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FEMS Microbiology Letters 140 (1996) 209–214

FEMS MICROBIOLOGY LETTERS

The effect of the length of a malarial epitope on its antigenicity and immunogenicity in an epitope presentation system using the *Pseudomonas aeruginosa* outer membrane protein OprF as the carrier

Rebecca S.Y. Wong, Robert E.W. Hancock *

Department of Microbiology and Immunology, #300-6174 University Boulevard, University of British Columbia, Vancouver, B.C. V6T 1Z3, Canada

Received 6 February 1996; revised 12 April 1996; accepted 30 April 1996

Abstract

This study showed that the antigenicity of a malarial epitope increased with the length of the epitope when inserted at positions aa^{26} (amino acid position 26) and aa^{196}, but not at aa^{213}, of the *Pseudomonas aeruginosa* major outer membrane protein OprF (326 amino acids). Immunization studies showed that a 19-aa epitope was significantly more immunogenic than a 7-aa epitope when inserted at aa^{26} of OprF, while neither an 11- nor a 19-aa epitope fused to the C-terminus of glutathione *S*-transferase was immunogenic.

Keywords: Antigen expression; OprF; Malarial epitope; Immune response

1. Introduction

Outer membrane protein OprF is the major outer membrane protein of *Pseudomonas aeruginosa*. The protein functions as a porin to allow the passage of small hydrophilic molecules [1,2], and it also has a structural role in maintaining cell shape and outer membrane integrity [3,4]. Purified OprF has been shown to be a B cell mitogen [5]. Immunizing animals with purified or partially purified OprF preparations protects the animals from subsequent challenge with *P. aeruginosa* in various models. These findings suggested that OprF is immunogenic and a potential candidate for a *P. aeruginosa* vaccine [6,7]. The proposed membrane topology model of OprF suggested that the protein consists of 15 or 16 transmembrane anti-parallel β-sheets joined by surface-exposed loops and periplasmic turns [8].

Our previous linker-insertion mutagenesis study identified 'permissive' sites in the protein that can accommodate extra amino acid residues without grossly affecting the production and stability of the protein [9]. A subsequent epitope insertion study, using the repeating epitope (NANP) of the malaria parasite *Plasmodium falciparum*, suggested the potential use of OprF as a carrier in an epitope presentation system [10]. The study also demonstrated that the epitope displays different antigenicity when

* Corresponding author. Tel.: +1 (604) 822 2682; Fax: +1 (604) 822 6041; E-mail: bob@cbdn.ca

PII S0378-1097(96)00182-6

inserted at different positions of OprF [10]. The other protein carrier used in this present study, glutathione *S*-transferase (GST), was originally identified in *Schistosoma japonicum* [11] and is a commonly used affinity tag for the purification of fusion proteins as well as a carrier to induce immune response against protein antigens [12,13]. In this paper, we report the effect of the length of the malarial epitope on its antigenicity at three different insertion sites of OprF. We also present the results of our immunization studies which compared the immunogenicity of two different lengths of the malarial epitope in the OprF and GST carrier systems.

2. Methods and materials

2.1. Plasmid constructions

The OprF::malarial epitope hybrid proteins were constructed by inserting a linker carrying *Xho*I and *Xba*I sites into the *Pst*I site of the *oprF* alleles generated by linker-insertion mutagenesis [9], followed by the insertion of hybridized oligonucleotides encoding different lengths of the repeating malarial epitope. The GST::malarial epitope fusion proteins were constructed by inserting hybridized oligonucleotides encoding the malarial epitope into the *Bam*HI and *Eco*RI sites of the pGEX-1N cloning vector (Pharmacia, Canada).

2.2. Protein expression

OprF::malarial epitope hybrid proteins were prepared from *E. coli* strain C158 (*ompA, ompC, phoE*) [14] grown in Luria broth supplemented to a final concentration of 1.7% (w/v) NaCl and 0.1% (w/v) glucose to suppress the production of OmpF and LamB respectively. To maintain plasmid selection, the media were supplemented with 75 μg ml^{-1} of ampicillin. *E. coli* outer membrane samples containing the OprF::malarial epitope hybrid proteins were prepared by the sucrose gradient centrifugation method as described by Hancock and Carey [15]. The GST::malarial epitope fusion proteins were purified from *E. coli* by affinity chromatography using glutathione agarose beads as described by Smith and Johnson [16].

2.3. Antigenicity study

The antigenicity of the inserted epitope in OprF was measured by ELISA as described previously [10]. Briefly, different dilutions of the outer membrane samples (100 μl) were used to coat the bottom of 96-well plates by incubation at 4°C for 16 h. For each experiment, two parallel assays were done using either a rabbit anti-OprF antiserum [8] or a malarial epitope-specific monoclonal antibody (mAb) pf2A.10 [17] as primary antibody. Horseradish peroxidase-conjugated secondary antibodies were used and 3,3',5,5'-tetramethylbenzidine (TMB) (Pierce Chemical Co., USA) was used as a chromogenic substrate. To normalize for the expression levels of the hybrid proteins, each index was the ratio of the A_{450} readings when pf2A.10 was used as the primary antibody to the A_{450} readings when the OprF-specific polyclonal antibody was used as the primary antibody. For each experiment, a plot of A_{450} readings versus the concentrations of coating antigen was drawn for each antibody and only values that corresponded to the linear portion of the binding curve were used for the calculation of antigenicity indices. Each experiment was repeated three times. The relationship between the antigenicity and the length of the epitope was analyzed by linear regression where r (correlation coefficient) > 0 indicates a positive linear relationship. Antigenicity of the GST::malarial epitope fusion proteins was demonstrated by ELISA using different amounts of the affinity-purified fusion proteins as coating antigens and different titrations of pf2A.10.

2.4. Immunogenicity study

To examine the effect of the length of the epitope on its immunogenicity, two OprF::malarial epitope hybrids, OprF::ME7aa26 and OprF::ME19aa26, and the two GST::malarial epitope fusion proteins, GST::ME11 and GST::ME19, were used as immunogens in an immunization study. Groups of 6–8-week-old female C57BL/6J mice (5 animals per group) were immunized subcutaneously with 20 μg of immunogens on days 0 and 21, and with 10 μg of immunogens on day 35. Adjuvax™ (Alpha-Beta Technology, Worcester, MA), 200 μg per injection, was used as the adjuvant in 200 μl total volume.

Serum samples were obtained by tail-bleeding on days 0 and 28 and by a whole body bleed on day 45. Control groups included animals injected with wild-type OprF, GST or PBS. The anti-OprF titer in serum samples was determined by ELISA using FPLC-purified OprF from *P. aeruginosa* as the coating antigen (500 ng ml^{-1}). The anti-malarial epitope titer was determined by using affinity-purified GST::ME19 (2 μg ml^{-1}) or gel-purified OprF::ME19aa26 (1 μg ml^{-1}) as the coating antigens. GST::ME19 and OprF::ME19aa26 were chosen because these proteins demonstrated highest binding to the malarial epitope-specific mAbs in ELISA as compared to the corresponding proteins carrying the shorter versions of the epitope.

3. Results and discussion

3.1. OprF::malarial epitope and GST::malarial epitope hybrid proteins

The OprF::malarial epitope hybrid proteins were designated as OprF::ME(X)aa(Y), where ME referred to malarial epitope, X referred to the number of amino acids in the inserted epitope, and Y referred to the amino acid position of the insertion. Table 1 summarizes the insertion sites and the length of the inserted epitope in the series of OprF::malarial epitope hybrid proteins constructed. The apparent molecular mass of the hybrid proteins increased corresponding to the increase in length of the inserted epitope (Fig. 1). The GST::malarial epitope fusion proteins, GST::ME11 and GST::ME19, expressed 11 amino acids {$P(NANP)_2NA$} and 19 amino acids {$P(NANP)_4NA$} respectively at the C-terminus of the carrier protein.

3.2. Antigenicity study

Antigenicity, the ability to interact with antibodies, was determined by ELISA with specific antibodies and expressed as an antigenicity index. Western immunoblot analysis indicated that there was little or no degradation of the hybrid proteins. The antigenicity index of the malarial epitope insertions at aa^{26} (amino acid position 26) and aa^{196} increased with the length of the epitope, while that at aa^{213} did

Table 1
Summary of OprF::malarial epitope hybrid proteins and the antigenicity of the inserted epitope

Insertion site	Hybrid protein	Amino acids inserted [a]	Antigenicity indices from three independent experiments (*r values*) [b]		
Ala-26	OprF::ME7aa26	PAARNPNANPNLDAGP	0.25	0.16	0.26
	OprF::ME11aa26	PAAR(NPNA)$_2$NPNLDAGP	0.80	0.60	0.48
	OprF::ME15aa26	PAAR(NPNA)$_3$NPNLDAGP	0.94	0.84	1.01
	OprF::ME19aa26	PAAR(NPNA)$_4$NPNLDAGP	1.09	0.94	1.30
			(*0.931*)	(*0.959*)	(*0.988*)
Ala-196	OprF::ME7aa196	PAARNPNANPNLDAGP	0.53	1.27	0.81
	OprF::ME11aa196	PAAR(NPNA)$_2$NPNLDAGP	0.73	1.46	0.91
	OprF::ME15aa196	PAAR(NPNA)$_3$NPNLDAGP	0.80	1.72	1.52
	OprF::ME19aa196	PAAR(NPNA)$_4$NPNLDAGP	1.26	1.82	1.71
			(*0.945*)	(*0.988*)	(*0.961*)
Arg-213	OprF::ME7aa213	TCTRNPNANPNLDCRS	1.17	1.55	3.48
	OprF::ME11aa213	TCTR(NPNA)$_2$NPNLDCRS	1.42	2.00	4.01
	OprF::ME15aa213	TCTR(NPNA)$_3$NPNLDCRS	1.21	1.86	3.65
	OprF::ME19aa213	TCTR(NPNA)$_4$NPNLDCRS	1.37	1.54	3.61
			(*0.415*)	(*−0.096*)	(*0.017*)

[a] The amino acid residues corresponding to the malarial epitope are underlined. The flanking amino acids PA_GP and TC_RS are the results of the previous linker-insertion mutagenesis procedures [9].

[b] Each column represents indices from one experiment. The four indices corresponding to different lengths of the epitope in each set of the hybrids were analyzed by linear regression. The correlation coefficient, r, of each set of four values is shown below each set in parentheses. $0 < r < 1$ indicates a positive linear relationship, the closer r is to 1, the stronger the relationship.

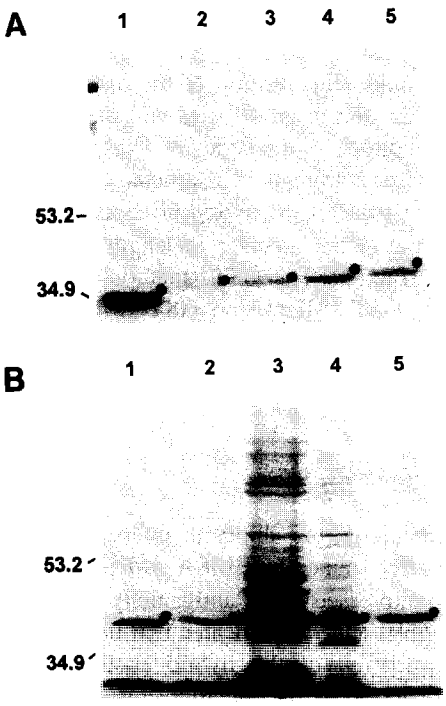


Fig. 1. SDS-7.5% PAGE of outer membrane preparations carrying insertions at (A) Ala-26 and (B) Arg-213 of OprF. Samples were heated at 37°C for 10 min in solubilization buffer (2% SDS, 10% glycerol, 62.5 mM Tris-HCl pH 6.8) before loading. The gels were stained with Coomassie blue after electrophoresis. Lane 1, OprF with no insert; lanes 2–5 represent hybrids carrying 7, 11, 15 and 19 amino acids of the epitope respectively. Due to the presence of inclusion bodies in the outer membrane preparations containing the hybrids carrying insertion at aa^{26}, the corresponding samples were obtained by octyl-polyoxyethelene extraction of the outer membrane preparations. The positions of relevant molecular mass standards (kDa) are indicated on the left. Bands corresponding to OprF or OprF hybrid proteins are marked with solid circles.

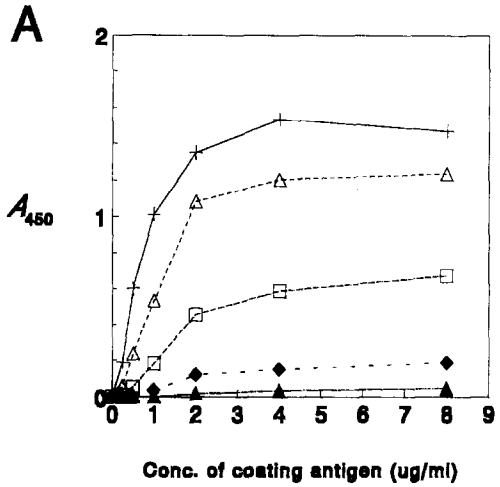
not (Table 1). These findings suggested that the effect of length on the antigenicity of the epitope inserted in OprF was site-specific. It was previously demonstrated that a 10-aa (10 amino acid) epitope inserted at aa^{213} was comparatively more antigenic than the same 10-aa sequence inserted at aa^{26} and aa^{196}, probably due to the better exposure of the epitope at this site [10]. Thus the shortest version of the malarial epitope inserted at aa^{213} might already have been adequately accessible for antibody binding. Therefore, the increase in length did not lead to a significant improvement in antigenicity. On the other hand, in the case of insertion sites aa^{26} and aa^{196}, we propose that the longer inserted sequence increased the antigenicity by improving the exposure of the epitope. This seemed to be particularly obvious at aa^{26}, where a more than two-fold increase in antigenicity was observed as the length of the inserted epitope was increased from 7 to 11 amino acids. In contrast both of the GST-malarial epitope fusion proteins demonstrated similar binding affinity for the malarial epitope-specific mAb pf2A.10 (Fig. 2), while GST alone did not bind to the antibody (data not shown).

3.3. Immunogenicity study

A significant anti-OprF response was detected in all three groups of animals immunized either with OprF or with the OprF::malarial epitope hybrid proteins. A significant anti-malarial epitope response was detected in all of the mice immunized with OprF::ME19aa26, a weak anti-malarial epitope response was detected in only one of the five mice immunized with OprF::ME7aa26 (Table 2), and no anti-malarial epitope response was observed in the native OprF controls. Characterization of the antisera by Western immunoblotting demonstrated the presence of anti-OprF and anti-malarial epitope antibodies in the antisera that showed a corresponding positive antibody response in ELISA (data not shown). Neither the pre-immune sera nor antisera taken from the PBS control group showed any significant anti-OprF or anti-malarial epitope response. These results suggested that the 19-aa epitope inserted at aa^{26} was significantly more immunogenic than the 7-aa epitope inserted at the same site. Animals immunized with GST or the GST fusion proteins developed a significant anti-GST response. However, little or no anti-malarial epitope response was observed in the groups immunized with the fusion proteins or GST (Table 2).

The difference in immunogenicity of the two versions of malarial epitope inserted at the same position of OprF could be due to a number of factors. For instance, the increase in length of the epitope might have enhanced the accessibility of the epitope for binding to B cell surface receptor, as was indicated by the results of the antigenicity study which demonstrated a positive correlation between

length and antigenicity at aa[26]. Alternatively, since B cell activation requires the crosslinking of B cell antigen receptors, the poor immunogenicity of the shorter version of the epitope might reflect its poor ability to elicit B cell receptor signalling. Moreover, the longer version of the epitope might have represented a higher 'dosage' of the repeating unit, and hence induced a dosage effect on immunogenicity. Since an immune response involves a network of mechanisms, it is possible that a number of factors have come into play to affect the immunogenicity of the epitope.



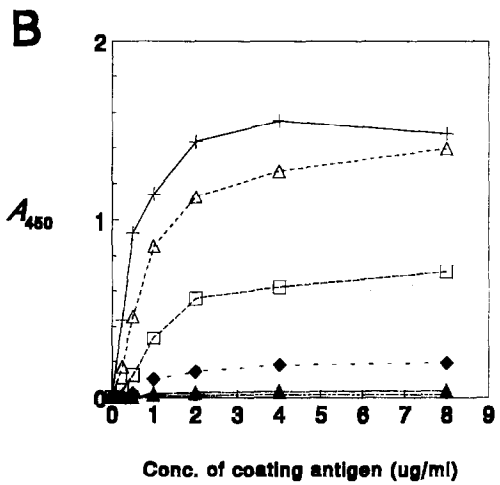


Fig. 2. Binding of GST::malarial epitope fusion proteins GST::ME11 (A) and GST::ME19 (B) with the malarial epitope-specific mAb pf2A.10. Curves represent different dilutions of the antibody: +, 1:1000; △, 1:5000; □, 1:25000; ◆, 1:125000; ▲, 1:625000.

Table 2
Summary of antibody responses induced in mice immunized with OprF and GST and their malarial epitope fusion derivatives

Immunogen	Log ELISA titers after 3 injections [a]		
	Anti-OprF	Anti-GST	Anti-malarial epitope
OprF	5.49±0.16	–	<2
OprF::ME7-aa26	5.25±0.18	–	<2 [b]
OprF::ME19-aa26	5.59±0.08	–	3.51±0.20
GST	–	4.53±0.17	<2 [c]
GST::ME11	–	4.26±0.19	<2 [d]
GST::ME19	–	4.20±0.10	2.04±0.12 [e]

[a] Titers are reported as the log of dilutions of antisera that gave twice the A_{655} readings of the pre-immune serum at 100-fold dilutions. Antisera were taken after three injections. The reported values are the mean values ± standard error from three independent assays on five mice. For the mice receiving OprF derivatives, the anti-OprF and anti-malarial epitope titers were determined by using FPLC-purified OprF and affinity-purified GST::ME19 as coating antigens respectively. For the mice receiving GST derivatives, the anti-GST and anti-malarial epitope titers were determined using affinity-purified GST and gel-purified OprF::ME19aa26 respectively.
[b] One of the five mice gave a slight response of 2.30±0.00.
[c] One of the five mice gave a slight response of 2.15±0.21.
[d] One of the five mice gave a slight response of 2.10±0.17.
[e] One of the five mice gave no response, whereas the other four gave marginal responses.

Despite the antigenicity of the two versions of the malarial epitope in the GST fusion proteins, neither of them was immunogenic. The length of the epitope should not be a limiting factor in this case because the length of the epitope in GST::ME19 was identical to that in OprF::ME19aa26. One possible explanation could be that the conformation of the epitope in the GST::malarial epitope fusion proteins prevents its interaction with the components of the immune system. Since antigen processing requires cleavage of the original proteins by proteases, one may speculate that the folding of the epitope in vivo might have rendered the protease cleavage site inaccessible for antigen processing, resulting in its failure to elicit an efficient antibody response. Alternatively, GST may be a poorer carrier than OprF for the malarial epitope used in this study.

In conclusion, this study demonstrated that the

effect of the length of the epitope on its antigenicity in the OprF system was site-specific. Using the OprF and the GST carrier systems, it was shown that the antigenicity of the epitope in vitro did not necessarily reflect its immunogenicity in test animals. The ability of OprF to promote immunogenicity of a foreign epitope and its potential as a vaccine against *P. aeruginosa* infections suggested that it has the possibility for the development of a multivalent vaccine. The use of the OprF and GST systems in a complementary fashion represented a simple and flexible approach to induce and monitor an anti-peptide response without the use of synthetic peptides.

Acknowledgements

This work was sponsored by a research grant from the Medical Research Council of Canada (MRC), and a studentship from MRC to R.S.Y. Wong. We thank R.A. Wirtz for kindly providing the malarial epitope-specific monoclonal antibody pf2A.10.

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