



Short communication

Immunogenic mimics of *Brucella* lipopolysaccharide epitopes

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ABSTRACT

Brucella melitensis and *Brucella abortus* are responsible for brucellosis in bovine and ovine species and for Malta fever in humans. The lipopolysaccharide (LPS) of *Brucella* is an important virulence factor and can elicit protective antibodies. Because of their potential importance in vaccine design and in serological diagnosis, we developed peptides mimicking the antigenic properties of distinctive antigenic determinants of *Brucella* LPS. These peptides were selected from several phage display random peptide libraries for their ability to bind monoclonal antibodies directed against the A- or C-type epitopes of *Brucella* LPS. Plasmids encoding for two of the isolated peptides induced, after DNA immunization, LPS-specific antibody responses. Although these responses were only moderate in extent, these data further suggest the feasibility of using peptide mimics of carbohydrate epitopes as immunogens, a property which may be useful in the design of novel anti-*Brucella* vaccines.

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1. Introduction

Brucellosis, one of the most common zoonoses in the world, is of great concern for human health [6]. The disease is caused by facultative intracellular bacteria belonging to the genus *Brucella*, mainly by *Brucella melitensis* and *Brucella abortus* [6]. The lipopolysaccharide (LPS) component of the cell wall of these bacteria is the most important virulence determinant and several monoclonal antibodies (mAbs) directed against the O-chain of LPS were shown to protect against infection [2,8,9]. Despite the desirable properties of these antibodies, there is currently some concern that the presence of O polysaccharide in vaccine strains may interfere with the serological diagnosis of infection [6]. Therefore, the development of reagents selectively mimicking distinct epitopes of the O-chain of *Brucella* LPS may be useful in circumventing these problems and in developing novel vaccination strategies and serological tests. At least three distinct epitopes, named A, M, and C (or “common”) can be found on the *Brucella* LPS O-chain [7]. The LPS from the *B. abortus* W99 strain is an example of an “A rich” LPS, while the *B. melitensis* Rev1 strain, which is used to immunize sheep and goats, is “M rich”. MAb 4F9 strongly reacts against W99 LPS and is considered to be A-specific, while mAb 12B12 is considered to be C-specific and reacts against both W99 and Rev1 LPS [7]. We explore here the possibility of using peptides

mimicking the A or C epitope as immunogens to induce anti-LPS antibodies. These mimics, which were obtained from phage display random peptide libraries by selecting with mAbs 4F9 or 12B12, may be useful in setting up novel strategies for controlling brucellosis.

2. Materials and methods

2.1. Reagents

We used four previously generated random peptide libraries of 9, 12 or 15 amino acids, named pVIII-9aa, pVIII-12aa, pVIII-12aa-Cys and pVIII-15aa [5,11]. These libraries carry random inserts encoding peptides fused to the N-terminal region of pVIII, the major coat protein of the M13 filamentous phage. Purified mAbs, W99 LPS, Rev1 LPS and *Escherichia coli* LPS were obtained from X. De Bolle and J.J. Letesson (University of Namur, Namur, Belgium) [3].

2.2. Peptide selection

Phage particles were selected from the libraries by three rounds of affinity selection using 1 µg/ml of each mAb and 10¹⁰ transducing units of each library, as described [12,4]. Positive phage clones were identified through plaque immunoscreening followed by enzyme immunoassay as described [12]. To verify specificity of binding, the mAbs were pre-incubated for 1 h with 1 µg/ml of W99 LPS or Rev1 LPS. All ELISA-positive clones were sequenced using the dideoxy-mediated chain termination method,

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Cy5-5' primers and the AlFexpress sequencer (Amersham), following manufacturer's instructions.

2.3. DNA constructs

To generate plasmids for DNA vaccination, we used a previously described expression vector designated as pT.neo [1]. The antigenic peptide sequences were inserted at the 3' EcoRI/XbaI flanking site of the tetanus toxin C subunit-derived T helper epitope sequence of pT.neo. For co-immunization, we used a previously described plasmid encoding for murine interferon- γ (mIFN- γ) [10]. After transforming the DH5 α *E. coli* strain, the plasmids were purified using EndoFree Plasmid Giga kits (Qiagen). Each lot of plasmid DNA had an A_{260}/A_{280} ratio ≥ 1.8 , as determined by UV spectrophotometry, endotoxin content ≤ 0.1 EU/ μ g DNA, as determined by *Limulus* Amebocyte Lysate test (Associates of Cape Cod) and a predominantly supercoiled form.

2.4. Immunization

For DNA immunization, BALB/c mice (5–7-week old, Charles River) were immunized in the quadriceps muscle with 100 μ g of purified DNA in a total volume of 50 μ l of PBS. Mice were injected on day 0, 21 and 42 and tail vein bled on day 56. In the co-immunization studies, 70 μ g of pmlFN- γ was mixed with the immunizing plasmid and injected in a total volume of 50 μ l.

2.5. Enzyme immunoassay

Sera from immunized mice were diluted 1:100 and tested on flat-bottom 96 well plates (Nunc MaxiSorp) coated with LPS (1 μ g/ml). After overnight incubation with sera, the wells were incubated for 2 h with alkaline phosphatase-conjugated goat anti-mouse IgG antibody (diluted 1:5000; Sigma) using *p*-nitrophenyl phosphate as substrate. Plates were read in a microplate reader (Labsystems Multiskan) at 405 nm after 40 min of incubation at 37 °C.

3. Results

3.1. Peptide selection and screening

To obtain peptides mimicking the A-type epitope, we isolated four phage clones from different libraries using mAb 4F9 as a selection agent (Table 1). All clones, but not the negative control pC89 clone, reacted, to different degrees, with the selected mAb (Fig. 1A). Moreover, this binding was totally inhibited by purified A antigen-rich W99 LPS, which reacts with mAb 4F9 [3] (Fig. 1A). These data indicated that the peptides competed with W99 LPS for

Table 1

Amino acid sequences of peptides mimicking *Brucella* LPS.

	Clone	Library	Peptide sequence
4F9	MB1.3	pVIII-12aa	WTEIHDWEAAME
	MB1.4	pVIII-12aa	HVTDMMEYLTLG
	MB2.2	pVIII-12aa.cys	CEFRDGAGSICG
	MB9.1	pVIII-9aa	YPAIVETGL
11B12	MB4.1	pVIII-12aa	DMYGDRLTGLI
	MB4.2	pVIII-12aa	CDGWMMLGECILV
	MB4.3 MB4.4	pVIII-12aa	DYMGNIHQDFDT
		pVIII-12aa	NTYGDFFMVKSPN
	MB6.2	pVIII-15aa	DSFGNWRLWAPIG
	MB6.3	pVIII-15aa	DLFGNVLLGQRLDQG

binding to mAb 4F9 and therefore mimicked the *Brucella* LPS A-type epitope. In further experiments, we sought to obtain peptides mimicking the C epitope of *Brucella* LPS using mAb 12B12 as bait. After selection, we obtained six clones, four from the pVIII-12aa library and two from the pVIII-15aa library, which reacted with mAb 12B12 (Table 1). The nucleotide sequence of the MB6.2 clone (isolated from the pVIII-15aa library) contains a 6-nt deletion in the insert, resulting in a variant 13-aa peptide sequence in the recombinant protein. With all clones, such binding was totally inhibited by the C antigen-rich Rev1 LPS, except for MB6.2 phages, whose binding was only partially inhibited (Fig. 1B). The amino acid sequences of the isolated peptides, obtained after sequencing PCR-amplified phage inserts, differed from each other and bore no discernible consensus motif (Table 1). In summary, we isolated four and six different peptides mimicking, respectively, A- and C-type *Brucella* LPS epitopes.

3.2. DNA immunization

To ascertain whether the identified mimics could induce anti-LPS antibodies after immunization, we used a previously described DNA vaccination strategy [1,10]. Peptide-encoding oligodeoxynucleotides were cloned in the pT.neo mammalian vector at the 3' end of a T helper epitope sequence to produce the vectors described in Table 2. Sera from mice immunized with single vectors, with or without co-immunization with the pmlFN- γ vector, were assayed for the presence of antibodies directed against *Brucella* LPS. Fig. 2A shows reactivity with W99 LPS in sera from mice immunized with mini-genes encoding for peptides mimicking the A-type epitope. Mice immunized with pT.MB1.3 (with or without pmlFN- γ -co-immunization), showed higher IgG levels than those observed in animals immunized with the empty pT.neo vector or with pmlFN- γ alone. In contrast, only one of seven pT.MB9.1 + pmlFN- γ immunized animals had a significant IgG

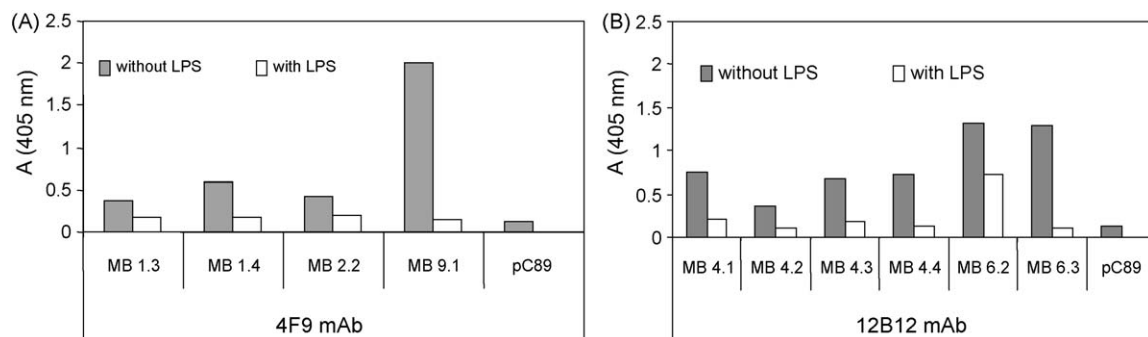


Fig. 1. Binding of phage clones to the anti-*Brucella* LPS mAbs 4F9 (left panel) or 12B12 (right panel). Plates were sensitized with a mAb directed against phage protein III (1 μ g/ml) and 100 μ l of purified phage clones (10^{10} PFU/ml) were added. After 1-h incubation mAbs 4F9 or 12B12 (1 μ g/ml) were added in the presence (white columns) or in the absence (grey columns) of *Brucella* LPS. Binding was detected by using AP-conjugated anti-mouse IgG. Phage pC89 displaying wild-type pVIII was used as negative control. Data represents the means $\pm$ SD of three determinations.

bind to specific antibodies. Interestingly, the amino acid sequences of the isolated peptides differed from each other and bore no discernible consensus motif. This is not surprising, since previous studies have demonstrated the ability of different peptide sequences to recognize a single mAb [4,12]. Such property likely reflects the ability of random peptide libraries to provide more than one structural solution for binding to a given antibody combining site. In a previous study using different libraries [3] De Bolle et al have described peptide mimics of *Brucella* LPS A- and M-type epitopes, while we studied here mimics of A- and C-type epitopes. The A-type mimics described in that previous study showed an overrepresentation of proline or tryptophan residues and bear no homology with the ones described here. Moreover, in that previous investigation, the identified peptides were incompletely characterized for their immunogenic activity, a feature that was examined in detail in the present study, taking advantage of the ease and versatility of a recently described DNA immunization approach [1,10].

The ability of two of the mimics (i.e. MB1.3 and MB6.3) described here to induce anti-LPS responses after DNA immunization was of particular interest. It is not surprising that, of the nine peptides tested, only two showed some ability to induce anti-LPS antibodies since previous experience also indicated that only a minority of peptide mimotopes shows the ability to induce an immune response directed against the “nominal” antigen [12]. The lack of immunogenicity of most peptides could be related to their tendency to take numerous different conformations, only few of which may mimic the tridimensional structure of relevant epitopes. It is also noteworthy that immunizations with 12B12-derived mimotopes lead to a bimodal distribution of the humoral response reported in Fig. 2B for the majority of the reactivities recorded with Rev1 LPS, by comparison with the negative control. Interestingly, in the present study, the “non-immunogenic” peptide mimics were able to induce anti-peptide antibody elevations and did not differ, in this respect, from those induced by the “immunogenic” ones, indicating that only a fraction of the anti-peptide antibodies cross-reacted with the relevant *Brucella* LPS epitopes.

The importance, in terms of protection in humans or animals, of the antibody response observed in our studies remains, of course, to be established. It should be noted, in this respect that a fraction of the animals vaccinated with the genes encoding for the MB1.3 and MB6.3 peptides failed to produce detectable anti-LPS responses. However, recently refined techniques for delivery of DNA vaccines, such as gene gun or electroporation, may increase the magnitude of the anti-LPS response and/or the frequency of responders. Moreover, this effect could also be reached by using

second-generation mimics designed on the basis of existing peptides and/or guided by detailed structural analysis of the interaction between the nominal antigen and the antibody combining site [13].

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