264. These four proteins reacted variously with the mAbs and permitted accurate identification of the carboxy-terminal boundaries for mAb reactivities. Our data serve to identify the following domains of mAb reactivities: (i) mAb POR.1 reacts with some amino acids between Ala-104 and Phe-139; (ii) mAbs POR.2, POR.3, POR.4, and POR.5 react with some amino acids between Gly-162 and Val-174; and (iii) mAbs POR.6 and POR.7 react with some amino acids between Glu-267 and Phe-341. From our results with flow cytometry, we propose that domain (i) is either buried in the membrane or is on the periplasmic face of the outer membrane, that domain (ii) is a surface-exposed loop, and that domain (iii) or, more likely, part of this sequence is also oriented as a surface-exposed loop. Because domains (i) and (iii) are long stretches of 36 and 75 amino acids, respectively, further fine-structure mapping of these regions is warranted. Domain (ii) of 13 amino acids is close to the minimum length of sequences required for antibody recognition.

Coinciding with our success in generating and characterizing surface-reactive mAbs, there appeared another report in which mAbs against OmpP2 were isolated and used for essentially epidemiological purposes (Martin *et al.*, 1990). Most of the mAbs that were isolated in their study also appeared to be surface-reactive. No studies were reported that related to the biological effects of the mAbs, i.e. bactericidal tests or animal-protection studies. It was of interest that their mAbs reacted with encapsulated bacterial clones previously assigned to phylogenetic division I (Musser *et al.*, 1990) whereas none reacted with clones in primary division II.

Some recent mapping of B-cell epitopes on OmpP2 identified four distinct immunogenic and antigenic regions (Martin *et al.*, 1991). We have used computer-assisted methods (PC-Gene) to predict antigenic determinants on OmpP2. The three highest points of hydrophilicity are Lys-326–Lys-331 (average hydrophilicity, Ah = 2.43), Lys-161–Arg-166 (Ah = 2.03), and Lys-165–Lys-170 (Ah = 2.03). Six of our seven mAbs map to these regions and some of their mAbs map to these regions.

A surprising result of our studies is that even though six of the mAbs were surface-reactive, none was able to mediate complement-dependent lysis of Hib. This lack of bactericidal effects was not attributable to some steric hindrance of the capsule as was previously shown for anti-P1 antibodies (van Alphen *et al.*, 1986), since flow cytometric analysis demonstrated binding of mAbs both to capsulated and non-capsulated *H. influenzae*; nor were the mAbs immunoprotective in an infant rat model of bacteraemia. It is possible that antibodies against other surface-located antigenic determinants on porin of Hib might elicit different biological effects compared with those we observed. On the other hand, some revision of the conclusions of Munson *et al.* (1983) — that anti-porin antibodies are bactericidal and protective —may be necessary. Recombinant porin will be of value in extending our observations.

Experimental procedures

Bacterial strains and culture conditions

H. influenzae type b (Hib) strains ATCC9795 (Vachon *et al.*, 1985), Eagan (Moxon *et al.*, 1974) and RH3527 (also designated 760705; van Alphen *et al.*, 1983) were used in our studies. They are of OMP subtypes 1H, 1L, and 3L, respectively, and therefore have the same primary sequences of porin (Hansen *et al.*, 1989a; Munson *et al.*, 1989), a result confirmed by our nucleotide sequencing. A spontaneously isolated variant of RH3527 is designated RH3528 and is devoid of capsular polysaccharide. Hib strain DL42/2F4$^-$ was constructed by insertion mutagenesis of the porin gene *ompP2* and was characterized as porin-deficient (Cope *et al.*, 1990). The identity of all strains of *H. influenzae* was confirmed by their growth requirements for haemin and NAD$^+$. The capsular serotype was determined by the latex agglutination kit, commercially available from Wellcome Laboratories. Strains were grown either on chocolate agar supplemented with 1% Isovitalex (BBL) or in brain–heart infusion medium supplemented with Fildes, Enrichment Agar (Becton Dickinson).

Preparation of antigens

Vesicles containing OMPs from Hib were prepared using the lithium chloride extraction procedure (Johnston *et al.*, 1976). Alternatively, OMPs were extracted from whole cells of Hib with 2% cetyl trimethyl ammonium bromide (CTB). For purification of the 38 kDa porin, the CTB extract was subjected to chromatography on DEAE-Sepharose (Vachon *et al.*, 1985). Porin was further purified by FPLC using a MonoQ HR 5/5 column (Pharmacia LKB Biotechnology Inc.). The protein solution from the DEAE-Sepharose column containing 50 mM Tris-HCl pH 8.0, 10 mM ethylenediaminetetraacetic acid (EDTA), and 0.05% Zwittergent Z-3,14 was washed with 80% ethanol and suspended in 50 mM Tris-HCl pH 8.0, 0.05% Zwittergent Z-3,14. A sample of 30 mg in 10 ml was loaded on to the column. The salt concentration of the elution buffer was raised to 0.35 M using a 2 ml NaCl gradient. A number of protein-containing peaks were eluted at this salt concentration. When no other proteins could be eluted at 0.35 M NaCl, the salt concentration was raised in a gradient fashion to 1.0M. A major peak which contained highly purified porin was eluted at 0.65M NaCl.

Anti-porin monoclonal antibodies (mAbs)

BALB/c mice were injected intraperitoneally with 10 μg of FPLC-purified porin in Freund's incomplete adjuvant (Gibco Laboratories). At two weeks and four weeks after primary immunization, animals received injections of 10 μg of porin, also in incomplete adjuvant. Four days before fusion, a booster dose of 30 μg of OMPs in 0.01 M phosphate buffer, pH 7.5, 0.85% NaCl (PBS) was given intravenously. Spleen cells from immunized mice were fused in a ratio of 10:1 with non-secreting hypoxanthine-guanine phosphoribosyl transferase-deficient SP2/0 myeloma cell line in a solution containing 50% (w/v) polyethylene glycol (PEG1450; Eastman Kodak) in Dulbecco's modified Eagle's medium (Flow Laboratories). Fused cells at a concentration of 10^5 cells ml^{-1} were distributed into 96-well tissue-culture plates which already contained a feeder layer of 4×10^3 murine macrophages. On day 10–12, hybridoma supernatants were tested for reactivity against vesicles containing OMPs. Antibody-secreting cells were subcloned by limiting dilution. The class and subclass of mAbs were determined with reagents prepared by Southern Biotechnology Associates.

Purification of mAbs using affinity chromatography

Mouse anti-Hib porin mAbs were purified by using a rat anti-mouse kappa mAbs 187.1 (Yelton *et al.*, 1981) column. Ascites fluid was diluted five times with PBS. The sample was passed through a filter of 0.45 μm pore size (Nucleopore), loaded onto the column, and washed with PBS. Bound anti-porin mAbs were eluted with 0.1 M sodium acetate pH 2.8, 0.15 M NaCl. The absorbance (at 280 nm) of the collected fractions was measured and peak fractions were pooled. After addition of 1 M Tris-HCl pH 8.2 to neutralize the pH, the pooled fractions were dialysed against PBS. The total protein was measured using the dye binding assay. The mAbs were then brought to a concentration of 0.5 mg ml^{-1}. Dilutions ranging from 1/100 to 1/100 000 were used in ELISA and in Western immunoblotting analysis.

Flow cytometry

Bacteria from fresh overnight cultures were washed in Tris-buffered saline and suspended in the same buffer to 10^9 cells ml^{-1}. To 2×10^8 bacteria was added affinity-purified mAb at 1/100 dilution, corresponding to 5 μg ml^{-1} of antibody (Bentley and Klebba, 1988). After incubation for 60 min at 37°C, cells were pelleted by brief centrifugation in a microcentrifuge, washed, and further incubated with fluorescein-labelled anti-mouse Ig and analysed using a FACScan (Becton Dickinson) with Consort 30 software. Acquisition gates were set on forward scatter and side scatter to exclude lysed cells and cell aggregates. To minimize background noise, all solutions including the sheath fluid were filtered through a 0.22 μm filter. Twenty thousand cells were analysed for each sample.

Cleavage of Hib porin by cyanogen bromide

Two-microgram amounts of FPLC-purified Hib porin were solubilized in 25 μl of 70% acetic acid. Cyanogen bromide (250 μg) (Pierce) was added to each reaction vial and incubated at room temperature in the dark for 18 h (Gross, 1967). Upon completion of the reaction acetic acid and cyanogen bromide were removed from the reaction medium by vacuum concentration. The protein pellets were resuspended in 25 μl of sample buffer, boiled for 5 min, and analysed by SDS–PAGE.

Hydroxylaminolysis of Hib porin

Aliquots of 5 μg of FPLC-purified Hib porin were subjected to SDS–PAGE and the gel was stained with Coomassie Brilliant Blue. Gel pieces were excised, transferred to 1.5 ml vials and washed four times with 5% methanol at 4°C to remove SDS. The gel pieces were brought almost to dryness in a vacuum concentrator. To the vials containing the gel pieces was added 300 μl of cleavage solution; the samples were incubated at 45°C for 3 h. Cleavage solution was 2 M hydroxylamine hydrochloride (Baker), 6 M guanidine hydrochloride, and 15 mM Tris in 4.5 M LiOH pH 9.0 (Bornstein and Balian, 1977). The reaction was stopped by adding 150 μl of 70% acetic acid to each vial. The gel pieces were washed four times with 5% methanol at 4°C to remove the cleavage solution. The gels were brought to near dryness; 100 μl of sample buffer was added to each vial and boiled for 5 min. Peptide fragments were released from the gels by analysis on a second SDS–PAGE. For amino-terminal sequencing and for staining of fragments with Coomassie Brilliant Blue, the above protocol was repeated with 50 μg of porin as starting material.

Limited trypsin digestion of Hib porin in solution or in gels

Two-microgram amounts of FPLC-purified Hib porin in 5 μl of 50 mM Tris-HCl pH 8.0, 0.05% Zwittergent Z-3,14 were placed in reaction vials. TPCK-treated trypsin (200 ng) (Sigma) in 10 μl of 50 mM Tris-HCl pH 8.0 was added to each vial and the vials were incubated at 37°C for up to 16 h (Smyth, 1967). Sample buffer (25 μl) was added; boiling for 5 min terminated the reaction. Peptide fragments resulting from the digestion were analysed by SDS–PAGE. For amino-terminal sequencing, 20 μg of porin was digested with 2 μg of TPCK-treated trypsin.

Alternatively, FPLC-purified Hib porin was immobilized in acrylamide gels and subjected to trypsin digestion. Gel pieces containing 5 μg amounts of porin were incubated in 300 μl of 50 mM Tris-HCl pH 8.0 containing 500 ng of TPCK-treated trypsin. The reaction was at 37°C for 1 h. For sequencing purposes, 50 μg of Hib porin was treated with 5 μg of TPCK-treated trypsin. Gel pieces were prepared and the digestion products analysed by the same protocols as in hydroxylaminolysis.

Amino-terminal sequencing of peptide fragments

Cleavage products were subjected to SDS–PAGE and fragments transferred electrophoretically onto Immobilon-P (Millipore) membranes. The membranes were washed several times with distilled water to remove traces of glycine from the transfer buffer. Bands were cut out and subjected to gas-phase sequencing

(Hewick *et al.*, 1981) in a PI 2090E Integrated Micro-Sequencing System (Porton Instruments Inc.). Edman degradations were conducted according to procedures recommended by the manufacturer. PTH-amino acid analysis employed gradient elution (Solvent A: triethylamine/acetio acid/tetrahydrofuran pH4.0: solvent B: acetonitrile) from a reversed-phase Hewlett Packard Amino Quant (200 × 2.1 mm) column with a flow rate of $0.2\,\mathrm{ml\,min^{-1}}$ at 42°C. Five sequential cycles of the amino terminus of each fragment were sequenced.

Molecular cloning of ompP2

Chromosomal DNA of Hib strain ATCC9795 was digested with *Pvu*II and size-fractionated by electroelution from an agarose gel. A library of chromosomal fragments was constructed in M13 mp18 that had been digested with *Sma*I. The library was screened using an oligonucleotide (5′-GCTGTTGTTTATAA-CAACGAAGGG-3′) complementary to amino acids at the amino terminus of Hib porin. A *Pvu*II fragment containing the entire porin sequence but lacking codons for the signal sequence was isolated as a 2.0 kbp insert. The Hib porin gene was excised from the RF form of M13 by digestion with *Kpn*I and *Ssp*I as a fragment of 1130 bp and subcloned into pBluescript (Stratagene) that had been restricted with *Kpn*I and *Sma*I. The resulting plasmid was designated pACC01. The restriction map of the insert matched the information reported by Hansen *et al.* (1989a) and by Munson and Tolan (1989).

Improved baculovirus expression vectors were recently developed to expedite screening of recombinants (Vialard *et al.*, 1990) and a recent version of this class of vectors, pJV.P10Z, was used in our experiments. This vector contained promoters derived from the P10 and the polyhedrin genes of *Autographica californica* nuclear polyhedrosis virus. P10 promoter directed synthesis of a β-galactosidase and the polyhedrin promoter controlled the synthesis of the *ompP2* gene. The unique *Nhe*I cloning site of this plasmid necessitated modification of the 5′- and 3′ sequences of the porin gene. To generate the *Nhe*I ends on *ompP2*, a synthetic adaptor was added to the 5′ end and consisted two oligonucleotides: 5′-CTAGCTATAAAT$\underline{\text{ATG}}$GGT-AC-3′ and 5′-CCATATTTATAG-3′. When these two oligonucleotides were annealed, they generated *Nhe*I and *Kpn*I sites at their 5′ and 3′ ends: internal sequences introduced an AT-rich region just upstream of the $\underline{\text{ATG}}$ start site. The AT-rich sequence may serve as a polymerase-binding site. This sequence also corresponds to those necessary for maximal levels of expression of another foreign gene (Matsura *et al.*, 1987).

pACC01 was digested with *Kpn*I and *Bam*HI to excise the entire Hib porin gene. The DNA fragment containing *ompP2* was gel-purified and ligated with annealed adaptor at a molar ratio of 10:1 (adaptor:porin). The adaptor-modified fragment with *Nhe*I–*Bam*HI ends was force-cloned into pBR322 cut with *Nhe*I and *Bam*HI, thereby creating pACC02 as an intermediate plasmid containing *ompP2*. pACC02 was digested with *Bam*HI, backfilled with the Klenow fragment of DNA polymerase I, and ligated to an 8 bp *Nhe*I linker that was added in 100-fold molar excess. The linker-ligated porin gene was then subjected to excess *Nhe*I digestion to remove multiple linkers from the 3′ end of the *ompP2* gene. This linker-ligated fragment was subcloned into pBR322 cut with *Nhe*I and was designated pACC03 (5523 bp). The *Nhe*I insert of pACC03 (1161 bp) was ultimately transferred into the 13 kbp baculovirus transfer plasmid, pJV.P10Z, creating pACC33. Two primers that were complementary to the vector's 5′-promoter sequences (5′-CGTAACAGTTTTGTAATAAA-3′) and 3′-end sequences (5′-GTGAGTTTTTGGTTCTTGCC-3′) were used for DNA sequencing to confirm the orientation of the porin gene with respect to the polyhedrin promoter and to confirm the fidelity of the subcloning through intermediate plasmids.

To generate a series of deletion mutants of *ompP2*, pACC03 was digested with *Dra*I or *Sna*BI or *Eco*RI or *Ase*I. Restriction with the first two enzymes yielded blunt ends; restriction with the latter two enzymes generated cohesive ends that were back-filled with the Klenow fragment of DNA polymerase. Each of the linearized, blunt-ended plasmids was ligated with the omega cassette derived from pHP45 (Prentki and Krisch, 1984). This cassette of 2 kbp contains the spectinomycin/streptomycin-resistance genes and in either orientation provides a large stable stem-loop structure, making it an efficient transcription terminator. The blunt-end ligation of the cassette resulted in the recircularization of the plasmid, now increased in size to 7.5 kbp. The cassette-modified *ompP2* genes were purified as *Nhe*I fragments and ligated into pJV.P10Z cut with *Nhe*I.

DNA transfections, plaque assays, and isolation of recombinant virus

pACC33 and the four transfer vectors containing omega-modified *ompP2* genes were purified by CsCl gradient ultracentrifugation and were separately transfected into Sf9 cells together with wild-type viral DNA (Summers and Smith, 1987) by using the calcium phosphate precipitation technique. Plaque assays were performed on culture plates, such that the infected cells were overlaid with 1% Sea Plaque agarose in Grace's medium and containing 150 μg of Bluo-Gal per ml of overlay. Blue plaques, easily recognized after incubation for 5 d at 27°C, were picked and diffused overnight in Grace's medium. Plaque assays were repeated on 10-fold dilutions of diffused virus, again using Bluo-Gal in the overlay. Three rounds of plaque assays were sufficient to generate recombinant virus free of wild-type virus. Isolated recombinant virus was finally amplified to a titre 10^7 p.f.u. $\mathrm{ml^{-1}}$.

Microbiological tests

Bactericidal activity was tested as described earlier (Käyhty *et al.*, 1988). Bacteria were incubated for 60 min, 37°C in microwells with dilutions of serum or mAbs and 5 or 25% human serum as a complement source. The complement source did not kill the bacteria without added antibody. After incubation, aliquots of the bacterial suspensions were plated in duplicate on a chocolate agar plate and incubated overnight at 37°C in a CO_2 incubator. The last dilution that gave 50% killing was taken as the bactericidal antibody titre.

To determine whether passively given mAbs were protective *in vivo*, infant rat experiments were performed as described earlier (Smith *et al.*, 1973). The antisera or mAbs were given intraperitoneally (i.p.) 2 h before the challenge Hib which was also given i.p. Bacteraemia was detected 24 h later by plating 20 μl of neat or 100 μl of 1:10 and 1:100-diluted blood.

Analytical procedures

Sodium dodecyl sulphate/ polyacrylamide gel electrophoresis (SDS–PAGE) was used to assess the purity of Hib porin following FPLC, to resolve fragments derived from chemical or enzymatic digestions of Hib porin, and to resolve proteins in samples of outer membrane vesicles or insect cell lysates. The gel compositions were adopted from protocols of Lugtenberg *et al.* (1975). Gels were stained for proteins with Coomassie Brilliant Blue or by the more sensitive method of Morrissey (1981) for silver staining. The molecular mass standards were from Pharmacia LKB Biotechnology Inc. Alternatively, gels were silver stained to identify lipopolysaccharide (LPS) by the procedure of Tsai and Frasch (1982). The amounts of LPS that remained bound to Hib porin samples were estimated by comparing the staining pattern to standards of 50, 100, 200 and 300ng on the same gels.

Following SDS–PAGE, proteins were transferred electrophoretically (Towbin *et al.*, 1979) to nitrocellulose paper (Schleicher & Schuell, Inc.). Rainbow markers (Amersham Canada Ltd) were also electrophoretically transferred and used on immunoblots to estimate the sizes of reactive fragments. The nitrocellulose paper was blocked with 1% (w/v) skimmed milk or with 1% bovine serum albumin in Tris-saline, and the immobilized proteins were incubated with (i) hyperimmune serum, or (ii) concentrated culture supernatants or (iii) affinity-purified monoclonal antibodies. The developing antibodies were alkaline phosphatase-labelled anti-mouse immunoglobulins (Cappel Laboratories). The enzymatic reactivity of alkaline phosphatase was detected by addition of substrates NBT and BCIP (BioRad Laboratories).

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SUMMARY

Haemophilus influenzae (Hib) is a leading cause of pediatric bacterial meningitis. Past efforts to develop effective immunization materials against Hib have been centred on the capsular polysaccharide, which has been established to be important for virulence as well as being a target in the immune response to this pathogen. Although vaccines utilizing the capsular polysaccharide have been developed and used in the past, they are not optimal. The construction of vaccines efficacious in very young infants is a valid scientific goal. Recent research has been directed towards OM proteins of Hib that may prove to be more successful vaccine components.

The outer membrane of gram-negative bacteria is relatively permeable to low molecular weight hydrophilic substances even though it is essentially a lipid bilayer. This is due to the presence of porins in the outer membrane which serve as molecular channels for the passage of small molecules. Several porins have been identified in a variety of organisms. Most appear to be non-specific, allowing the passage of small molecules across the cell envelope.

The P2 protein/porin is the only known pore forming protein of Hib. Vachon *et al.* (1985, 1988) established a value of 1400 Da as the molecular weight exclusion limit and an estimated pore diameter of 0.9nm for this protein. Munson *et al.* (1983) established that rabbit polyclonal antibodies against porin protected infant rats from infection by Hib. Murphy and Bartos (1988) later isolated a monoclonal antibody directed against the porin protein of a nontypable Hib. This antibody recognized a surface exposed epitope and exhibited bactericidal activity *in vitro*. Thus the potential value of the porin protein in immunoprotection and its essential role as the sole pore forming protein of this organism, warrant a complete investigation of the antigenic, biochemical, and physiological characteristics of this protein. The gene for porin, *ompP2*, has been cloned and sequenced (Hansen *et al.*, 1989; Munson and Tolan, 1989). The availability of sequence information has led to the opportunity to study the role that porin may play in the immune response to Hib and in the pathogenesis of the organism. The expression of stable recombinant porin has assisted in this endeavour to better understand this protein.

This thesis documents the generation of a series of recombinant plasmids in pBR322 and in the baculovirus transfer vectors, pJVETLZ and pJVP10Z. Each contains either the full-length porin gene or an omega-adapted porin gene which could result in the generation of carboxy-terminal deletion proteins. These were co-transfected with wild type DNA into insect cells and pure recombinant virus was recovered. Recombinant porin was found to co-migrate with native bacterial porin of Hib. The recombinant proteins were stable and appeared not to be subject to endogenous proteolysis, as observed by the lack of breakdown products in Western analysis. More importantly, recombinant porin reacted with an anti-porin monoclonal antibody. Thus the behaviour of recombinant porin by SDS-PAGE and Western analysis concurs with that of bacterial porin.

The yield of the recombinant full-length protein was estimated by comparison to prepared porin standards on a Western blot. The staining of recombinant porin was compared to the staining of prepared porin standards. In this manner the yield of recombinant porin was appraised to be 1.0 ug by 4.8 X 10^6 Sf9 cells (Coulton *et al.*, 1992). In many reported cases higher yields of recombinant proteins were observed (50 mg/L of 2 x 10^6 cells is not uncommon). This was not always the case however, as with our experience. The yield obtained varied widely depending upon the gene product. It is still unclear as to why certain proteins are expressed at extremely high levels while others are not. It has been postulated that these variations in production might be a result of mRNA instability or due to post-translational effects, but they are unlikely to be a result of transcriptional deficiencies (Matsura *et al.*, 1987).

The recombinant porin was tested for its pore-forming properties by incorporation into planar lipid bilayers. It is suggested that LOS plays a role in the formation of functional pores in the cell membrane. As the recombinant porin was synthesized in a LPS free environment we were able to address this concern. It was shown by this analysis that the recombinant porin could form stable pores with a single channel conductance equivalent to porin isolated from bacterial cells. The average value for single channel conductance was found to be 0.89 nS for Hib porin compared to 0.88 nS for the recombinant porin. The conformation which recombinant porin assumed in the planar black lipid membrane was therefore similar to that of the Hib porin. As recombinant porin contains no LOS it is also reasonable to assume that, although LOS is intimately associated with bacterial porin, it is unlikely to play a key role in the construction of a functional porin channel.

Porin's role in the immune response to Hib needs to be better understood. In order to study the role of Hib porin in immune recognition, it is necessary to identify regions of the protein that are surface exposed. This information will assist in determining which epitopes are responsible for eliciting protective activity. To achieve this a panel of monoclonal antibodies was generated. The recombinant carboxy-terminal deletion proteins were tested for reactivities to the monoclonal antibodies. The results of these experiments were used to establish the boundaries of three antigenic determinants that were recognized by monoclonals: domain(i), amino acids 104-139; domain (ii), amino acids 162-174; and domain(iii), amino acids 267-341. The further identification of the specific sequences reactive to antibodies would allow for the synthesis of peptides which could form the basis of a new generation of semi-synthetic vaccines. In the past, immunization programs against Hib have involved the use of the capsular polysaccharide. A major drawback to this has been the relatively low immunogenicity of these polysaccharide antigens. Hib conjugate vaccines comprised of the polysaccharide covalently bound to proteins are providing more promising tools against Hib disease. The development of these new vaccines is contributing to our development of model microbial vaccines against diseases produced by other encapsulated bacteria including pneumococci, meningocci, and group B streptocci. Further studies to enhance such semi-synthetic vaccines will only augment our understanding of the mechanisms of the immune response. The use of the porin protein or portions of the protein in the form of synthetic peptides conjugated to the capsular polysaccharide could form the basis of such a vaccine.

The identification of surface-exposed regions is also important in understanding how the protein is folded in the membrane. Much research has been conducted in the past on the tertiary structure of porins. The structure of the pores of bacterial outer membrane has been studied using electron microscopy and x-ray diffraction. Such studies have been hampered by the presence of LOS which results in very low resolution of the image constructions (Benz, 1988). The availability of purified BEVS recombinant porin, which is LOS free, will allow for the crystallization of porin and hence a study of the topology of the protein. This information will be essential in the understanding of the structure and its importance in the function of the pore in the outer membrane. The availability of BEVS truncated porin proteins may also assist in the identification of functional domains of the protein. For example, reconstitution studies using the deletion mutants may establish boundaries in the amino acid sequence which are essential for pore formation and function.

The recombinant full-length and carboxy-terminal deletion porin proteins reported here have already provided valuable insight into this protein and have great potential for future studies. LOS has been shown to be intimately associated with Hib porin. By whole cell radioimmunoprecipitation (RIP) procedures LOS was observed to co-precipitate with Hib porin. In fact mAbs which were previously thought to be Hib porin specific were actually directed against Hib LOS (Gulig and Hansen, 1985). RIP analysis showed that the LOS specific mAB immunoprecipitated only with Hib porin and not to any of the other major OMP. This interaction of LOS/LPS with outer membrane proteins is not uncommon. Several other OMPs have been shown to require LOS/LPS for functional activities. For example LPS was shown to be essential for the receptor activity of bacteriophage T4 by OmpA of *E. coli* K-12 (Schweizer and Henning, 1977). Schindler and Rosenbusch (1981) observed that the restoration of the channel forming ability of an *E. coli* porin in planar lipid bilayers required the presence of LPS. Also, Kuusi *et al* (1981) demonstrated that LPS is required in the preparation of mAbs directed against *Salmonella typhimurium* outer membrane porin, as the absence of LPS contaminants in the preparations resulted in the loss of protective activity. These are just a few examples of evidence which support an interaction between porins and LOS/LPS.

To date, studies using recombinant porin which is devoid of LPS have provided some evidence that the interaction of porin with LOS is not essential for reconstitution in planar black lipid membranes. In addition the panel of mAb appear specific to Hib porin and not to LOS as indicated by the similar reactivities of Hib porin and recombinant porin (devoid of LOS) with the mAb (Srikumar *et al*, 1992). The interaction of LOS/LPS with porins may differ somewhat amongst different organisms. The interaction of Hib porin with LOS requires further study, and recombinant LOS free porin should assist in this endeavour.

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