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Induction of anti-carbohydrate antibodies by phage library-selected peptide mimics

One of the prerequisites for the development of polysaccharide subunit vaccines is the induction of an efficient immune response to carbohydrate antigens like lipopolysaccharide (LPS) or capsular polysaccharide antigens of pathogens. In an attempt to overcome the problems that arise from the T-independent immune response induced by such antigens, selecting peptide sequences that mimic protective carbohydrate epitopes has been proposed. In this study, we investigate a new selection strategy for immunogenic peptide mimics using the phage-displayed peptide library technology. Two monoclonal antibodies (mAb) of the A isotype (mIgA), mIgA C5 and mIgA I3, specific for the O-antigen (O-Ag) part of the human pathogen *Shigella flexneri* serotype 5a LPS and protective against homologous infection were used to screen two phage-displayed nonapeptide libraries in pVIII. Using mIgA C5, 13 different specific clones were selected, and 6 using mIgA I3; 5 of the latter also interacted in enzyme-linked immunosorbent assay with the first mAb. All of the 19 clones selected were separately used to immunize mice, but only 2 of them, p100c (mIgA I3-specific) and p115 (interacting with both mIgA) were able to induce anti-O-Ag antibodies. The immune response was specific for the O-Ag of the *S. flexneri* serotype 5a, and also selectively recognized the corresponding bacterial strain. The amino acid sequences of p100c and p115 immunogenic peptide mimics were YKPL-GALTH (flanked by two Cys residues) and KVPPWARTA, respectively. These results are the first example of immunogenic mimicry of carbohydrates by phage-displayed peptides, and indicate a new strategy of selection of immunogens for the development of anti-polysaccharide vaccines.

1 Introduction

The main targets of the protective immune response against bacterial infection are the capsular polysaccharide as well as the O-Ag carbohydrate moiety of the LPS (for a review, see [1]). Carbohydrate antigens are T cell independent, inducing weak antibody responses associated with the lack of a strong B cell memory response [2], and vaccine strategies have thus been mainly focused on the development of either polysaccharide-protein conjugates or anti-idiotypic vaccines based on mimicking the carbohydrate structure [3]. The difficult steps of the former approach are the purification of the polysaccharide (especially when starting from LPS, which must be devoid of any residual lipid A-related endotoxic activity) and the loss of immunogenicity of the carbohydrate moiety during coupling to the protein carrier. Carbohydrate synthesis may diminish the problems associated with antigen purification, but nonetheless it remains a limited solution due to the overall difficulties of carbohydrate chemistry. The latter strategy, based on the mimicry of carbohydrate antigens by anti-idiotypic antibodies, is not a simpler alterna-

tive, since obtaining these antibodies is relatively time-consuming, and their use in humans is still a matter of debate. Therefore, polysaccharide-protein conjugates remain, despite difficulties, the only viable strategy for human vaccination against bacterial polysaccharide antigens investigated till now.

Over the last years, phage-displayed peptide libraries have been widely screened with antibodies as well as non-antibody molecules, leading to the identification of new ligands which do not necessarily resemble the natural ones, but display similar binding capacity (for reviews see [4–7]). The identification of peptides that mimic carbohydrate structures has also been reported [8–13], and this approach might be an alternative to the use of anti-idiotypic antibodies as mimics [14]. However, the immunogenicity of phage-displayed peptides that mimic the carbohydrate structures involved in the protective immune response against pathogens has not been reported so far. We decided to investigate whether peptide mimics selected through such a strategy could act as immunogenic mimics able to induce anti-carbohydrate antibodies.

The pathogen *Shigella flexneri* has been selected for this study. This gram-negative bacterium is responsible for the endemic form of shigellosis, a dysenteric syndrome characterized by bacterial invasion of the human colonic mucosa [15]. LPS and certain protein antigens are the targets of the humoral systemic as well as local immune responses [16, 17]. Protection is specific for the serotype of the strain [18, 19], which is defined by the structure of the repeated saccharide unit constituting the O-Ag of LPS [20, 21]. In addition, we recently reported that a monoclonal (m) mIgA directed against a serotype-specific epitope of the O-Ag, mIgA C5, present in local secretions before infec-

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tion, confers protection, thus showing the fundamental role played by both the carbohydrate O-Ag (especially the serotype-specific determinants) and the local humoral response against this pathogen [22]. Similar results were obtained with mIgA I3, another mIgA specific for the O-Ag of *S. flexneri* serotype 5a, whose affinity was similar to that of mIgA C5 (A. Phalipon, unpublished data). Preliminary results of the characterization of the saccharide epitope recognized by each of these mIgA indicated that they probably do not recognize exactly the same carbohydrate determinant on the O-Ag (L. Mulard and A. Phalipon, unpublished data).

We report here the identification of peptide mimics of protective carbohydrate epitopes capable of inducing specific anti-carbohydrate antibodies by combining these two *S. flexneri* serotype 5a specific mIgA and phage-displayed peptide technology.

2 Materials and methods

2.1 Phage-displayed peptide libraries and selection of peptide mimics by biopanning

The two phage peptide libraries used in this study, pVIII-9aa [23] and pVIII-9aa.Cys [24], were previously constructed by our group. Both are derived from approximately 10^8 independent clones carrying 9-amino acid-long random peptide inserts in the N-terminal region of the phage major coat protein (pVIII); in pVIII-9aa.Cys, the random inserts are flanked by two cysteine residues and hence can be cyclically constrained. Specific phage clones were isolated from the libraries by two rounds of affinity selection according to previously described biopanning procedures [23, 25].

In the first round, the mAb was incubated at 1 μ M concentration overnight at 4°C with 10^{10} Amp^R transducing unit (TU) of library in a total volume of 10 μ l. The mixture was incubated with 0.25 μ g of a biotin-conjugated goat anti-mouse IgA secondary antibody (α -chain specific, Sigma, St. Louis, MO), which was previously pre-adsorbed overnight at 4°C with 2×10^{11} phage particles of UV-killed M13K07 to prevent nonspecific binding, and then the phage-mAb-secondary Ab complexes were tethered on streptavidin-coated dishes. The second round of affinity selection was carried out in the same way, but using 10 nM or 0.1 nM concentrations of mAb and proportionally lower amounts of the secondary antibody. Positive phage clones were identified through plaque immunoscreening [24, 26] and further characterized by ELISA [27, 28].

2.2 Immunization of mice

Six-week-old female BALB/c mice (Janvier, France) were immunized i.p. six times at 15-day intervals for the first three injections, and at 30-day intervals for the last three ones, using 10^{12} phage particles per immunization, purified on a CsCl gradient [27]. A group of ten mice was used for each of the phage clones used as immunogen. Preimmune sera were individually recovered from every mouse and used as a negative control when testing the presence of anti-*S. flexneri* LPS antibodies in each of the correspond-

ing immune sera. For each of the clones inducing a positive response, another group of ten mice was also immunized i.p. to test the reproducibility of anti-carbohydrate antibody induction; i.v. immunizations were also assessed.

2.3 Immunoblotting of LPS

Briefly, 2 μ g per lane of purified LPS diluted in Laemmli sample buffer were run on an SDS-15 % polyacrylamide gel [29] in the presence of 4 M urea. After transfer to nitrocellulose, the anti-*S. flexneri* LPS antibodies in the serum of mice immunized with each of the different phage clones were revealed using horseradish peroxidase-labeled goat anti-mouse IgG as secondary antibody (dilution 1:5000; Sigma, St. Louis, MO) and visualized by enhanced chemiluminescence (Amersham International, Amersham, GB).

2.4 ELISA

ELISA was performed as previously described [30]. Briefly, either 1 μ g of *S. flexneri* LPS purified according to Westphal et al. [31] or 10^{10} p100c or p115 phage particles were coated per well. Binding of specific antibodies was revealed using alkaline phosphatase-conjugated goat anti-mouse IgG as secondary antibody (dilution 1:5000; Bioss, Compiègne, France). Antibody titers were defined as the dilution of serum specimens leading to an absorbance value twice that of the negative control (*i.e.* preimmune sera), except for the measurement of the anti-LPS titer in which incubation of sera of mice immunized with wild-type phage (pwt) (cross-reacting with the *Shigella* LPS core moiety) was used as the negative control. Specific inhibition of recognition of O-Ag by p100c- or p115-induced antibodies was performed under the same conditions, except that various concentrations of p100c and p115 phage particles were incubated with the p100c and p115 immune sera before adding the sample to the well.

2.5 Labeling of bacteria

Freshly grown bacteria were centrifuged onto coverslips ($700 \times g$ for 10 min) and fixed with 3.7 % paraformaldehyde in PBS for 20 min at room temperature. Labeling was performed as described [32] with immune sera of mice immunized with either p110c, p115, or pwt particles (dilution 1:20). Rhodamine-conjugated goat anti-mouse immunoglobulin G (Sigma) was used as secondary antibody (dilution 1:200). The labeled preparations were observed using a conventional fluorescence microscope (BH2-RFCA, Olympus Optical Co., Ltd.).

3 Results

3.1 Selection and features of phage-displayed peptides mimicking protective carbohydrate epitopes of the *S. flexneri* serotype 5a O-Ag

Both mIgA C5 and mIgA I3 specific for the O-Ag of the *S. flexneri* serotype 5a LPS (previously shown to be protec-

Table 1. Phage-displayed nonapeptide sequences interacting with the antigen binding site of mIgA C5 and/or mIgA I3 specific for the O-Ag of *S. flexneri* serotype 5a⁽¹⁾

clone	peptide sequence	recognition by mIgA I3	recognition by mIgA C5
100c	YKPLGALTH	0.85	0.05
121	KVPAWARRL	0.18	0.51
6	HIPAYATHV	0.05	1.40
115	KVPPWARTA	0.51	1.36
19	EHFWEQRPR	0.06	1.11
5c	TRGHFLQNR	0.05	1.79
12	HYLVQSPFW	0.05	1.14
21	QSHFLQGT	0.05	1.12
22	KRHFLSQRQ	0.06	1.23
11c	RRHFLDQRG	0.05	1.92
20	HFLSQNFFG	0.05	1.66
18c	SPHFFNQIR	0.06	1.26
16	WGPFQYAAG	0.05	1.98
148c	SQGRWPPWR	0.31	0.66
8	LLRQARERP	0.05	0.96
160c	GSPLRQRSS	0.20	0.36
143c	GSPLRQRSL	0.19	0.50
14	PPLSQRRAL	0.05	1.46
9	TRQQNPER	0.05	0.90
pwt		0.05	0.05

a) The number of the phage clone and the corresponding sequence displayed by it are indicated. Recognition of these phage clones by mIgA C5 and/or mIgA I3 is indicated by the absorbance value obtained during screening of the nonapeptide libraries as described in Sect. 2.4. "c" following the number of a clone indicates that this clone was selected from the cysteine-constrained nonapeptide library, thus in the pVIII recombinant protein the peptide insert is flanked by two cysteine residues.

tive *in vivo* against *Shigella* infection [22]; A. Phalipon, unpublished results) were used to screen phage-displayed nonapeptide libraries, and clones interacting with these antibodies were isolated as described in Sect. 2.1. Six different clones were selected with mIgA I3, and thirteen with mIgA C5. Five of the clones selected with mIgA I3 were shown by ELISA to interact also with mIgA C5.

To select relevant peptide mimics of the carbohydrate epitopes, the phage clones were then tested for their ability to compete with the antigen for binding to the antibody. Binding in ELISA of each mIgA to the selected phage clones was measured in presence of various concentrations of the *S. flexneri* serotype 5a LPS. The binding of all the phage clones to the antibody(ies) they interacted with was inhibited by LPS (data not shown). The peptide sequences of the inserts of the phage clones mimicking carbohydrate determinants are summarized in Table 1. In total, 19 peptide sequences mimicking protective carbohydrate epitopes of the O-Ag were selected by the use of two different phage-displayed peptide libraries.

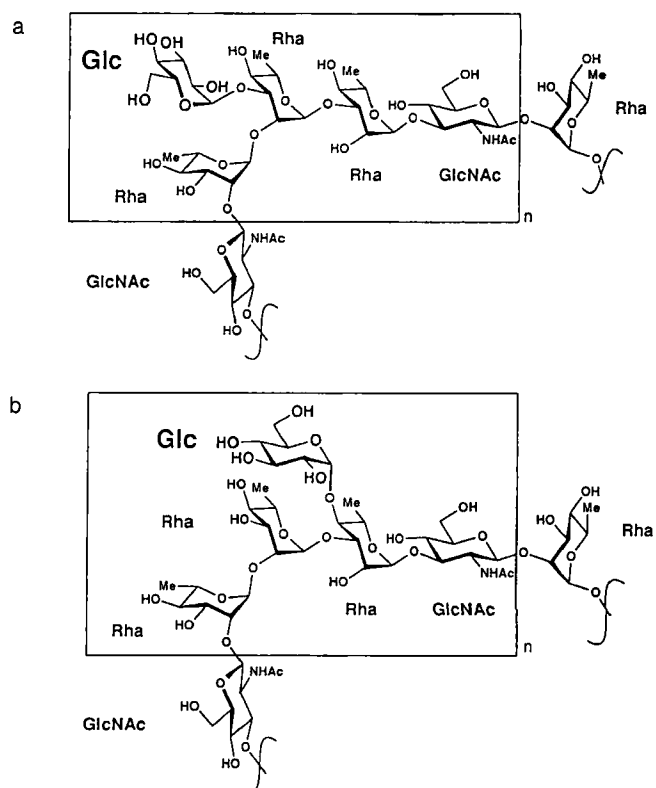


Figure 1. Structure of the repeated saccharide unit of the O-Ag of *S. flexneri*. The basic structures of serotype 5a (a) and serotype 2a (b) are shown. The saccharide unit is boxed and "n" indicates that it is repeated n times to constitute the O-Ag. The GlcNAc and Rha residues outside the surrounding are part of the (n – 1) and (n + 1) unit, respectively.

An interesting common feature of all the sequences was the high frequency of aromatic amino acid residues [tyrosine (Y), tryptophan (W), phenylalanine (F)] or residues having cyclic side chains [proline (P), histidine (H)]: a large part (82 %) of the positive insert sequences comprised at least two of these residues, more than half (55 %) at least three, and clone 12 five out of nine.

3.2 Immunogenicity of the peptide mimics

If the peptide sequences that have been selected mimic the protective carbohydrate epitopes, they could be expected to induce antibodies specific for the O-Ag of the *S. flexneri* LPS. The basic structure of the saccharide unit which is repeated to form the O-Ag of the *S. flexneri* species is three rhamnose (Rha) and one N-acetylglucosamine (GlcNAc) with a glucosyl residue (Glc) that specifies the serotype. For instance, Glc linked to the central Rha residue specifies serotype 5a (Fig. 1a), whereas Glc branched to the Rha linked to the GlcNAc specifies serotype 2a (Fig. 1b). Usually, no anti-O-Ag antibodies specific for serotype 2a are elicited following natural infection or experimental immunization with bacteria of serotype 5a and vice versa. As both mIgA used for the selection of the peptide mimics are serotype 5a-specific, the peptide

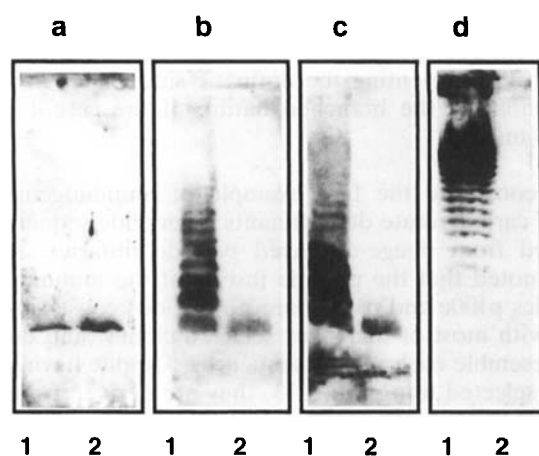


Figure 2. Specificity of the peptide-induced antibodies. Western blots were performed using purified LPS of serotype 5a (lane 1) or serotype 2a (lane 2), and incubated with sera of mice (dilution 1:50) immunized with the phage clones pwt (a), p100c (b), or with mIgA C5 (dilution 1:1000) (d).

mimic-induced antibodies should therefore be specific for this serotype.

To test the immunogenicity of the peptide mimics, each of the 19 selected phage clones were used to immunize BALB/c mice, as described in Sect. 2.2. The anti-carbohydrate antibody response induced was tested by immunoblotting using purified LPS from the *S. flexneri* serotypes 5a and 2a. Among the 19 clones previously selected, p100c (mIgA I3-specific) and p115 (recognized by both mIgA), carrying the sequences YKPLGALTH and KVPPWARTA, respectively, induced anti-O-Ag antibodies, which were specific for the serotype 5a (Fig. 2b and c, respectively). The observed ladder, which is a feature of the recognition of the O-Ag by specific antibodies, is constituted by the repeats of the basic saccharide unit. Interestingly, the average molecular weight of the O-Ag molecules recognized by the peptide-induced antibodies

was different from that of those recognized by mIgA C5 or mIgA I3 (Fig. 2d) which were used to select the immunogenic peptide mimics. The p100c- and p115-induced antibodies recognized O-Ag molecules of molecular mass ranging from 950 (1 unit) to 10 450 (11 units) (Fig. 2b), and from 950 (1 unit) to 16 150 (17 units) (Fig. 2c). A common pattern of recognition was similarly observed for mIgA C5 or I3, but these antibodies also recognized O-Ag molecules of higher molecular mass (Fig. 2d).

The lowest band, corresponding to the LPS core moiety, was detected by the p100c- and p115-induced antibodies (Fig. 2b and c, respectively) as well as by sera of mice immunized with pwt (Fig. 2a). As phage preparations may contain traces of *E. coli* LPS, whose core region is very similar to that of *Shigella*, the recognition of the *S. flexneri* LPS core region probably reflected the cross-reactivity of anti-*E. coli* LPS antibodies induced following immunization with the phage particles.

3.3 p100c- and p115-induced anti-LPS and anti-phage antibody titers

The antibody response induced by the two immunogenic peptide mimics was further analyzed by ELISA. As immunizations were performed with the purified phage particles, the anti-LPS antibody response induced by p100c and p115 was mainly of the G isotype. As shown in Fig. 3 (b and c), for both immunogenic mimics the anti-LPS and anti-phage antibody titers were 1:100 and 1:10 000, respectively. A similar anti-phage response was observed with the pwt (Fig. 3a), whereas, as expected, no anti-LPS antibody response was detected. Binding of the mimic-induced antibodies to LPS in the presence of the phage clones p100c, p115 or pwt was also tested in ELISA. Inhibition of binding of p100c-induced antibodies was observed in the presence of p100c but not p115 or pwt; similar results were obtained with p115 and the p115-induced antibodies (data not shown).

3.4 Recognition of *S. flexneri* serotype 5a bacteria by peptide mimic-induced antibodies

If the antibodies play a role in protection against infection, thus disrupting the pathogenic process, they should recognize and bind to the bacteria. As could be expected, *S. flexneri* serotype 5a, but not serotype 2a bacteria were recognized by p115-induced antibodies (Fig. 4a and b). Similar data were obtained with p100c-induced antibodies (not shown). No labeling was observed when bacteria were incubated with pwt-induced antibodies (data not shown). These findings suggest that the peptide mimic-induced antibodies should be able to interact with the pathogen in an *in vivo* situation.

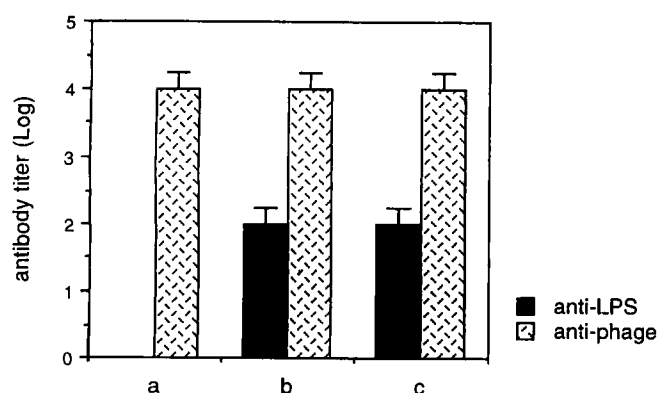


Figure 3. p100c- and p115-induced anti-LPS and anti-phage antibody titers. Three groups of ten BALB/c mice were immunized i.p. with the phage clones pwt (a), p100c (b), or p115 (c) as described in Sect. 2.2. Similar results were obtained following i.v. immunizations. The ELISA data are representative of three independent experiments. Anti-LPS and anti-phage antibodies were estimated in individual sera. Titers are given as the mean $\pm$ SD of individual samples.

4 Discussion

The results presented in this paper demonstrate that peptide sequences mimicking protective carbohydrate epitopes of pathogens selected through phage-displayed peptide libraries can act as immunogenic mimics and induce anti-carbohydrate antibodies. This approach represents a

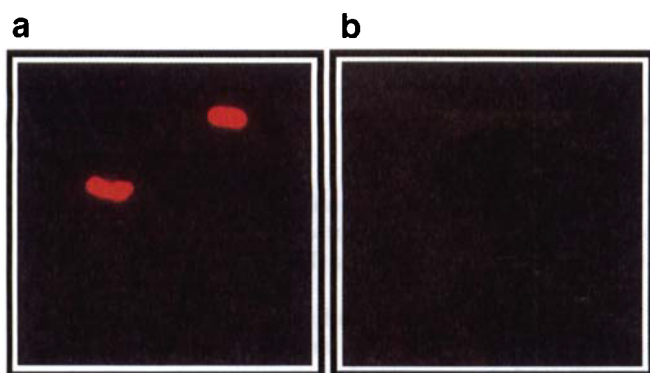


Figure 4. Labeling of *S. flexneri* bacteria with the peptide mimic-induced antibodies. Labeling of *S. flexneri* serotype 5a (a) or serotype 2a (b) bacteria, previously centrifuged and fixed onto coverslips, was performed with sera of mice immunized with pwt, p100c, or p115. Rhodamine-conjugated goat anti-mouse immunoglobulin G was used as secondary antibody (dilution 1:200). Results shown in this figure were obtained with p115-induced antibodies (dilution of sera 1:20).

practical alternative to the use of anti-idiotypic antibodies as mimics of carbohydrate structures. It is also a much simpler way to select peptide mimics of carbohydrates, since only two tools are required, *i.e.* a protective monoclonal antibody specific for the carbohydrate epitope to be mimicked, and phage-displayed random peptide libraries, which are now widely available. The anti-idiotypic antibody approach appears to be a more difficult and time-consuming task. As the anti-idiotypic antibody molecule in its entirety is unsuitable for repeated immunization, the characterization and use of its complementarity-determining region (CDR) as immunogenic peptides to elicit anti-carbohydrate antibodies has recently been reported [14], representing an additional complication. In comparison, obtaining peptide mimics through the phage display technology is quite straightforward.

The peptide sequences selected as mimics of protective carbohydrate epitopes of the *S. flexneri* serotype 5a O-Ag by screening the phage-displayed nonapeptide libraries comprise a varying number of aromatic amino acids or amino acids having cyclic side chains, at least one per sequence. This is in accordance to previous data suggesting features that are common to peptides that mimic carbohydrates. Although the previously reported peptides mimic completely different carbohydrate structures, it appears that their sequences are similar. For instance, all members of a class of proteins which act as α -amylase inhibitors have the strictly conserved residues WRY [33–35]. Peptides binding Con A have been shown to contain the consensus sequence YPY [8, 9]. The amino acid sequence (APWLYGPA) of a peptide mimic of a carbohydrate epitope of the Lewis Y antigen has also been reported, which contains the residues PWLY, critical for peptide binding to an antibody specific for this antigen [10]. Interestingly, a similar result has been reported for mimicry of carbohydrate structures by anti-idiotypic antibodies. Westerink and al. [14] reported the presence of the YRY sequence in the variable region (CDR3) of an anti-idiotypic antibody mimicking the meningococcal group C capsular polysaccharide. How is this preference for aromatic amino acid residues explained? As discussed by Hoess et al. [10], they resemble sugar moieties in their size, cyclic shape, and par-

ticularly in the case of tyrosine, hydrogen bonding potential. Furthermore, the proline may serve an important structural role in orienting the aromatic side chains in a manner similar to the branched nature of the carbohydrates they mimic.

Our data constitute the first example of immunogenic mimicry of carbohydrate determinants by peptide sequences selected from phage-displayed peptide libraries. It should be noted that the peptide inserts of the immunogenic mimics p100c and p115 share no obvious consensus sequence with most of the other selected clones, and do not even resemble each other's sequences. Despite having been both selected using mIgA I3, they are derived from two different libraries (the p100c insert is Cys-flanked) and also have a different pattern of recognition with mIgA C5. More interestingly, p100c and p115 are both able to raise a specific anti-carbohydrate antibody response in mice, but p100c is not able to inhibit p115-induced antibody binding to LPS and vice versa.

Some of the other IgA-specific clones share sequence similarities with p100c or p115, but not their biological property of immunogenic mimicry of *S. flexneri* O-Ag. For example, p121 displayed a peptide sequence very similar to that of p115 (the sequence KVP-WAR – is common to both clones) yet it did not elicit a detectable anti-carbohydrate antibody response. This suggests that the remaining amino acid residues also play a significant role in carbohydrate mimicry, and consequently in the induction of the specific immune response.

The anti-O-Ag antibody response induced following immunization with p100c and p115 appeared to be slightly different from that induced by the whole bacteria or purified LPS, showing a weaker titer of anti-LPS antibodies and a lower molecular weight of the recognized O-Ag molecules. Similar differences in the recognition of the O-Ag molecules were observed for the peptide mimic-induced antibodies and mIgA C5 or mIgA I3, although a common range of O-Ag molecules were recognized. An explanation for the differences observed could be that the nonapeptide sequences, due to their conformational structure, were only partially mimicking the structural complexity of the carbohydrate epitopes. To test this hypothesis, phage libraries displaying longer peptides, for instance 15 amino acid residues long, will be screened with mIgA C5 and mIgA I3, and tested for their immunogenicity. Whether a better immunogenic peptide mimicry of the carbohydrate determinants could be obtained will be also tested through site-directed mutagenesis of p100c and p115 clones.

The strategy reported here could well be of interest for the development of a new type of anti-polysaccharide vaccine. Immunogenic peptide mimics of protective carbohydrate epitopes of the most frequent serotypes of the *Shigella* species responsible for either the endemic or epidemic form of shigellosis could be combined to develop a multivalent subunit vaccine. As phage particles might prove unsuitable for vaccination, the capacity of the mimics used as peptides to elicit anti-carbohydrate antibodies should be investigated. Preliminary results show that p115-synthetic peptide is recognized by both mIgA, and also by sera of mice immunized with p115-phage particles (data not

shown), suggesting a limited contribution of the pVIII amino acid residues flanking the peptide insert to the induction of anti-peptide antibodies, and therefore of anti-LPS antibodies. Immunogenicity of p115-synthetic peptide is currently tested. The protective role of the peptide-induced antibodies has not been reported in this study. The immunogenicity of the peptide mimics was assessed by using an immunization protocol leading to the induction of an IgG-mediated antibody response directed against the O-Ag. In the murine model of *Shigella* infection, however, protection has been shown to occur in the presence of an IgA-, but not IgG-mediated antibody response specific for the O-Ag ([22]; A. Phalipon, unpublished data). Therefore, further studies will be aimed at the induction of anti-O-Ag IgA antibodies and the study of their role in protection against experimental infection.

Finally, our system will serve as an additional model to study the molecular basis of carbohydrate mimicry by peptides. To achieve this goal, the characterization of the carbohydrate epitope recognized by mIgA C5 and mIgA I3 is under way, as well as the CDR sequencing for both mIgA. As reported for anti-idiotypic mimicry of protein antigens [36], it is reasonable to expect that the peptide sequence mimics the carbohydrate antigen by mimicking its binding interaction to the antibody.

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