

Antigenic Properties of Peptidic Mimics for Epitopes of the Lipopolysaccharide from *Brucella*

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The lipopolysaccharide (LPS) is up to now the only identified major virulence determinant of *Brucella*. This bacterium is responsible for brucellosis in animals and for Malta fever in humans. Several monoclonal antibodies (mAbs) directed against various LPS epitopes have been characterized. Two mAbs, named A15-6B3 and B66-2C8, directed against distinct LPS epitopes have been used to select peptides from 11 phage display libraries. The sequences of the selected peptides contain an overrepresentation of either proline or tryptophan residues when selected with either A15-6B3 or B66-2C8 mAbs, respectively. For the best binding peptides, competition with LPS for the binding to the mAb is detected, which suggests that the peptides bind to the paratope of the mAb. The phages selected from the libraries were used to immunise mice, and a weak antibody response directed against LPS has been observed. These data suggest that a subset of the selected peptides are mimotopes of the LPS epitopes.

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Keywords: phage display; mimotopes; lipopolysaccharide; *Brucella*

Introduction

The Gram negative bacteria *Brucella abortus* and *Brucella melitensis* are responsible for brucellosis in bovine and ovine species, and for Malta fever in humans (Adams, 1990). Brucellosis is a worldwide zoonosis that became a major concern for human health. The lipopolysaccharide (LPS) is the most important virulence determinant known for the *Brucella* species (Godfroid *et al.*, 1998). As for many other Gram negative bacteria, LPS is also a major target of immune humoral response in infected animals. The O-chain of the *Brucella* LPS is an homopolymer of 4,6-dideoxy-4-formamido- α -D-mannopyranose (or 4-formamido-perosamine) residues, linked by α 1,2 or α 1,3 bonds (Carroff *et al.*, 1984; Bundle *et al.*, 1987). In *B. melitensis* strains, approximately 20% of the linkages are α 1,3 bonds whereas in most biovars of the *B. abortus* strains,

this proportion is only 2% (Bundle *et al.*, 1989). The availability of monoclonal antibodies (mAbs) directed against the O-chain of the LPS of different species of *Brucella* (essentially *B. abortus* and *B. melitensis*) allowed the definition of various epitopes (Bundle *et al.*, 1989; Weynants *et al.*, 1997): the M and A epitopes, present on M and A dominant *Brucella* strains, respectively; the common (C) epitope, strictly specific for smooth *Brucella* spp., either A or M dominant; and the C/Y epitope, which is common to smooth *Brucella* spp. and *Yersinia enterocolitica* O:9. Recently, subdivision of the C and C/Y epitope into C(A = M), C(M > A), C/Y(A = M), C/Y(A > M) and C/Y(M > A) has been proposed (Weynants *et al.*, 1997).

The A, M, C and C/Y epitopes, defined by specific antibodies, are not clearly characterized at the structural level. Their chemical synthesis is therefore impossible.

It has been shown that several high affinity mAbs directed against the *Brucella* LPS are at least partially protective when used in passive immunisation protocols (Montaraz *et al.*, 1986; Limet *et al.*, 1987, 1989). In addition, rough *Brucella* strains (unable to synthesise the O-chain of the LPS) are attenuated in an experimental mouse infection model (Godfroid *et al.*, 1998). Altogether, these data suggest that LPS and particularly O-PS should

Abbreviations used: GAM-HRP, goat anti-mouse immunoglobulins coupled to horseradish peroxidase conjugate; LPS, lipopolysaccharide; O-PS, O-chain polysaccharide; TBS, Tris buffer saline; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay.

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be included in a subunitary vaccine for *Brucella* species. However, the weak immunogenicity of O-PS necessitates its coupling to a carrier protein. Given the difficulties inherent to the chemical synthesis of artificial O-chains, the isolation of peptidic mimics for epitopes of *Brucella* O-PS became an attractive alternative approach. This approach can be performed in the absence of structural information about the epitope recognized by the monoclonal antibody. This has already been performed using anti-idiotypic antibodies. It is now possible to isolate peptidic mimics of polysaccharide structures through the use of peptide libraries presented on the surface of filamentous phages (for a review, see Kay & Hoess, 1996). The phage display technology is indeed a powerful method that is well adapted to the selection of new peptidic ligands for monoclonal antibodies. Here, we describe the isolation of peptide mimics recognized by mAbs, either B66-2C8 or A15-6B3, that are directed against the A and M epitopes, respectively, of the LPS O-chain of *Brucella* spp.

Despite the considerable interest of peptides able to mimic epitopes of saccharidic LPS structures, only few authors reported (e.g. see Phalipon *et al.*, 1997; Pincus *et al.*, 1998) success in the isolation of peptidic mimics that elicit an immune response against the native saccharidic structure when used in an animal model. Together with the previous reports, the data presented here suggest that the obtainment of peptide mimics leading to an immune response directed to the original saccharidic structure is possible, even if considerable improvement will be required to include these peptides in a new generation of vaccine.

Results

The biopannings were performed with 12 different phage-displayed peptide libraries (Table 1) as described in Materials and Methods. The hybridoma culture supernatant containing the mAb, either B66-2C8 or A15-6B3, was incubated on dishes coated with protein A (or protein G), which allowed the immobilisation of the antibody-phage complexes.

The 12 libraries were pooled into four distinct groups, according to the mode of presentation of the peptides and to antibiotic resistance encoded by vectors in which the libraries were constructed. The first group contained all the libraries in which the peptide was presented in fusion with the g3p protein, in a "3" system (i.e. one recombinant gene 3) or a "33" system (i.e. two genes 3, one wild-type and one recombinant). The second group contained only the zinc fingers library. The third group contained the X9 and CX9 libraries, both being a "8 + 8" system, using the M13K07 helper phage. The last group contained libraries Cys0 to Cys6 (Cysx libraries), which are all "88" systems (two g8 genes, one wild-type and one recombinant).

Table 1. Peptide libraries presented on filamentous phages that were used in this study

Name	Recombinant protein	Nature and context of the peptide
X6	g3p	(X) ₆
Minibody	g3p	Ig-like mini-domain, two groups of six variable positions
Zinc fingers	g8p	Zinc finger mini-domain with five variable positions
X9	g8p	(X) ₉
CX9	g8p	C(X) ₉ C
Cys0	g8p	(X) ₅ CC(X) ₅
Cys1	g8p	(X) ₅ CXC(X) ₅
Cys2	g8p	(X) ₅ C(X) ₂ C(X) ₅
Cys3	g8p	(X) ₅ C(X) ₃ C(X) ₄
Cys4	g8p	(X) ₄ C(X) ₄ C(X) ₄
Cys5	g8p	(X) ₄ C(X) ₅ C(X) ₄
Cys6	g8p	(X) ₄ C(X) ₆ C(X) ₄

X indicates that the nature of the amino acid at this position is randomized. The g3p and g8p are the names of the proteins encoded by genes 3 and 8, respectively. The name Cysx stands for all libraries from Cys0 to Cys6.

Colony immunoblottings obtained after the second, third and fourth rounds of biopanning with A15-6B3 mAb revealed a large fraction of positive clones with the 88 libraries. Positive signals were detected in the eluates of the second and the third rounds of biopanning with the B66-2C8 mAb, for the 8 + 8 and the 88 mixes of libraries. None of the other libraries gave positive signals. The yield of the biopannings (i.e. the ratio between the number transducing units used in the input of the round and the number of transducing units observed in the eluate) with the 88 mixes was three times and six times higher for B66-2C8 and A15-6B3, respectively, compared to the biopannings that did not produce positive clones. The intensity of labelling of the colonies can be dramatically different from one colony to another (data not shown). This difference is reproducible, and could arise either because the mAb bear a different affinity for various peptides or because different peptides are presented in different proportion on the surface of the phage. Positive clones were isolated from the first round in which they were detectable (in eluates of the second or the third round) and tested again for their ability to be recognized by mAbs A15-6B3 or B66-2C8.

The recombinant gene 8 of the positive clones was amplified by PCR and the obtained PCR products were purified and sequenced as described in Materials and Methods. Among 30 different clones selected with A15-6B3, four different sequences were obtained. Forty phage clones selected with the B66-2C8 mAb in the 88 libraries yielded only two different sequences. The analysis of the clones selected with the B66-2C8 mAb in the 8 + 8 mix of libraries resulted in six different sequences. All deduced sequences are listed in Table 2.

All the sequences of the peptides selected in the 88 mix (of Cysx) libraries using the A15-6B3 mAb belong to the Cys5 library. This demonstrates that this mAb has a strong preference for the peptides from this particular library. For the B66-2C8 mAb, clones were isolated from the Cys6 (one clone), Cys3 (one clone), CX9 (two clones) and X9 (six clones) libraries.

The sequences of the clones selected with the A15-6B3 mAb, named 6B3[x] sequences, are rich in proline residues (17 proline in a total of 52 randomized residues), especially peptide 6B3[4], which contains six proline residues in a total of 13 randomized positions. The probability to find a proline by chance at a randomized position being 2/32 in the Cysx libraries, proline residues are extremely overrepresented in these sequences. A proline residue is always present in positions 2 and 12 (see Table 2). It is also noteworthy that these sequences do not contain acidic residues. Peptides 6B3[2], 6B3[4] and 6B3[6] contain a glutamine at position 4; this conservation suggests that this residue is involved in a specific contact with the antibody. Sequences of peptides 6B3[2] and 6B3[4] are very similar, especially in the region between the two cysteines, where three residues out of five are identical.

The sequences of the clones selected with the B66-2C8 mAb, the 2C8[x] sequences, also present a high content of proline residues, even if this is less obvious than for the peptides selected using the A15-6B3 mAb. Two major characteristics particular to the 2C8[x] are the abundance of tryptophan residues and the presence of a large number of negatively charged residues. Aliphatic residues leucine and valine are less frequent than expected, as well as positively charged lysine and arginine residues.

Bacteriophages carrying the selected peptides were used in an indirect ELISA test to determine their degree of recognition by the mAbs. Even if

the proportion of recombinant g8p at the surface of bacteriophages is unknown, this measure gives a rough idea of the avidity of the antibody for the peptides selected during the biopanning procedures. The phages were coated at different dilutions and their recognition by the mAbs has been measured by a classical indirect ELISA (data not shown). The apparent avidity of the A15-6B3 mAb for the different peptides can be ordered as follows:

$$6B3[6] \gg 6B3[1] > 6B3[4] > 6B3[2]$$

The same analysis was performed with B66-2C8, which yield the following order of apparent avidity:

$$2C8[7] > 2C8[1] = 2C8[4] \geq 2C8[5] > 2C8[3] \\ > 2C8[9] \geq 2C8[8] > 2C8[6]$$

The apparent avidity of the A15-6B3 mAb for peptides 6B3[1], 6B3[2] and 6B3[4] is very low, which also correlates with a low reactivity in colony immunoblottings, by contrast to 6B3[6] that was giving a strong signal in colony immunoblotting experiments. The same conclusion may be applied for the 2C8[x] peptides. In the indirect ELISA with B66-2C8 mAb, the strongest signals were obtained on peptides 2C8[1], 2C8[4], 2C8[5] and 2C8[7], which all contain a pair of cysteine residues potentially forming a disulphide bond. A peptide-mAb interaction is even detectable for concentrations of mAb at which LPS is not recognized, B66-2C8 being of quite low avidity for LPS. As for the 6B3[2] peptide, some 2C8[x] peptides do not give detectable signals in this indirect ELISA test.

All selected phage-displayed peptides that were positive in indirect ELISA were tested with five mAbs directed against the five epitopes of the *Brucella* LPS (A > M, M > A, C(A = M),

Table 2. Sequences of the peptides selected during the biopannings with A15-6B3 and B66-2C8 monoclonal antibodies

A. A15-6B3		
	Sequence of the peptide	Library
6B3[1]	PPSFCPNFIPCTDGL	Cys5
6B3[2]	APTQCSQTWPCPSHL	Cys5
6B3[4]	PPQCSCAWPCPPHT	Cys5
6B3[6]	GPGQCNTNRNPPCRPM	Cys5
B. B66-2C8		
	Sequence of the peptide	Library
2C8[1]	YGPWCDDTPQACRSWP	Cys6
2C8[3]	ESKHSQCQIWCDNML	Cys3
2C8[4]	CSIYGSTIAGC	CX9
2C8[5]	DWPAPIWRKQ	X9
2C8[6]	WPPPNMLTS	X9
2C8[7]	CHHSPEEYQPC	CX9
2C8[8]	PWLDISDFQ	X9
2C8[9]	PWRELWASR	X9

The invariant cysteine residues are underlined and indicated in bold. The name of the library corresponds to the one given in Table 1. The sequences correspond to the randomized portion of the g8p protein.

C(M > A) and C/Y epitopes). For all these peptides, only the mAb used for the selection gave a positive signal in indirect ELISA (Figure 1), the other anti-LPS antibodies giving signals close

to the one obtained with a control mAb (AK-13A2) recognizing an unrelated epitope (on the F protein of the bovine respiratory syncytial virus). When the six mAbs were tested in indir-

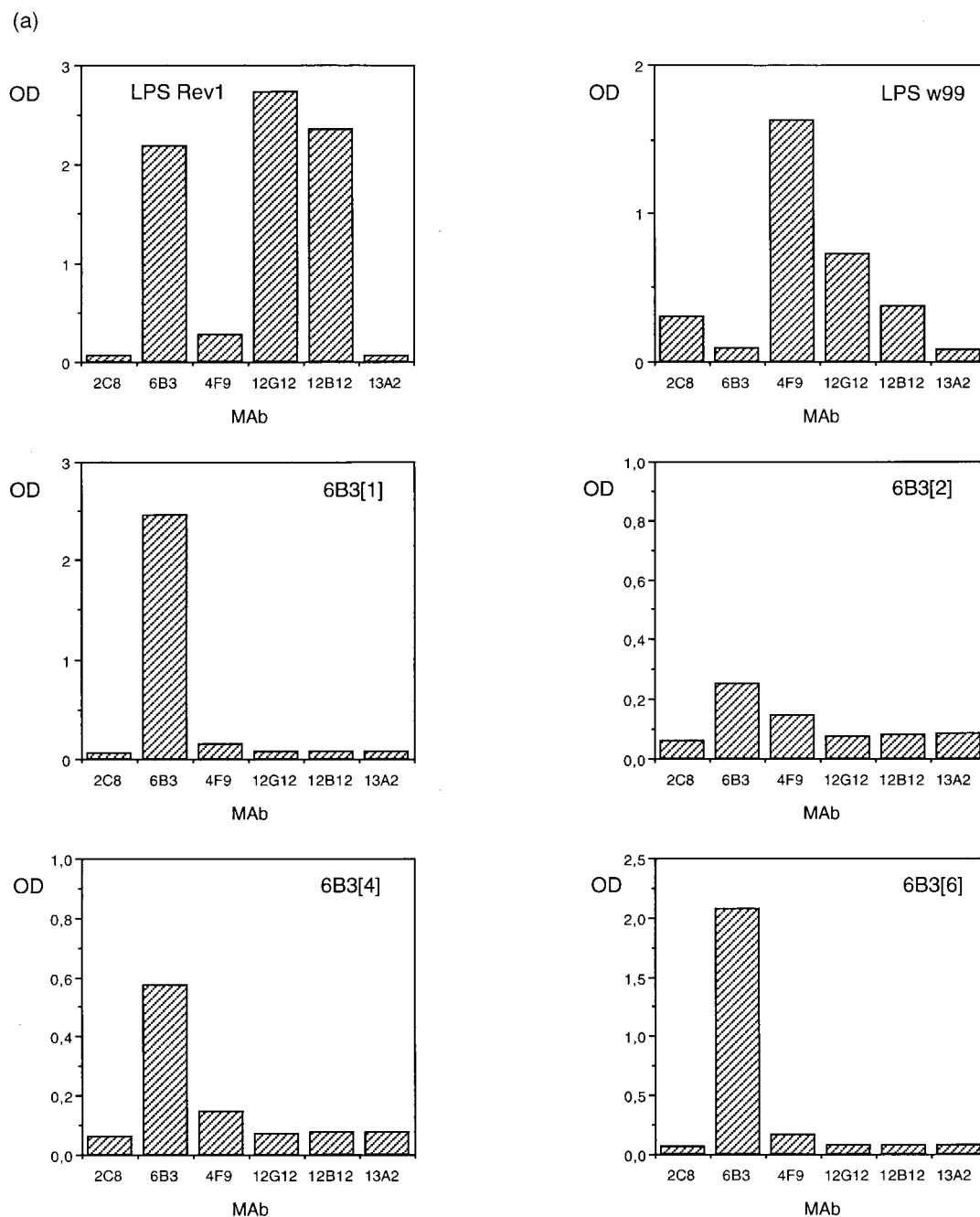


Figure 1. Indirect ELISA on selected phage-displayed peptides with various mAbs. Phage were coated on plates at a concentration of 10^{12} particles/ml, except the 6B3[6] phage that was coated at a concentration of 10^{11} particles/ml when assayed with the A15-6B3 mAb. When indicated, LPS was coated at 5 $\mu\text{g}/\text{ml}$ for B66-2C8 and AK-13A2, 1 $\mu\text{g}/\text{ml}$ for A15-6B3 and 0.1 $\mu\text{g}/\text{ml}$ for B66-4F9, A76-12G12 and A76-12B12 mAbs. The four first antibodies recognize various epitopes of the O-PS of *Brucella* spp., as indicated on the two top panels. The AK-13A2 mAb, that is directed against F protein of bovine respiratory syncytial virus, is used here as a negative control. The names of the peptides are indicated on the top right corner of the graphs. The sensitized wells were incubated with the mAb indicated below the graph and binding of the mAb was monitored with GAM-HRP. Given the differences in revelation time during the ELISA tests, chart comparison cannot be performed. The charts do not all have the same scale.

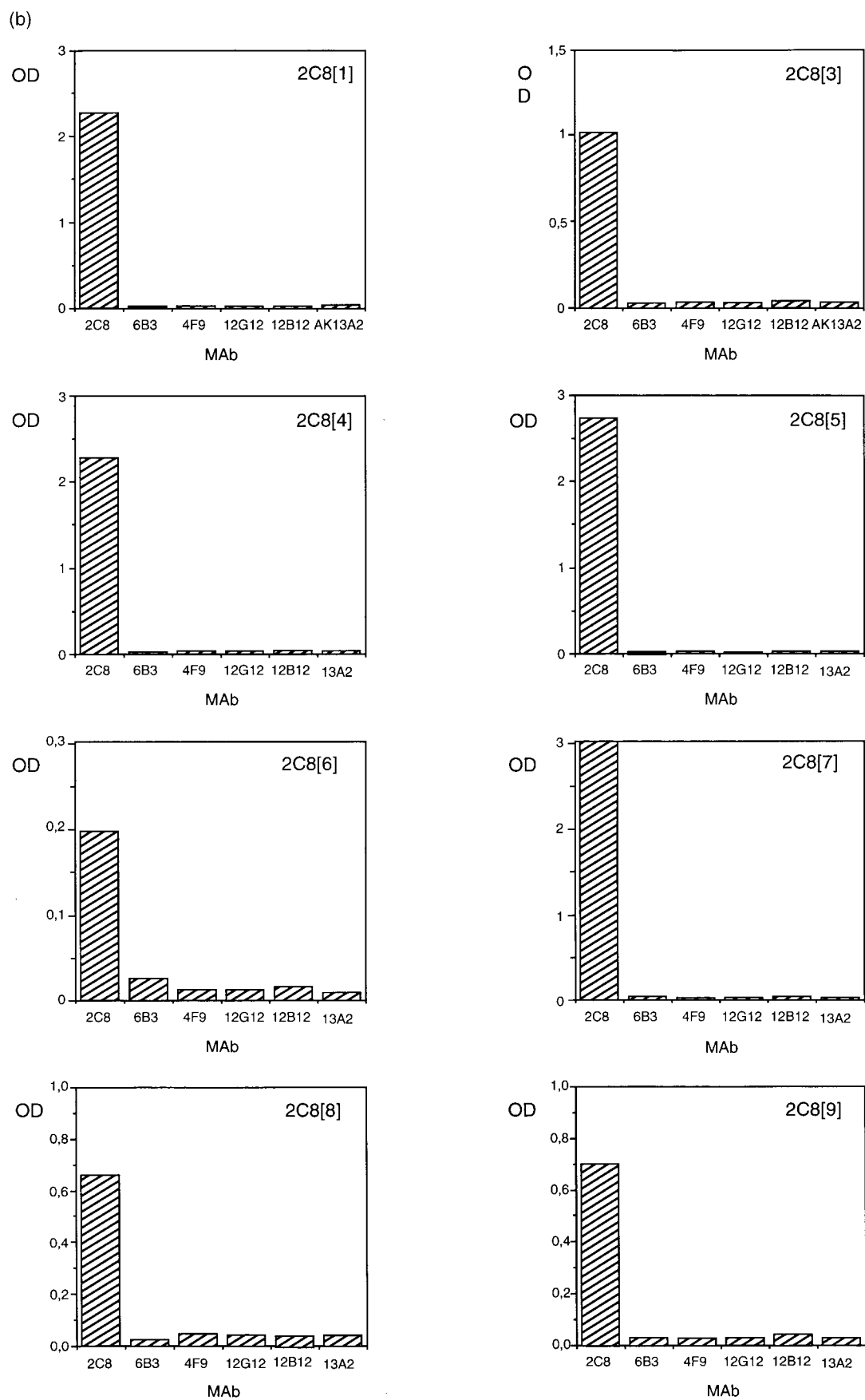


Figure 1 (legend opposite)

ect ELISA with wild-type or unrelated phages, the signals were usually very low, similar to the result obtained with AK-13A2 on all the phages analysed here (data not shown). The data presented in Figure 1 suggest that the peptidic mimotopes have some degree of specificity for the mAb used in the biopanning.

The phage clones obtained during the biopanning procedure were also analysed by ELISA to demonstrate the competition between the presented peptides and the LPS for the binding to the mAb used to select the phages (Figure 2). In a first protocol (Figure 2(a) and (b)), various phage concentrations are added to the mAb solution prior to the incubation in the LPS-coated wells, and detection of mAb binding is revealed by a classical indirect ELISA. In a second protocol (Figure 2(c) and (d)), mAb is mixed with LPS at different concentrations and added to wells coated with phage; here again, the binding of the mAb is detected by a classical indirect ELISA. In both protocols, signal extinction is therefore indicative of competition between LPS and the phage-displayed peptide. If there is competition between the mimotope and the natural antigen, it is likely that these bind to the same site on the mAb (the paratope).

As shown in Figure 2(a), there is a strong inhibition of the ELISA signal with the 6B3[6] peptide, whereas the other 6B3[x] peptides do not induce detectable decrease of the ELISA signal. These data indicate that 6B3[6] strongly competes with LPS for the binding to the A15-6B3 mAb. The 6B3[2] and 6B3[4] are members of the same family of sequence as 6B3[6] (Table 2). It is therefore likely that they bind on the same site on the mAb and that they do not induce competition only because the avidity of A15-6B3 mAb for these peptides is much lower than for 6B3[6]. When the second protocol of competition is used (Figure 2(c)), the Rev1 (M-dominant) LPS induces a detectable inhibition of the 6B3[1] and 6B3[6] phages recognition by the A15-6B3 mAb. These data suggest that 6B3[1] and 6B3[6] are antigenic mimotopes for A15-6B3; it also indicates that this second protocol of competition evaluation is less stringent than the first one.

Competition between 2C8[x] peptides and LPS for the B66-2C8 mAb reveals that the 2C8[3] peptide, that was not very well recognized in the indirect ELISA, allows a detectable inhibition of the LPS binding by the B66-2C8 antibody (Figure 2(b)). This is probably related to the poor avidity of the B66-2C8 mAb for LPS, its natural antigen, that probably makes competition with the peptides easier. The 2C8[1] and 2C8[7] peptides, that were of similar apparent avidity in indirect ELISA tests, are very different when compared in the competition test. Indeed, the 2C8[1] phage is an efficient competitor even when it is used at low concentration compared to the other phages. In the second protocol of competition ELISA, the LPS from the W99 (A-dominant) strain induces a strong inhibition of

the 2C8[9] recognition by B66-2C8 mAb, this inhibition being only partial for 2C8[5] and 2C8[6] phages, and not detectable for 2C8[4] and 2C8[7] phages. Thus, it seems that in this range of concentration, W99 LPS does not have a sufficient avidity for the B66-2C8 mAb to compete with peptides like 2C8[4] and 2C8[7]. Most of the competition data are related to the avidity of the mAb for the various antigens.

The competition ELISA and studies of the specificity of the peptide-mAb recognitions data indicate that the isolated peptides are antigenic mimics of the epitope on LPS recognized by the A15-6B3 and B66-2C8 mAbs, respectively. These data suggest that, if immunized with a high phage concentration, mice could be able to develop an immune response directed not only to the peptide, but also to (part of) the LPS structure recognised by the mAbs.

Preliminary immunization experiments (the protocol is detailed in Materials and Methods) suggest that 6B3[2], 6B3[4], 6B3[6] and 2C8[7] might act as weak immunogenic mimics of LPS. In order to better characterise these peptides, competition between LPS and free peptides has been performed. The results are illustrated in Figure 3. Analysis of the inhibition by free peptides suggests that the 2C8[7] peptide is specific for B66-2C8 mAb, and is able to inhibit the binding of this mAb to W99 LPS. The 6B3[2], 6B3[4] and 6B3[6] peptides were found to be able to inhibit binding of the A15-6B3 mAb to the Rev1 LPS. The 6B3[6] peptide, that was suspected to have the highest avidity for the A15-6B3 mAb when it is presented on the surface of a phage, is able to compete with LPS at a lower concentrations compared to 6B3[2] and 6B3[4] peptides (Figure 3).

Discussion

Peptides able to bind to A15-6B3 and B66-2C8 mAbs were selected from phage display libraries. These mAbs are, respectively, directed against M and A epitopes on the O-chain of *Brucella* LPS. The deduced sequences of the peptides selected with the A15-6B3 or B66-2C8 mAbs were enriched in proline or tryptophan residues, respectively. For some of the peptides, competition between peptide and LPS for the mAbs was demonstrated, suggesting that the peptides bind to the paratope of the mAbs. The phages bearing the various peptides selected were used to immunise mice, and a slight and unfrequent response has been observed against LPS, which suggests that some of the peptides, mainly those selected with A15-6B3 mAb, are immunogenic mimics of the LPS epitopes.

There was a strong preference for the libraries from which the peptides were selected for the two mAbs. For the B66-2C8 mAb, candidates were obtained from two groups of libraries, and peptides were belonging to the X9 (four peptides),

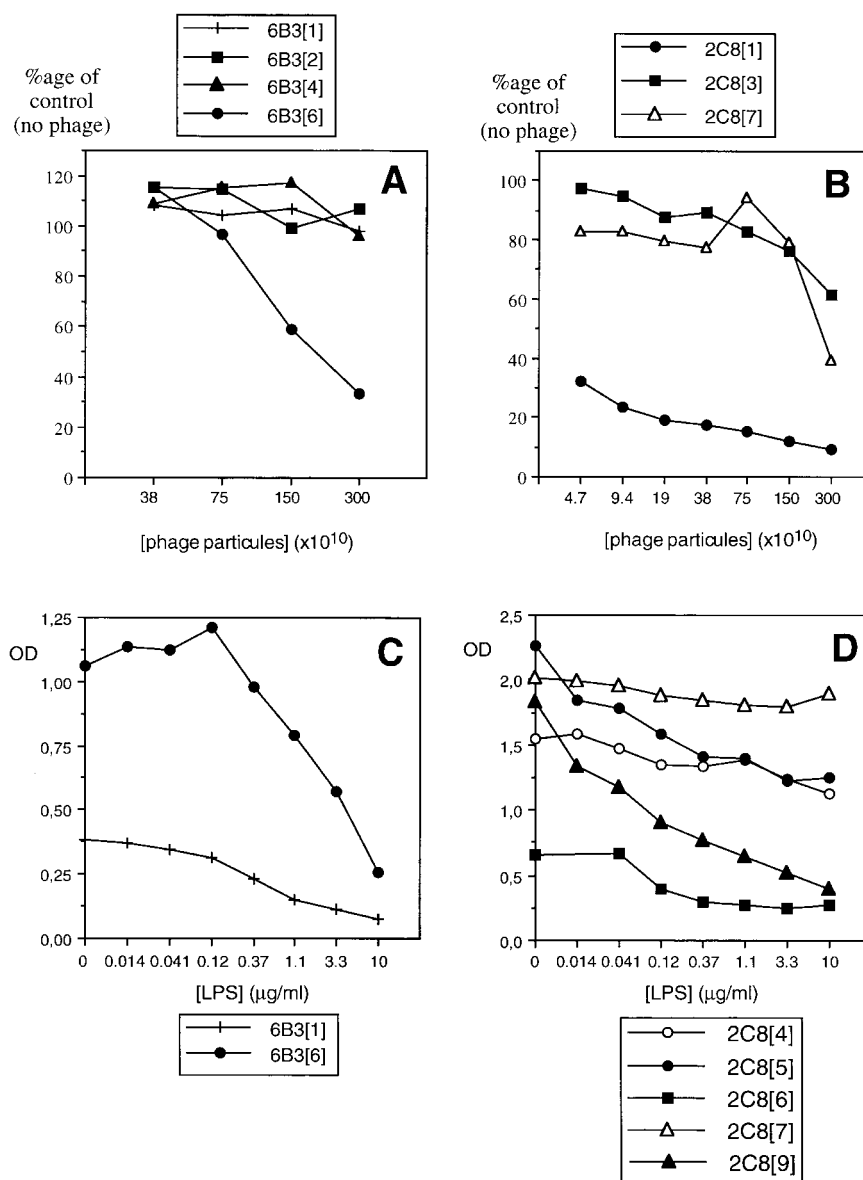


Figure 2. Competition between phages particles and LPS for the binding to A15-6B3 mAb and B66-2C8 mAb. In (a) and (b), the first protocol of competition ELISA was applied to the indicated phages. In this experiment, pre-incubation of the mAb with phage prevents it from binding to the LPS coated in the wells. The absorbance value for the control where phage is omitted is taken as 100 %. Dose-dependent inhibition of the mAb binding to the LPS indicates competition between the mimotope and the LPS for the paratope of the mAb. In (a), the LPS of the Rev1 *Brucella* strain was coated at 0.25 $\mu\text{g/ml}$. In (b), the LPS of the W99 *Brucella* strain was coated at a concentration of 2.5 $\mu\text{g/ml}$. In (c) and (d) the second protocol of competition ELISA was applied. A mix of mAb and LPS at the indicated concentrations was prepared and pre-incubated before its incubation in wells coated with phage. The coating concentrations of phage were as follows: 10^{12} particles/ml for 6B3[1], 10^{11} particles/ml for 6B3[4], 6B3[5], 6B3[6], 6B3[7] and 6B3[9], and $5 \cdot 10^{11}$ particles/ml for 6B3[6].

CX9 (two peptides), Cys3 (one peptide) and Cys6 libraries (one peptide). There is, therefore, no obvious bias in favour of the disulphide-containing peptides, which would have been expected if a selection particularly stringent had occurred during the panning procedures. For the A15-6B3 mAb, the four selected peptides came from the same library

(Cys5), which suggests that this mAb is very stringent in its selection.

All the peptides selected with A15-6B3 mAb contain a high proportion of proline residues. The proline residues are overrepresented in all parts of the peptides (N-terminal, between cysteines and C-terminal). This observation suggests that proline residues could have a more general effect rather

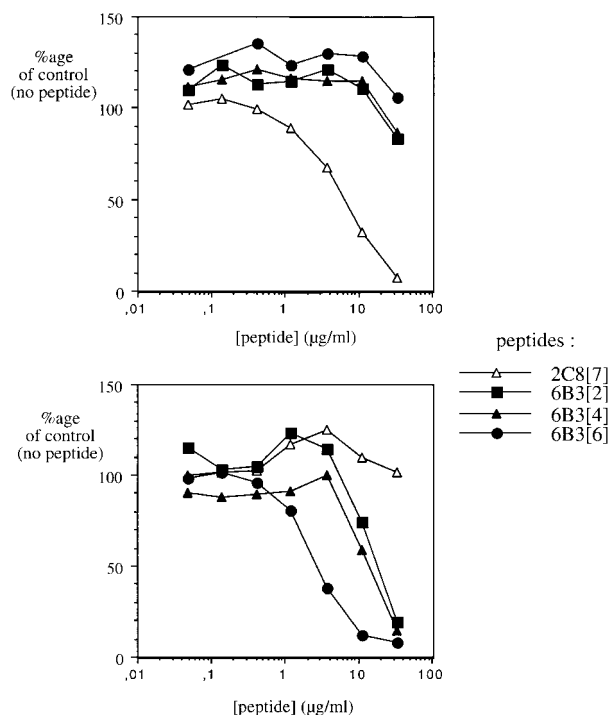


Figure 3. Inhibition of the LPS recognition by B66-2C8 (upper panel) and A15-6B3 (lower panel). ELISA tests in which LPS was coated (see the text for details concerning experimental procedure), and the recognition of LPS by mAbs was monitored by GAM-HRP. The data are presented as percentages of the control in which peptide is omitted. Competition between free peptide and LPS is detected by a decrease of the ELISA signal. Each point is the average of six measurements.

than being necessary to make a specific interaction. By reducing the flexibility of the free peptide, proline residues could reduce the entropic cost of the binding to the antibody and therefore favour formation of the mAb-peptide complex. Even though it could be a general phenomenon (proline-rich peptides are indeed also observed in other studies), our observations suggest that this may be more critical in the case of some particular mAbs, like A15-6B3.

The tryptophan residue is largely overrepresented in the sequences of the peptides selected with the B66-2C8 mAb. This is likely to be due to the abundance of aromatic residues (tryptophan, tyrosine and phenylalanine) at the bottom of the paratope in mAb. It is indeed known that aromatic residues make stable contacts between them. In the case of the 2C8[x] peptides, tryptophan is present in most of the peptides, and tyrosine is present in all the peptides where tryptophan is absent. Phenylalanine is always absent of the 2C8[x] peptides, demonstrating that the aromatic character of the residue is not sufficient to be selectable. This suggests that the aromatic residues of these peptides might also make additional contacts such as

hydrogen bonding when bound to the paratope of the mAb.

Analysis of the sequences of the peptides selected with mAbs gives interesting hypotheses about the possible interactions between the mimotopes and the paratopes. However, these hypotheses need to be supported by the resolution of the crystallographic structure of the mAb in complex with the mimotopes. This has been performed for a mAb directed against the *Cryptococcus neoformans* glucuronoxylomannan (Young *et al.*, 1997). As for the B66-2C8 mAb, the selected peptides are rich in aromatic residues and the peptide bound to the paratope of the mAb contain aromatic residues that are buried in the complex.

The 2C8[7], 6B3[2], 6B3[4] and 6B3[6] peptides are able to compete with binding of their corresponding mAb to LPS. This suggests that the environment of the peptide on the surface of the phage is not crucial for the recognition of the peptides by the mAb.

Preliminary immunization experiments suggest that some of the selected clones might act as weak immunogenic mimics of LPS (data not shown). The low immunogenic mimicry of the peptides for the LPS could be explained by the numerous possible conformations for the peptides, only a few of these conformations being able to generate an immune response directed against LPS. This response could potentially be strengthened by the use of second generation peptides designed on the basis of the existing peptides, but with a more constrained structure. Evans *et al.* (1994) suggested that anti-idiotypic antibodies generated using anti-LPS antibody (YsT9.1) are unable to efficiently mimic the antigen because, in the structure of a complex between two Fab molecules (anti-LPS and anti-idiotypic), the paratope of the anti-LPS mAb is not well filled by the anti-idiotypic antibody. This is due to the fact that the paratope of the anti-LPS antibody consists of a deep cavity. In the future, such problems may be avoided by the use of second generation peptides instead of anti-idiotypic antibodies.

In conclusion, it can be considered that the data presented here are the initial and crucial step in the design of a complete peptidic mimotope for the mAbs tested here. Obtaining efficient immunogenic mimicking of the LPS epitopes will need the selection of new peptides either from libraries generated on the basis of the peptides characterized here (second generation libraries) or designed using structural information about the complex between the peptides and the mAbs.

Materials and Methods

Phage libraries

All phage libraries are listed in Table 1. Libraries X6 and Cysx were generous gifts from G. P. Smith (University of Missouri, Columbia). X6 is a type 3 library (one recombinant gene 3, no wt gene 3), and

Cysx are type 88 libraries (one wt gene 8 and one recombinant gene 8). Zinc fingers, X9 and CX9 libraries were kind gifts from F. Felici (IRBM, Pomezia, Italy). Zinc fingers, X9 and CX9 are type 8 + 8 libraries, which means that recombinant gene 8 is located on a phagemid. The Minibody library (Martin *et al.*, 1994) is a type 3 library provided by M. Sollazo (IRBM, Pomezia, Italy). The X6, Cysx, X9 and CX9 libraries were amplified once before use.

Production of antibodies

The hybridoma producing the mAbs were grown in RPMI 1640 (BioWittaker, Walkersville, MD, USA) with 10% (v/v) FCS (Bethesda Research Laboratories, Bethesda, USA). Culture supernatants used for biopanning procedures were obtained by directly transferring exponentially growing hybridoma cells into RPMI 1640 without FCS, for a period of five to seven days. The supernatant was collected and cell debris were eliminated by centrifugation.

Biopanning procedures

Four cycles of biopanning were performed. During the first cycle, Nunclon[®] 35 mm diameter cell culture dishes (Nunc, Roskilde, Denmark) were coated overnight at 4°C with protein A (Sigma, St. Louis, USA) at 1 µg/ml 0.1 M sodium carbonate. After saturation, the antibody (0.4 ml of culture supernatant without FCS) was incubated on the protein A-coated dishes. After five washes with TBS (50 mM Tris, 150 mM NaCl (pH 7.5)) containing 0.5% (v/v) Tween 20, the phages (10¹¹ phage particules for each library, e.g. two 10¹¹ phage particules for a mix of two libraries) were added and incubated for four hours at 4°C. The dishes were then washed ten times with TBS-0.5% Tween 20, and bound phages were eluted 20 minutes at room temperature with a glycine buffer at pH 2.2. With the X9 and CX9 libraries, elution for two hours at 37°C with a solution containing 100 µg/ml of *Brucella* LPS (equal mix of LPS from W99 and Rev1 strains, A and M-dominant LPS, respectively) was performed. When needed, the eluate was immediately neutralized and used for amplification and titration of infectious phage particules. Strains of *Escherichia coli* used for amplification and titration were either K91Kan (from the G. P. Smith Laboratory) for the Cysx libraries or XL1-Blue (Stratagene) for X9 and CX9 libraries. For the following cycles of biopanning, 10¹¹ phage particules were incubated overnight with 0.4 ml of culture supernatant at 4°C. The phage-antibody mixes were then added to cell culture dishes coated alternatively with either protein G (Sigma, St. Louis, USA) or protein A: wash and elution steps were performed as described above.

Screening by colony or plaque immunoblottings

Petri dishes containing the required antibiotics (and 1 mM IPTG if necessary) with either fresh colonies or fresh plaques were blotted with nylon amphoteric membranes (Porablot NY amp, Macherey-Nagel, Düren, Germany) for two hours at 37°C. The membranes were subsequently saturated with 5% (w/v) skimmed milk in TBS and incubated with diluted hybridoma culture supernatants. The binding of the mAb to phage particules contained in colonies or plaques was revealed using GAM-HRP (Dako, Denmark) diluted 1000 times in saturation solution. The presence

of the secondary antibody was in turn detected using HRP colour development reagent (Bio-Rad, Hercules, USA). Several rounds of isolation, re-infections and colony immunoblottings were used to ensure isolation of pure phage clones.

Sequence determination of selected peptides

The sequence of the selected peptides was determined using a two steps procedure. In the first step, the recombinant gene 8 was amplified by polymerase chain reaction (PCR) using oligonucleotides annealing in the tac promoter (5'-CCCCATCCCCCTGTTGACAAT-3') or in the trpA terminator (5'-ATTAGGCGGGCTGGTATC-3'), these sequences being specific for the recombinant gene 8 in the Cysx libraries (see Table 1). The recombinant gene 8 of the X9 and CX9 libraries was amplified by PCR with oligonucleotides annealing upstream (5'-ATTCTAGAGATACGCC-3') and downstream (5'-TGCTGCAAGGC-GATTAAGTT-3') the region coding for the presented peptide. The PCR were carried out with the *Pwo* polymerase (Boehringer Mannheim, Mannheim, Germany) in order to reduce possible risks or errors during amplification. The PCR products were sequenced using the T7 Sequenase PCR product sequencing kit (Amersham Pharmacia Biotech) using the M13-40 Forward primer (5'-GTTTCCCCAGTCACGAC-3') for the X9 and CX9 libraries or an internal primer (5'-ACGCTTTAGAAGT-GAAT-3') for the Cysx libraries. Sequences were analysed with the DNA Strider 1.2 software.

Enzyme-linked immunosorbent assay (ELISA) with phages particules

Indirect ELISAs were used to monitor the binding of the mAbs to the selected phage-displayed peptides. Various concentrations of phage particules, suspended in TBS, were coated overnight at 4°C on Maxisorp multiwell plates (Nunc, Roskilde, Denmark). The plates were then saturated with 3% (w/v) skimmed milk in TBS for two hours at room temperature and washed five times (with TBS-0.1% (v/v) Tween 20). The mAb (culture supernatant diluted 1.5 to three times) was then incubated in the coated wells for one hour at room temperature, washed five times, and GAM-HRP (Dako, Denmark) was added to detect the binding of the mAb to the phages. After five washings, the peroxidase activity was monitored by addition of K-Blue[®] substrate (Neogen, Lexington, USA) at room temperature. The reaction was stopped with 4N H₂SO₄ and the absorbance at 450 nm-630 nm was recorded.

Competition ELISAs were performed following two different protocols. In the first protocol, multiwell plates (reference number 269620; Nunc, Roskilde, Denmark) were coated overnight at 4°C with *Brucella* LPS (see the Figure legends for LPS concentration and *Brucella* strains). After two hours of saturation at 37°C with 3% skimmed milk in TBS, mixes of mAb with various concentrations of phage (preincubated for one hour at 37°C) were added to the wells and incubated for one hour at 37°C. After five washes with TBS-0.1% Tween, the binding of mAb was revealed with a GAM-HRP conjugate and K-blue[®] at room temperature. The reaction was stopped with 4N H₂SO₄.

In the second protocol, Maxisorp plates were coated overnight at 4°C with phage at various concentration (as indicated in the legend to Figure 2) in 0.1 M NaHCO₃ solution. The wells were saturated with 3%

skimmed milk in TBS for two hours at 37°C and mixes of mAb with various concentrations of LPS were added for one hour at 37°C. The fixation of the mAb was detected as in the first protocol described for competition ELISA.

Competition between free peptides and LPS for the binding to the mAbs has also been measured by indirect ELISA. Free peptide synthesis was performed by Syntem (France), and the sequences were as follows: 2C8[7], (NH₂-AEGEFCHHSPEEYQPCGPAK-CONH₂); 6B3[2] (NH₂-AAPTQCSQTWPCPSHLPAG-CONH₂); 6B3[4], (NH₂-APPPQCSKAWPCPPTHPAG-CONH₂); and 6B3[6], (NH₂-AGPGQCNTNRCPRMP-PAEG-CONH₂). Mixes of various concentrations of free peptide were incubated with a fixed concentration of mAb in TBS. A control without peptide was also performed. After a one hour incubation at room temperature, the mixes were added onto plates coated overnight with LPS (0.25 µg/ml Rev1 LPS for A15-6B3 mAb or 2.5 µg/ml W99 LPS for B66-2C8 mAb) and saturated for two hours with 3% skimmed milk in TBS. The binding of the mAb to the LPS was detected by a GAM-HRP conjugate, as described for the other indirect ELISA.

Mice immunisation and detection of anti-LPS response by ELISA

Groups of six to nine BALB/c mice, eight-week old females, were injected intraperitoneally with 10¹² phages particules in a 500 µl volume containing 50% (v/v) at 2% Alhydrogel (Superfos, Frederikssund, Denmark). Afterwards, four intramuscular injections were performed at three week intervals with 10¹² phages particules in a 100 µl volume containing 50% (v/v) Alhydrogel at 2%. A last intramuscular injection was performed three weeks before the bleeding from which sera were collected. In the ELISA tests used to screen the mice sera, LPS preparation from *B. melitensis* Rev1 and *B. abortus* W99 strains were separately coated overnight at 4°C on multiwell plates (reference number 269620; Nunc, Roskilde, Denmark) at a concentration of 1 µg/ml. The plates were saturated with 5% (w/v) skimmed milk in TBS, washed five times (with TBS-0.1% Tween 20) and the sera (diluted 20 to 160 times) were then incubated in the coated wells (or empty wells as control). Pre-immune sera and a sera obtained with a mouse immunized with irrelevant phage were used as negative controls, and gave very low signals.

Acknowledgements

The authors thank Armelle Phalipon (Institut Pasteur, Paris) and Franco Felici (University of Roma 2, Italy) for helpful and stimulating discussions. The libraries used in this work were kindly provided by Professors G. P. Smith (University of Missouri, Columbia), M. Sollazo (IRBM, Pomezia, Italy) and F. Felici, that are gratefully acknowledged. This research was funded by the DGTRÉ (Direction Générale des Technologies de la Recherche et de l'Energie) of the Région Wallonne (convention number 3160).

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Edited by J. Karn

(Received 28 May 1999; received in revised form 4 October 1999; accepted 4 October 1999)