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Behring Institute Research Communications

Behring Inst. Mitt., No. **98,** 283–290 (1997)

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R. E.W. HANCOCK, R. WONG

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia V6T 1Z3

Summary

Outer membrane protein OprF is the major outer membrane of Pseudomonas aeruginosa, *and has been expressed to a similar high level in* Escherichia coli *from the cloned gene. It contains conserved surface epitopes, and antibodies against these epitopes can protect mice from* P. aeruginosa *infections. To develope the oprF gene as a carrier for foreign epitopes, linker insertion mutagenesis has been performed to introduce 12 nucleotide inserts marked by a unique* PstI *site. Nine such sites can accept and express a foreign epitope within the surface loop regions of OprF on the surface of* E. coli. *The antigenicity at a given insertion site, and the influence of the length of a model repeating malarial epitope on antigenicity, have been shown to be site-specific and apparently dependent on the nature of the surrounding amino acids at the insertion site. Immunization of mice with OprF containing a highly antigenic inserted epitope led to an epitope-specific antibody response. These data suggest that OprF has potential for use as a carrier for foreign epitopes.*

Introduction

Two of the major modern issues in vaccinology are the use of "cocktails" of vaccine components directed against a variety of pathogenic bacteria or viruses, and the use of appropriate conjugants and/or adjuvants to enhance immunogenic potential. Both of these issues can potentially be addressed through the use of genetically-constructed bivalent (or multi-valent) vaccines. A bivalent vaccine is one that gives protection to two organisms. In practice it can involve a mixture of two separately manufactured vaccine components. However new biotechnological approaches provide the potential for a single vaccine to elicit an immune response to more than one organism. This has been achieved by cloning antigens from one pathogen into an attenuated version (live vaccine strain) from a second pathogen (*Clement* and *El-Morshidy,* 1984; *Schorr* et al., 1991). Alternatively one can create a chimeric protein containing protective epitopes from two different pathogens (*Hofnung,* 1991). Both of these approaches can result in "conjugation" of the antigenic epitopes to molecules that provide T-cell help and/or improved antigen presentation to the immune response. In this review we discuss the potential for outer membrane protein OprF in forming bivalent vaccines using the above-mentioned chimeric approach.

OprF is the most predominant (major) outer membrane protein of *Pseudomonas aeruginosa.* It has two important cellular functions, as the major non-specific porin of *P. aeruginosa* (*Bellido* et al., 1992), and a structural role in maintenance of normal cell length and ability to grow in low osmolarity medium (*Woodruff* and *Hancock,* 1989). It has no homology to the superfamily of non specific porins related to OmpF of *Escherichia coli* (*Jeanteur* et al., 1991), but its C-terminal half can be aligned with a large collection of diverse proteins including *E. coli* OmpA (which is also functionally related to OprF), the PAL (peptidoglycan associated lipoprotein) proteins, the *Neisseria* serum blocking protein PIII, and the *Bacillus subtilis* flagellum rotation protein MotB (*DeMot et al.,* 1994). OprF is 326 amino acids long, comprises 56% β-sheet struc-

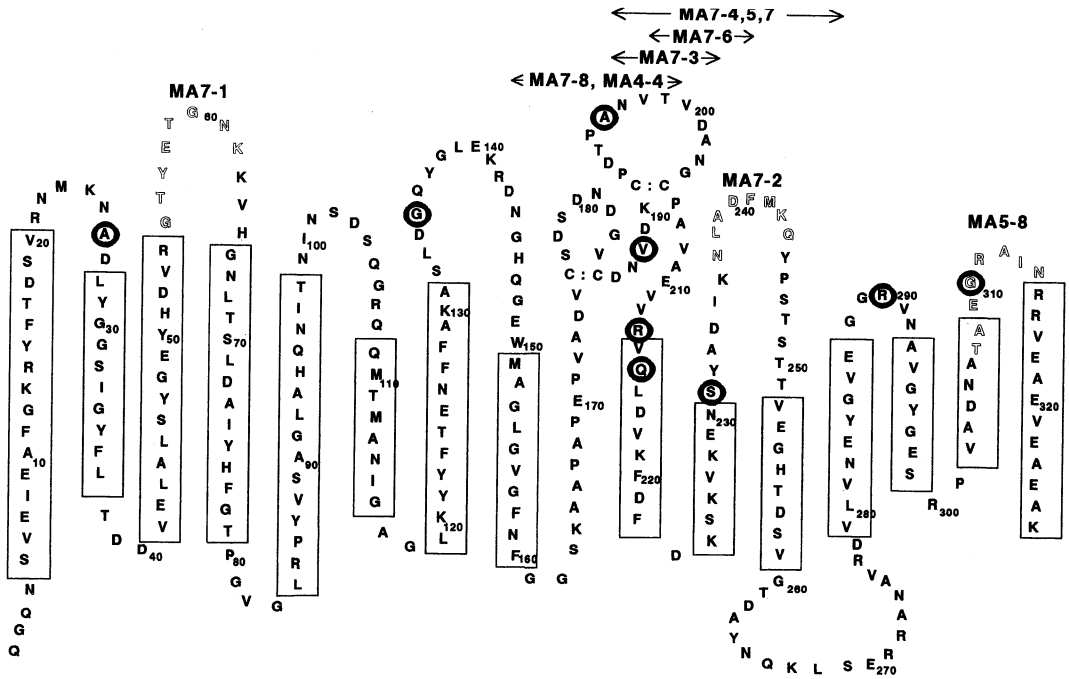

Fig. 1: Membrane topology model of OprF, modified from *Rawling* et al., 1995, and *Wong* et al., 1995. Boxed areas represent the putative membrane spanning β-strands. The top represents that portion of OprF facing the exterior. Circled amino acids are those that present malarial epitope on the surface of cells. The unfilled letters with MA designations above them represent linear epitopes for the given monoclonal antibodies, whereas the approximate location of the conformational epitopes for 7 other monoclonal antibodies are marked above the OprF sequence.

ture and contains two intrachain disulphide bands. A wide variety of studies, some of which are described here in overview, have led to the model shown in Figure 1. However it must be pointed out that the assignment of residues 260-278 to a large loop in the periplasm is controversial, since *Hughes,* et al. (1992), in contrast to *von Specht* et al. (1995), have demonstrated that antibodies raised against a synthetic peptide from this region are surface reactive. Another as-yet unconfirmed part of our model is the actual pairing of cysteine residues into disulphides.

Vaccine Potential of OprF

OprF is a highly conserved antigen

Southern hybridization of the *oprF* gene with DNA from 59 clinical and serotyping strains, indicated only one restriction endonuclease polymorphism observed in 2 of these strains (*Ullstrom* et al., 1990). However despite 16 nucleotide sequence differences between representatives of these two patterns (Genbank accession numbers P13794 and M94078), the encoded amino acid sequences were identical (*Martin* et al., 1993). Similarly, probing of 46 isolates of *P. aeruginosa* from a variety of sources, with ten different OprF-specific monoclonal antibodies, indicated an identical pattern of reactivity with all of these strains (*Martin* et al., 1993). We have examined dozens of strains by SDS-PAGE analysis of their outer membrane proteins and OprF is almost always present with identical molecular weight and heat-and 2-mercaptoethanol-modifiability (*Hancock* et al., 1985; *Mutharia* and *Hancock*, 1984). One exception is a multidrug resistant clinical isolate, se-

lected by quinolone therapy, that was OprF deficient (*Piddock* et al., 1992).

OprF also shows some immunological cross reactivity across the entire rRNA homology group I (fluorescent) *Pseudomonadaceae,* and limited sequence comparisons with OprF from *P. fluorescens* (*DeMot* et al., 1994) and *P. syringae* (*Ullstrom* et al., 1990) indicate reasonably high sequence conservation, especially at the C-terminus. Even OmpA from *E. coli* has 33% identity to OprF in its C-terminal half, and a recent membrane topology model is rather similar to that shown here for OprF (*Stathopoulos*, 1995).

OprF is immunogenic

Antibodies to OprF have been observed in animals immunized with purified OprF (*Gilleland* et al., 1988; *Wong* and *Hancock,* 1996), with intact bacteria (*Lam* et al., 1983), and with peptide fragments of OprF (*Hughes* et al., 1995). In addition examination of 239 sera from 52 patients with cystic fibrosis complicated by *Pseudomonas aeruginosa* lung infection, demonstrated antibodies to OprF (*Hancock* et al., 1985). Studying the development of antibodies to OprF in immunized animals as a function of number of immunizations indicated that substantial OprF-specific responses occurred upon primary injection, and increased to a maximal level after two injections of OprF. Epitope scanning techniques have indicated that there is a relative paucity of antibodies against linear epitopes within both rat and mouse antisera (*Rawling* et al., 1995).

OprF contains surface-exposed epitopes

It has been observed amongst a number of outer membrane proteins (e.g. *Rutz* et al., 1991; *van Alphen* et al., 1991) that surface exposed epitopes vary considerably. In contrast when we examined 10 monoclonal antibodies, each of which had separable but conserved epitopes (*Rawling* et al., 1995), nine of these monoclonal antibodies reacted with the surface of *P. aeruginosa* as demonstrated by indirect immunofluorescence and opsonization studies (*Martin* et al., 1993).

OprF-specific antibodies promote phagocytosis

Both monoclonal antibodies (*Martin* et al., 1993) and polyclonal antisera to OprF, or peptides derived therefrom (*Hughes* et al., 1992) are able to promote uptake and killing of *P. aeruginosa* by macrophages and/or neutrophils. The result is a 2 to 7-fold increase in phagocytosis over that observed in controls, and phagocytosis was complement independent (*Hancock,* et al., 1985).

OprF-specific antibodies protect animals against Pseudomonas *infections*

Gilleland et al.(1984) first demonstrated that purified OprF could be used as a protective vaccine in the peritoneal infection model, and followed up by showing protection against chronic pulmonary infections of rats (*Gilleland* et al., 1988) and in the mouse burn model (*Matthews-Greer* and *Gilleland,* 1987). Similarly we demonstrated passive protection by monoclonal antibodies in the mouse peritoneal infection, mouse burn (*Hancock* et al., 1985) and rabbit corneal infection (*Moon* et al., 1988) models.

Insertion of Foreign Epitopes into OprF

Assets of OprF as a vehicle for epitope insertion

Outer membrane proteins usually contain antiparallel transmembrane β-strands connected by short periplasmic turns, and longer surface exposed loops (e.g. Figure 1). Generally speaking, the surface loops are able to accept the insertion and deletion of amino acids (*Agterberg* et al., 1990; *Cowan* et al., 1990; *Hofnung,* 1991) and over evolution, related porins vary in length and composition almost exclusively in these regions (*Jeanteur* et al., 1991). In contrast, the transmembrane β-

strands do not readily accept such alterations (*Struyve* et al., 1991). For this reason we considered OprF to be a reasonable candidate for mutagenesis. Other assets of OprF include the fact that it has been cloned and expressed as a major outer membrane protein in *E. coli* (*Woodruff* and *Hancock,* 1989), in which bacterium OprF is more easily genetically manipulable, and the observation that only the N-terminal 161 amino acids are required for expression of a product [cf. the porins which require the entire protein to produce a β-barrel (*Struyve* et al., 1991)]. In addition OprF is highly expressed and thus relatively easy to purify and can be formulated into liposomes for use as a vaccine (*E. Mouat* and *R. Hancock,* unpublished). Also it is a known B-cell mitogen (*Chen* et al., 1980).

Epitope insertions and fusions with OprF

To investigate permissive sites for insertion mutagenesis of OprF, linkers encoding 4 amino acids were semi-randomly inserted into OprF. Ten permissive sites were identified and as a result of the method of mutagenesis utilized, were marked within the *oprF* gene sequence by unique *Pst1* sites (Figure 1). Nine of these were able to accept an oligonucleotide encoding a malarial epitope PNANPNA in either single or multiple repeat copies (*Wong* et al., 1995). In each case the malarial epitopes inserted into these permissive sites were shown to be surface localized by indirect immunofluorescence with an epitope-specific monoclonal antibody. Other permissive epitope insertions that have been performed included the random cloning of pieces of the *fha* gene of *B. pertussis* into site aa^{188} of OprF (*A. Siebers, R. S. Y. Wong, R. E. W. Hancock* and *B. B. Finlay,* manuscript in preparation), and the insertion of an oligonucleotide encoding a superantigen epitope of the toxic shock syndrome toxin of *S. aureus* into site aa^{213} (*A. Chow* and *I. Sharif,* unpublished data).

A second type of epitope insertion involved the fusion of longer sequences to OprF. Thus an N-terminal-less alkaline phosphatase gene was randomly transposed into the OprF gene, and permissive clones expressing alkaline phosphatase fused to the N-terminus of OprF were identified by enzymatic and Western immunoblot analysis (*Finnen* et al., 1992). Also a synthetic sequence encoding a cationic peptide was fused to site aa^{188} of OprF (*Piers* et al., 1993), and expression confirmed by Western immunoblot with specific antisera. The maximum length of sequence that we were able to insert into OprF was 68 amino acids. However no such restriction seemed to apply to epitope fusions since the entire *phoA* gene could be fused to OprF. Therefore a good diversity of sequences has been presented by OprF on the cell surface.

Antigenicity and surface exposure of the foreign epitope

Only limited studies were done on the above sequences, except for the malarial epitope insertions which are described below. To determine antigenicity in a quantitative fashion, ELISA, using as the antigen the outer membranes of cells expressing the malarial epitope insertions, and whole cell dot blots were performed (*Wong* et al., 1995; *Wong* and *Hancock,* 1996). Data were normalized for differential expression of the OprF hybrids by dividing the titres obtained with a malaria epitope specific antibody by the titres obtained using an OprF-specific antiserum, to obtain an "antigenic index". The data (Figure 2) showed that (a) the different insertion sites varied in their ability to present for reaction with antibodies the malarial epitope in single copy, and (b) whereas most sites were equivalent in presenting epitope regardless of whether outer membranes or whole cells were used as antigen sources, the malarial epitope inserted at aa^{26} was significantly more reactive with the epitope specific antibody (i.e. more antigenic) when assayed in the context of

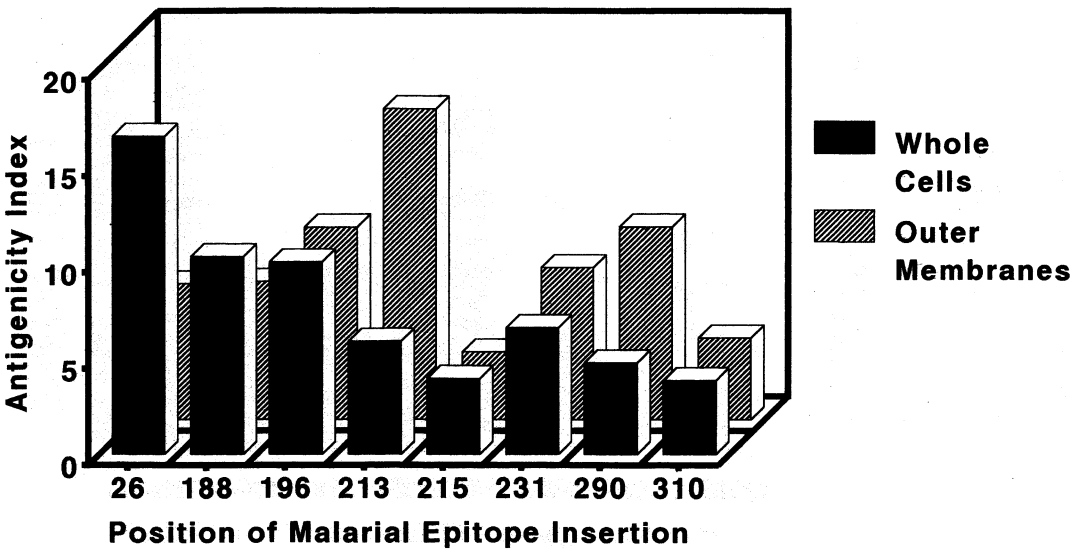

Fig. 2: Influence of amino acid position of a malarial epitope insertion NANPNANPNA on antigenicity (i.e. relative ability to react with a specific antibody). Both whole cells and isolated outer membranes were used as antigens. The positions of insertion correspond to the circled amino acids in Figure 1. The antigenicity index is corrected for the differential expression of the various constructs.

whole cells, whereas those at aa^{213} and aa^{290} were more antigenic when assayed in the outer membrane (presumably due to differential presentation and/or masking by other molecules such as LPS; *Wong* et al., 1995).

An attempt was made to correlate the nature of the insertion site with the antigenicity of single copy malarial epitope insertions. By computer analysis of the surrounding amino acids it was found that sites were better able to present the malarial epitope if the neighboring residues tended to adopt an extended conformation with a more flexible secondary structure (*Wong* et al., 1995).

In the malarial parasite *Plasmodium falciparum* the (NANP) motif from the circumsporozoite protein is repeated 37 times (*Ballou* et al., 1985). Therefore we wished to determine the influence of the number of the repeats of this motif on the antigenicity of the epitope. It was demonstrated that the antigenicity of the epitope increased with the length of the epitope for insertions at aa^{26} and aa^{196} (Figure 3). The maximal antigenicity was achieved when 19 amino acids encoding $(NPNA)_4NPN$ was inserted. This seemed to be due to enhanced antigen exposure rather than increased binding affinity for the antibody, since a similar series of insertions at aa^{213} showed no influence of increased length on antigenicity, and since the antigenicity of the smallest insertion encoding $(NPNA)_1NPN$ at this site showed an antigenicity index equivalent to or higher than the 19 amino acid insertion into aa^{26} or aa^{196} (Figure 3).

Immunogenicity

We have done limited studies on the immunogenicity of malarial epitope inserted at aa^{26}. Three injections of the purified malarial epitope insertion variants of OprF in Adjuvax™ as an adjuvant were required to produce an immune response in all animals (although two of five responded after 2 injections), as assessed by ELISA of serum antibodies. Only the OprF variant with a 19 amino acid epitope

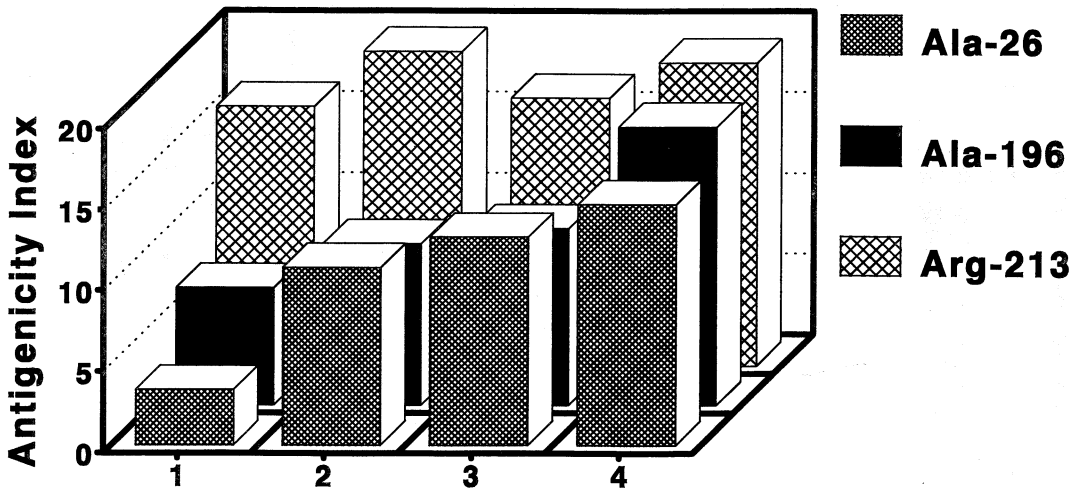

Fig. 3: Influence of the number of repeats of the malarial epitope on antigenicity at 3 separate sites in OprF. Actual inserts were: 1 = NPNANPN, 2 = $(NPNA)_2$ NPN, 3 = $(NPNA)_3$NPN, 4 = $(NPNA)_4$NPN.

insertion $(NPNA)_4$NPN gave a positive response leading to geometric mean ELISA titre of 3.3×10^3 against the malarial epitope as antigen. The less antigenic 7 amino acid epitope insertion (Figure 3) gave only a marginal response in one of 5 mice, whereas the negative controls [OprF with no insertion and glutathione-S-transferase (GST) with no insertion] showed the expected lack of antibodies specific for malarial epitope. As a positive control, OprF and both epitope insertion variants resulted in a vigorous polyclonal response to OprF sequences, with antibodies observable by ELISA even after a single injection. After 3 injections, mean ELISA titres of 2.9×10^5 were achieved.

Controls were done to indicate that OprF was a preferential expression vehicle for malarial epitope since 3 injections with genetic constructions of GST fused to 11 or 19 residue malarial epitopes (at the C-terminus) yielded at best marginal antibody responses, despite the strong antigenicities of the epitopes when assessed by malarial epitope specific antibodies and the strong immune response to GST (mean ELISA titres of 1.8 to 3.4 $\times 10^4$) after immunization with these constructs. Thus OprF is clearly a preferential carrier for epitope presentation in an immunogenic form.

Potential and limitations of bivalent vaccines

The above data attest to our ability to utilize OprF as a carrier for malarial and other epitopes. In its most antigenic form, the malarial epitope is also immunogenic. However these data are clearly incomplete since we have done a limited number of immunogenicity studies and have as yet not studied whether the antibodies produced are functional (i.e. protective). Thus we consider that while this system has reasonable potential for constructing bivalent vaccines, it is by no means perfected as yet. While the insertion of small epitopes into OprF is of potential interest, we are concerned about its utility since a monospecific response (i.e. against a single epitope) may be insufficient to give rise to protection. An alternative possibility would be to use two sites simultaneously for separate insertion of epitopes (either from different pathogens or the same pathogen). It is worth noting that all of our epitope insertion vectors carry a unique *Sal* site at the position encoding aa^{188} permitting simultaneous insertion of different epi-

topes into the unique *PstI* site elsewhere on the protein and into the *SalI* site. However with this approach we would be concerned about plasmid stability which can be a concern with some of the more altered constructs. An alternative approach, and one deserving of some attention, is the fusion of larger antigenic regions of vaccine proteins to the C-terminus of OprF, as described above, and demonstrated for the related protein OmpA (*Schorr* et al., 1991).

Acknowledgement

Financial assistance from MRC in the form of a grant to RH and a graduate studentship to RW is gratefully acknowledged.

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