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# Immunological Evidence for Functional Rather than Structural Mimicry by a *Shigella flexneri* Y Polysaccharide-Mimetic Peptide<sup>∇†</sup>

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**An approach to vaccine design is the use of molecules that mimic the immunogenic element of interest. In this context, the interaction of MDWNMHAA, a peptide mimic of the *Shigella flexneri* Y O polysaccharide (PS), with an anti-carbohydrate monoclonal antibody, as studied previously by X-ray crystallography, suggested the presence of functional rather than structural mimicry and a bound peptide conformation that was not represented significantly in the free-ligand ensemble. The antibody response elicited by an MDWNMHAA-carrier protein (tetanus toxoid [TT]) conjugate has now been investigated in BALB/c mice. The mice were immunized following a homologous prime/boost strategy using MDWNMHAA-TT as the immunogen. The mice showed anti-peptide antibody (immunoglobulin G [IgG]) titers that increased after being boosted. High anti-lipopolysaccharide (LPS) (IgG) titers were observed after the last boost. A faster immune response, with cross-reactive titers, was observed with a peptide conjugate with 30% more copies of the peptide. The binding of anti-peptide polyclonal antibodies to LPS could be inhibited by LPS, PS, MDWNMHAA, and MDWNMHAA-bovine serum albumin, as assessed by inhibition enzyme-linked immunosorbent assay. Conversely, mice immunized with PS-TT showed IgG anti-peptide titers. These data demonstrate the cross-reactivity of the antibody response and support the hypothesis that functional, as opposed to structural, mimicry of the *S. flexneri* Y O PS by MDWNMHAA or the underrepresentation of the bound ligand conformation in the free-ligand ensemble does not compromise immunological cross-reactivity. Prime/boost strategies were performed with a heterologous boost of PS-TT or MDWNMHAA-TT. They led to high anti-LPS titers after only three injections, suggesting alternatives to improve the immunogenicity of the carbohydrate-mimetic peptide and confirming the antigenic mimicry.**

Shigellosis, caused by *Shigella* species (gram negative), is a prominent, and the most infectious, diarrheal disease. *Shigella flexneri*, the species responsible for the highest mortality rate, is endemic in most developing countries (23). The 13 serotypes of *S. flexneri*, with the exception of serotype 6, result from structural modifications of the O-antigen polysaccharide (PS), the outer portion of the lipopolysaccharide (LPS) on the bacterial surface, which is both an essential virulence factor and a protective antigen (10). The basic O-antigen PS repeat, common to the 12 serotypes, is referred to as serotype Y. A murine immunoglobulin G3 (IgG3) monoclonal antibody, SYA/J6, specific for the PS O antigen of the *S. flexneri* Y LPS, was developed by Bundle (7) and Carlin and coworkers (8). The O-antigen Y PS is a linear heteropolymer with a tetrasaccharide repeating unit [ $\rightarrow$ 2)- $\alpha$ -L-Rhap-(1 $\rightarrow$ 2)- $\alpha$ -L-Rhap-(1 $\rightarrow$ 3)- $\alpha$ -L-Rhap-(1 $\rightarrow$ 3)- $\beta$ -D-GlcpNAc-(1 $\rightarrow$ )] and is recognized by the monoclonal antibody SYA/J6 (7, 9). The oligosaccharides of *S. flexneri* serotype Y have been well studied by nuclear magnetic resonance techniques, which have provided a three-dimensional model of the determinant in solution (4, 30, 31, 32) and the identity of the biological repeating unit (9).

Glycoconjugate vaccines to prevent shigellosis, focused on oligosaccharide analogues related to *Shigella flexneri* 2a and

other serotypes, have been synthesized and evaluated (3, 10, 29, 38). An interesting approach to vaccine design is the use of molecules that mimic the immunogenic element of interest (11, 20, 28). Carbohydrate-mimetic peptides have potential as surrogate ligands for traditional carbohydrate vaccines, providing more discriminating immune responses (19, 20, 28). However, there are few examples of immunological responses with peptide-based PS mimics (18, 20, 28). Therefore, the requirements for cross-reactivity are not fully understood and are certainly not predictable, because of the limited data set available.

In order to further exploit this principle, a carbohydrate-mimetic peptide of the *S. flexneri* Y O-antigen PS, MDWNMHAA, cross-reactive with the anti-*S. flexneri* Y O-PS monoclonal antibody, SYA/J6, was identified by phage library screening (15). The structures of complexes of the antibody SYA/J6 Fab fragment with synthetic deoxytrisaccharide and pentasaccharide ligands, related to the *S. flexneri* Y O antigen, and with the carbohydrate-mimetic peptide have been determined by X-ray crystallography (17, 33, 34, 35). The structure of the Fab complex with MDWNMHAA revealed differences, and few similarities, with respect to the oligosaccharide complexes (34), providing the first evidence that the modes of binding of the pentasaccharide and octapeptide differ considerably and that few aspects of structural mimicry exist (34).

Furthermore, for a peptide to be immunogenic, it might be necessary that a sufficient population of a bound conformation be displayed in the conformational ensemble of the free peptide (20). Since the  $\alpha$ -helix adopted by NMHAA in the C terminus of MDWNMHAA is present only in the bound con-

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formation and not in the free peptide, we questioned whether immunization with an MDWNMHAA conjugate would lead directly to a cross-reactive response against the corresponding PS. We questioned further whether prime/boost strategies would strengthen the immune responses already induced by the PS epitopes, as shown recently with a peptide mimic of the capsular PS of *Cryptococcus neoformans* (2). Therefore, it was of interest to test the immunogenicities and cross-reactivities of antibodies elicited by immunizations with MDWNMHAA conjugates to probe the hypothesis that predisposition of the  $\alpha$ -helix motif in the free-peptide conformational ensemble might be necessary for immunological peptide-carbohydrate cross-reactivity.

The synthesis of MDWNMHAA-based conjugates to bovine serum albumin (BSA) and to tetanus toxoid (TT) and of the *S. flexneri* O-PS glycoconjugate (PS-TT), together with their immunochemical evaluation with SYA/J6, was reported recently (16). It is noteworthy that a search of the human genome revealed that the sequence MDWNMHAA is not present (NCBI protein database). We now report the investigation of the immunogenicities of these conjugates in mice, together with the cross-reactivity of the immune sera with PS. The results are compared to those from our previous study of peptide-carbohydrate cross-reactivity with a peptide mimic of the cell-wall PS of group A *Streptococcus* (6). Evidence is presented that cross-reactive immunological responses can be elicited even with functional rather than structural peptide mimics, where the former refers to binding of the two ligands using different functional groups on the antibody and the latter to the engagement of the two ligands using the same functional groups (19).

#### MATERIALS AND METHODS

**PS antigens, monoclonal antibody SYA/J6, and synthesis of peptide MDWNMHAA, MDWNMHAA-TT and MDWNMHAA-BSA conjugates, and O-PS-TT conjugate.** The *S. flexneri* Y LPS, used as a solid-phase antigen for enzyme-linked immunosorbent assay (ELISA), and the *S. flexneri* Y O PS (Fig. 1, compound 1), used to generate the TT conjugate and as an inhibitor, were generous gifts from D. R. Bundle, prepared and purified as previously described (1). The monoclonal antibody SYA/J6 was also generated, characterized, purified, and provided by D. R. Bundle (7, 8) and was used as an ELISA control.

The PS-mimetic peptide MDWNMHAA (Fig. 1, compound 2) was synthesized using the 9-fluorenylmethoxy carbonyl solid-phase strategy and linked via the amino terminus to a bifunctional linker, diethylsquarate, and then conjugated to TT or BSA as an immunogenic carrier, as described previously (16) (Fig. 1, compounds 3 and 4). The levels of incorporation of the peptide on TT were 53% for the batch of conjugate used in the first study, (MDWNMHAA)<sub>32</sub>-TT, and 83% for the batch used in the second study, (MDWNMHAA)<sub>50</sub>-TT, while that on BSA was 100% (Fig. 1). The peptide MDWNMHAA and its conjugates bound to the antibody SYA/J6, as evaluated by ELISA (16).

The *S. flexneri* Y O-PS glycoconjugate (PS-TT) (Fig. 1, compound 5), which comprises 10 repeating units (tetrasaccharide), was prepared based on a reductive amination of the PS at the reducing end with 1,3-diaminopropane, followed by linking the aminated PS to diethylsquarate to form an adduct that was subsequently conjugated to TT (16) and used as an immunogen in this study.

**Experimental groups of mice and immunization protocols.** Female BALB/c mice (6 to 8 weeks old) were obtained from the Charles River Breeding Laboratories (Montreal, Quebec, Canada) and were maintained in our Animal Care Facility following the animal care guidelines. Groups of five mice each were injected subcutaneously with the conjugates, Alhydrogel (2%; Superfos Brenntag Biosector, Frederikssund, Denmark), or controls. The mice were bled every 2 weeks for 8 weeks, their sera were analyzed, and mean reciprocal endpoint titers and standard deviations were determined.

(i) **Study 1.** Control groups received 0.5  $\mu$ g of PS-TT (group 4 [G4]) and 100  $\mu$ g of Alhydrogel or 100  $\mu$ g of TT (G5) plus 100  $\mu$ g Alhydrogel, in weeks 0, 2, 4,

and 6. Mice from G1 received 100  $\mu$ g Alhydrogel and 100  $\mu$ g of (MDWNMHAA)<sub>32</sub>-TT in weeks 0, 2, 4, and 6 (homologous prime/boost strategy).

In G2 and G3, a heterologous-homologous prime/boost strategy was implemented. G2 mice were primed with 100  $\mu$ g (MDWNMHAA)<sub>32</sub>-TT and boosted with 0.5  $\mu$ g PS-TT in week 2 and received a final boost with 100  $\mu$ g of (MDWNMHAA)<sub>32</sub>-TT in week 6. G3 mice were primed with 0.5  $\mu$ g PS-TT and boosted with 100  $\mu$ g (MDWNMHAA)<sub>32</sub>-TT in week 2 and were given a final boost with 0.5  $\mu$ g of PS-TT in week 6. Mice from both groups received 100  $\mu$ g Alhydrogel per immunization.

(ii) **Study 2.** Control groups received 2  $\mu$ g of PS-TT (G5) and 100  $\mu$ g of Alhydrogel or 100  $\mu$ g of TT (G6) plus 100  $\mu$ g Alhydrogel in weeks 0, 2, 4, and 6. Mice from G1 received 100  $\mu$ g Alhydrogel and 100  $\mu$ g of (MDWNMHAA)<sub>50</sub>-TT, in weeks 0, 2, 4, and 6 (homologous prime/boost strategy). Mice from G2 received 100  $\mu$ g of (MDWNMHAA)<sub>50</sub>-TT and 100  $\mu$ g Alhydrogel in weeks 0, 2, and 6 (homologous prime/boost strategy).

In G3 and G4, an extended heterologous prime/boost strategy was implemented. G3 mice were primed with 100  $\mu$ g (MDWNMHAA)<sub>50</sub>-TT and boosted with 0.5  $\mu$ g PS-TT in weeks 2 and 6. G4 mice were primed with 0.5  $\mu$ g PS-TT and boosted with 100  $\mu$ g (MDWNMHAA)<sub>50</sub>-TT in weeks 2 and 6. Mice from both groups received 100  $\mu$ g Alhydrogel per immunization.

**ELISA.** Antibody titers to MDWNMHAA and LPS in sera from mice were determined before vaccination and 2 weeks after each vaccination by ELISA as described previously (39). The titer was defined as the highest dilution yielding an absorbance of  $\geq 0.1$  after twice the average background reading was subtracted. The negative control consisted of wells without serum. The positive control was the mouse SYA/J6, appropriately diluted.

**Competitive ELISA with LPS, PS, MDWNMHAA, and MDWNMHAA-BSA as inhibitors.** Sera from those mice that had the highest anti-MDWNMHAA antibody titers cross-reactive to LPS in week 8 (mice 1 from G1, G2, and G3 from study 1 or mouse 3 from G1, 4 from G2, 3 from G3, and 2 from G4 from study 2) were used. The sera were incubated in duplicate at a final dilution of 1:200 with twofold serial dilutions of inhibitors: *S. flexneri* Y PS and MDWNMHAA (Fig. 1, compounds 1 and 2), MDWNMHAA-BSA (Fig. 1, compound 3), and LPS in phosphate-buffered saline-Tween 20, starting at an initial concentration of 200  $\mu$ g/ml, for 1 h at room temperature. Then, 100  $\mu$ l (each) of the mixtures was transferred to plates coated with antigens and incubated at room temperature for 3 h. The plates were washed, and the bound IgG was detected by ELISA.

**Statistical analysis.** Within groups, comparisons of titers at different times were analyzed with the Wilcoxon matched-pairs test (36). Between groups, comparisons were analyzed with the Mann-Whitney *U* test (26). The *P* values were corrected for multiple comparisons (5). A *P* value of  $< 0.05$  was considered to indicate a significant difference.

#### RESULTS

The individual mouse antisera (study 1, five mice per group and five groups of mice, G1 to G5; study 2, five mice per group and six groups of mice, G1 to G6) were evaluated for the relative amounts of IgG antibody they contained that bound to MDWNMHAA-BSA (immunogenicity) and to LPS (cross-reactivity). Preimmune sera screened by ELISA, using MDWNMHAA-BSA or LPS as a solid-phase antigen, showed low background activity (Fig. 2 and 3, studies 1 and 2, week 0). The immunization schedules are described in Tables 1 and 2.

**Immunogenicities of the peptide conjugates: antibody titers to MDWNMHAA after a homologous prime/boost strategy (studies 1 and 2).** Mice primed with 100  $\mu$ g of the (MDWNMHAA)<sub>32</sub>-TT conjugate responded with IgG antibody titers against MDWNMHAA-BSA ( $P = 0.03$ ; week 2) (Fig. 2, study 1, G1, week 2). Increases were seen after repeated boosting of mice from G1 (Fig. 2) in weeks 2, 4, and 6 ( $P = 0.05$ ; week 8). Control groups (G4 and G5) showed much lower mean endpoint titers to peptide at all time points, although titers against peptide were observed after boosting with PS-TT (G4) in weeks 6 and 8 ( $P = 0.03$ ) and were higher than G5 ( $P = 0.04$ ; week 6) (Fig. 2, study 1). Similar results were observed after the (MDWNMHAA)<sub>50</sub>-TT conjugate was injected into the

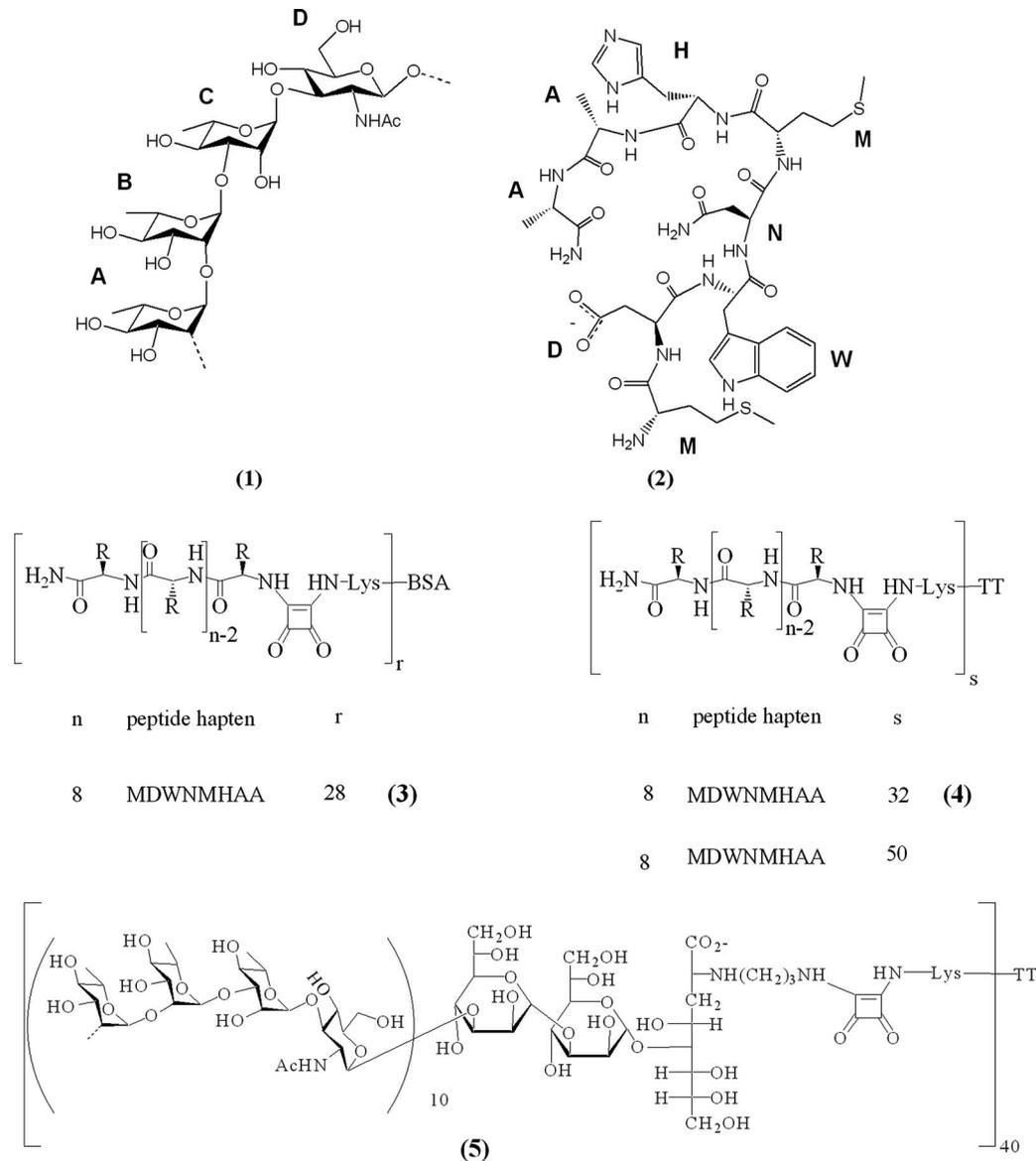


FIG. 1. Structures of compounds. Shown are the structures of the O PS of *S. flexneri* Y (compound 1, with monosaccharide residues of the repeating unit labeled A, B, C, and D) and its peptide mimic, the octapeptide MDWNMHAA (compound 2, with single-letter amino acid code), as well as the synthetic peptide conjugates MDWNMHAA-BSA (compound 3) and MDWNMHAA-TT (compound 4) and the *S. flexneri* Y O-PS conjugate (compound 5).

mice, although higher anti-peptide titers appeared in week 2 in study 2 ( $P = 0.03$ ), and increases were observed after each of the booster injections ( $P = 0.03$ ; weeks 2, 6, and 8) (Fig. 2, study 2, G1 and G2). The titers observed in mice from G1 were not significantly different from those from G2 in week 8 (corrected  $P = 1.0$ ) for study 2. These results indicate that the conjugates effectively promoted a strong and rapid thymus-dependent response to MDWNMHAA and showed a stronger response to (MDWNMHAA)<sub>50</sub>-TT.

**Immunogenicity of the peptide conjugate: antibody titers to MDWNMHAA after heterologous-homologous prime/boost strategies (study 1).** In order to improve the immune response to the weakly immunogenic peptide MDWNMHAA, we altered the immunization schedule with a PS-TT or (MDWNM

HAA)<sub>32</sub>-TT booster combined with the corresponding heterologous primer (Table 1). Thus, mice primed with 100  $\mu\text{g}$  of the (MDWNMHAA)<sub>32</sub>-TT conjugate (G2, study 1) responded with IgG antibody titers against MDWNMHAA-BSA ( $P = 0.03$ ; week 2) (Fig. 2, study 1, G2, week 2). Increases in titers were seen after boosting of mice from G2 with 0.5  $\mu\text{g}/\text{mouse}$  of PS-TT in week 2 ( $P = 0.03$ ; week 4) (Fig. 2, study 1, G2, weeks 4 and 6). Titers were observed after a homologous last boost of G2 mice with 100  $\mu\text{g}/\text{ml}$  (MDWNMHAA)<sub>32</sub>-TT in week 6 but were not significantly higher than those from week 6 ( $P = 0.14$ ) (Fig. 2, study 1, G2, week 8). Titers observed for G2 mice were not significantly different from those from G1 (corrected  $P = 1.0$ ) in week 8.

Mice primed with 0.5  $\mu\text{g}$  of the PS-TT conjugate (G3, study

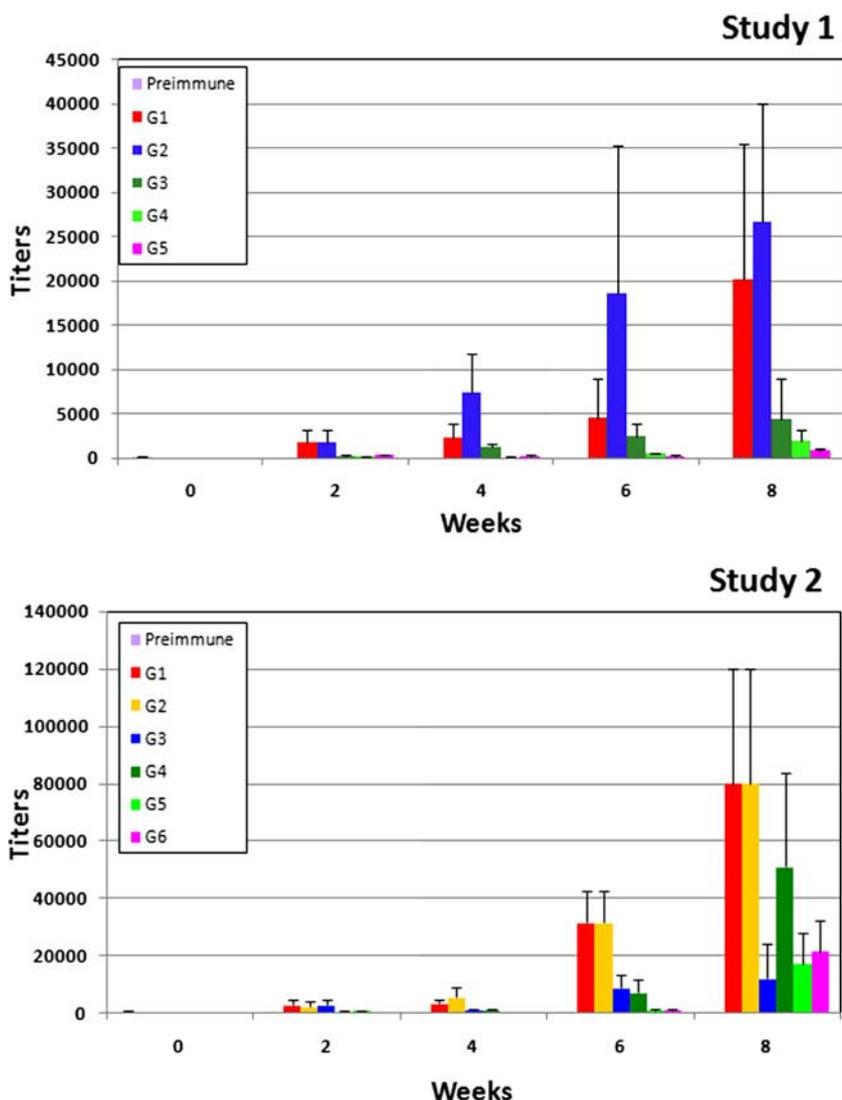


FIG. 2. Immunogenicities of the peptide conjugates: antibody titers (IgG) to MDWNMHAA. Plates were coated with MDWNMHAA-BSA (compound 3 in Fig. 1) (10  $\mu$ g/ml). Study 1 (Table 1 shows a detailed immunization schedule): G1, homologous prime/boost with (MDWNMHAA-TT)<sub>32</sub> (four times); G2, heterologous-homologous prime/boost (Table 1); G3, heterologous-homologous prime/boost (Table 1); G4, homologous prime/boost with PS-TT (four times); G5, TT (four times). Study 2 (Table 2 shows a detailed immunization schedule): G1, homologous prime/boost with (MDWNMHAA)<sub>50</sub>-TT (four times); G2, homologous prime/boost with (MDWNMHAA)<sub>50</sub>-TT (three times); G3, extended heterologous prime/boost (Table 2); G4, extended heterologous prime/boost (Table 2); G5, homologous prime/boost with PS-TT (four times); G6, TT (four times). The error bars represent standard deviations.

1) responded with IgG antibody titers against MDWNMHAA-BSA ( $P = 0.03$ ; week 2) (Fig. 2, study 1, G3, week 2). Increases in titers were seen after boosting of mice from G3 with 100  $\mu$ g/mouse of MDWNMHAA-TT in week 2 ( $P = 0.03$ ; weeks 4 and 6) (Fig. 2, study 1, G3, weeks 4 and 6). Titers were observed after a homologous last boost of G3 mice with 0.5  $\mu$ g/ml PS-TT in week 6 but were not significantly higher than those from week 6 ( $P = 0.30$ ) (Fig. 2, study 1, G3, week 8). Titers observed in mice from G3 were not different from those from G1 (corrected  $P = 0.25$ ) and G2 (corrected  $P = 0.1$ ) mice in week 8.

**Immunogenicities of the peptide conjugates: antibody titers to MDWNMHAA after extended heterologous prime/boost strategies (study 2).** In study 2, extended heterologous prime/

boost strategies were implemented (Table 2). Thus, mice primed with (MDWNMHAA)<sub>50</sub>-TT conjugate (G3) responded with IgG antibody titers against MDWNMHAA-BSA ( $P = 0.03$ ; week 2) (Fig. 2, study 2, G3, week 2). Increases in titers were observed after boosting of mice twice with 0.5  $\mu$ g PS-TT ( $P = 0.03$ ; weeks 6 and 8) (Fig. 2, study 2, weeks 6 and 8).

Mice primed with 0.5  $\mu$ g of the PS-TT conjugate (G4) did not respond with titers against MDWNMHAA-BSA ( $P = 0.79$ ; week 2) (Fig. 2, study 2, G4, week 2). Increases in titers were seen after these mice were boosted twice with 100  $\mu$ g/mouse (MDWNMHAA)<sub>50</sub>-TT ( $P = 0.03$ ; weeks 4, 6, and 8) (Fig. 2, study 2, weeks 4, 6, and 8). Titers from G3 mice were significantly lower than those from G1 (corrected  $P = 0.04$ ), G2 (corrected  $P = 0.04$ ), and G4 (corrected  $P = 0.03$ ) mice in week 8.

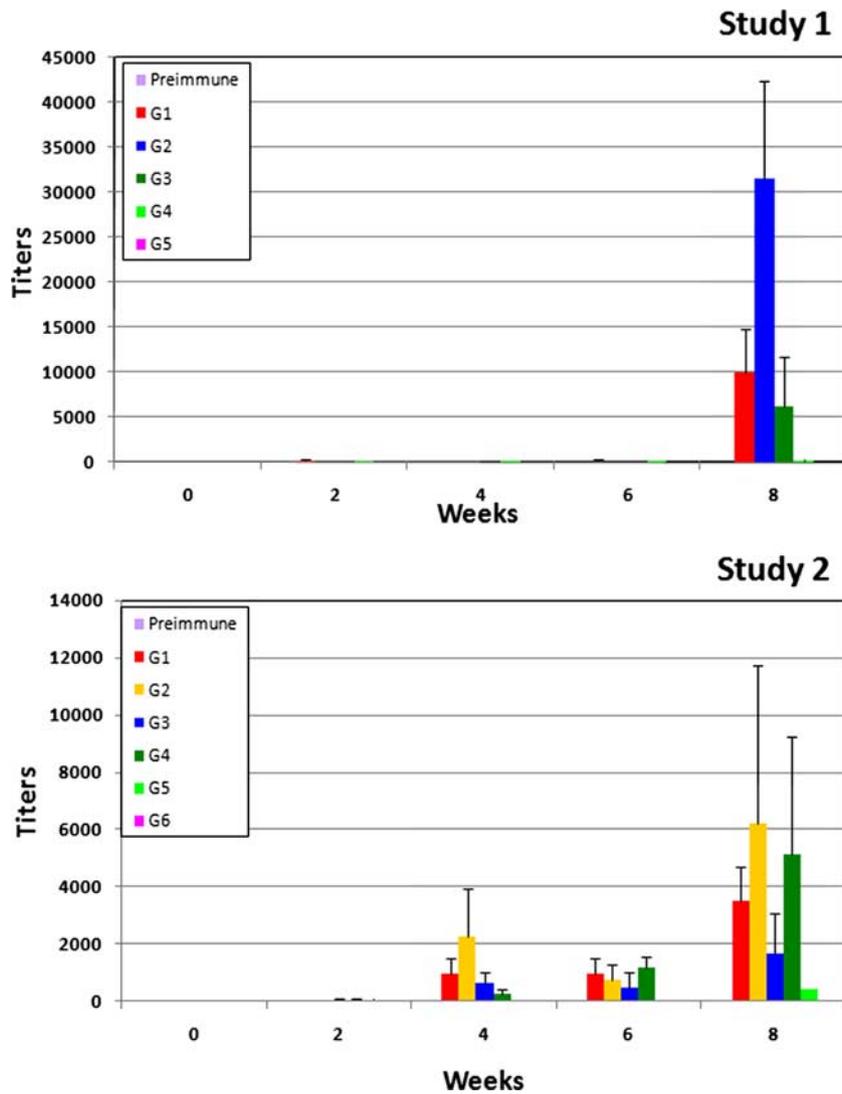


FIG. 3. Cross-reactivity: polyclonal antibodies (IgG) binding to LPS as the solid phase-antigen in ELISA (10  $\mu\text{g/ml}$ ). Study 1 (Table 1 shows a detailed immunization schedule): G1, homologous prime/boost with (MDWNMHAA-TT)<sub>32</sub> (four times); G2, heterologous-homologous prime/boost (Table 1); G3, heterologous-homologous prime/boost (Table 1); G4, homologous prime/boost with PS-TT (four times); G5, TT (four times). Study 2 (Table 2 shows a detailed immunization schedule): G1, homologous prime/boost with (MDWNMHAA)<sub>50</sub>-TT (four times); G2, homologous prime/boost with (MDWNMHAA)<sub>50</sub>-TT (three times); G3, extended heterologous prime/boost (Table 2); G4, extended heterologous prime/boost (Table 2); G5, homologous prime/boost with PS-TT (four times); G6, TT (four times). The error bars represent standard deviations.

**Cross-reactivity: anti-peptide antibodies bind to *S. flexneri* Y LPS after homologous prime/boost strategy (studies 1 and 2).**

In study 1, titers against *S. flexneri* Y LPS were obtained when high doses of the (MDWNMHAA)<sub>32</sub>-TT were administered

four times to mice, i.e., after four homologous prime/boost immunizations ( $P = 0.03$ ) (Fig. 3, study 1, G1, week 8). The subcutaneous immunization of mice with (MDWNMHAA)<sub>32</sub>-TT (100  $\mu\text{g/ml}$ ) resulted in a late anti-LPS IgG response (Fig. 3, week 8).

TABLE 1. Immunization schedule for study 1

Immunization strategy	Mouse group (n = 5)	Prime (wk 0) ( $\mu\text{g}/\text{mouse}$ )	First boost (wk 2) ( $\mu\text{g}/\text{mouse}$ )	Second boost (wk 4) ( $\mu\text{g}/\text{mouse}$ )	Second or third boost (wk 6) ( $\mu\text{g}/\text{mouse}$ )
Homologous	G1	MDWNMHAA-TT (100)	MDWNMHAA-TT (100)	MDWNMHAA-TT (100)	MDWNMHAA-TT (100)
Heterologous-homologous	G2	MDWNMHAA-TT (100)	PS-TT (0.5)	MDWNMHAA-TT (100)	MDWNMHAA-TT (100)
Heterologous-homologous	G3	PS-TT (0.5)	MDWNMHAA-TT (100)		PS-TT (0.5)
Homologous	G4	PS-TT (0.5)	PS-TT (0.5)	PS-TT (0.5)	PS-TT (0.5)
Homologous	G5	TT (100)	TT (100)	TT (100)	TT (100)

TABLE 2. Immunization schedule for study 2

Immunization strategy	Mouse group ( $n = 5$ )	Prime (wk 0) ( $\mu\text{g}/\text{mouse}$ )	First boost (wk 2) ( $\mu\text{g}/\text{mouse}$ )	Second boost (wk 4) ( $\mu\text{g}/\text{mouse}$ )	Second or third boost (wk 6) ( $\mu\text{g}/\text{mouse}$ )
Homologous	G1	MDWNMHAA-TT (100)	MDWNMHAA-TT (100)	MDWNMHAA-TT (100)	MDWNMHAA-TT (100)
Homologous	G2	MDWNMHAA-TT (100)	MDWNMHAA-TT (100)		MDWNMHAA-TT (100)
Extended heterologous	G3	MDWNMHAA-TT (100)	PS-TT (0.5)		PS-TT (0.5)
Extended heterologous	G4	PS-TT (0.5)	MDWNMHAA-TT (100)		MDWNMHAA-TT (100)
Homologous	G5	PS-TT (2)	PS-TT (2)	PS-TT (2)	PS-TT (2)
Homologous	G6	TT (100)	TT (100)	TT (100)	TT (100)

In study 2, IgG titers against *S. flexneri* Y LPS were observed after two injections of 100  $\mu\text{g}/\text{mouse}$  of (MDWNMHAA)<sub>50</sub>-TT ( $P = 0.03$ ; week 4) (Fig. 3, study 2, G1 and G2, week 4). These titers increased after the last boosting in week 6 ( $P = 0.03$ ; week 8). No titer increases against TT, used as control, were seen in any of the studies (Fig. 3, G5, study 1, and G6, study 6, weeks 2, 4, 6, and 8). The anti-LPS IgG titers appeared lower than those against peptide at the same time point in both studies (Fig. 2 and 3, week 8 for study 1 and weeks 4, 6, and 8 for study 2).

**Cross-reactivity: polyclonal antibodies bind to *S. flexneri* Y LPS after heterologous-homologous prime/boost strategies (study 1).** In study 1, priming with a high dose of (MDWNMHAA)<sub>32</sub>-TT and boosting with a low dose of PS-TT (Fig. 3, study 1, G2, week 2) resulted in no IgG titer increases against LPS (Fig. 3, G2, weeks 4 and 6). A high anti-LPS IgG response resulted after homologous boosting with a high dose of (MDWNMHAA)<sub>32</sub>-TT in week 6 ( $P = 0.03$ ; week 8) (Fig. 3, study 1, G2, week 8). These anti-LPS IgG titers were not different from those from G1 (corrected  $P = 0.1$ ) and G3 (corrected  $P = 0.08$ ) mice and appeared similar to those against peptide at the same time point (week 8).

Priming with a low dose of PS-TT and boosting with a high dose of (MDWNMHAA)<sub>32</sub>-TT in study 1 (Fig. 3, study 1, G3, week 2) did not increase the IgG anti-LPS titers. In contrast, an anti-LPS IgG response resulted after homologous boosting with a low dose of PS-TT in week 6 ( $P = 0.03$ ; week 8) (Fig. 3, study 1, G3, week 8). These anti-LPS IgG titers were not significantly different from those from G1 (corrected  $P = 1.0$ ) and G2 (corrected  $P = 0.08$ ) and appeared similar to those against peptide at the same time point (Fig. 2 and 3, week 8).

**Cross-reactivity: polyclonal antibodies bind to *S. flexneri* Y LPS after extended heterologous prime/boost strategies (study 2).** In study 2, priming with a high dose of (MDWNMHAA)<sub>50</sub>-TT and boosting with a low dose of PS-TT (Fig. 3, study 2, G3, week 2) resulted in IgG titer increases against LPS ( $P = 0.03$ ; week 4) (Fig. 3, G3, weeks 4 and 6). Small increases in anti-LPS titers were observed in week 8 ( $P = 0.05$ ) after a second heterologous boost (in week 6) with a low dose of PS-TT (Fig. 3, study 2, G3, week 8). G3 anti-LPS titers were not significantly different from those from G1 (corrected  $P = 0.4$ ), G2 (corrected  $P = 0.7$ ), and G4 (corrected  $P = 0.35$ ) in week 8.

In contrast, priming with a low dose of PS-TT and boosting twice with a high dose of (MDWNMHAA)<sub>50</sub>-TT (G4 in study 2) resulted in IgG anti-LPS titers ( $P = 0.03$ ; weeks 6 and 8) (Fig. 3, study 2, G4, weeks 6 and 8). These IgG anti-LPS titers

were not significantly different from those obtained after repeated homologous prime/boost immunizations, G1 (corrected  $P = 0.4$ ) and G2 (corrected  $P = 1.0$ ), in this study.

**Cross-reactivity: anti-PS (PS-TT) antibodies bind to MDWNMHAA-BSA (studies 1 and 2).** Mice immunized subcutaneously with 0.5  $\mu\text{g}/\text{mouse}$  of PS-TT in weeks 0, 2, 4, and 6 responded with titers against MDWNMHAA-BSA in study 1 ( $P = 0.03$ ; weeks 2, 6, and 8) (Fig. 2, study 1, G4, weeks 6 and 8). The titers increased after repeated boosting. The titers were higher than those corresponding to mice immunized with 100  $\mu\text{g}$  TT in week 6 ( $P = 0.04$ ), but not in week 8 ( $P = 0.12$ ) (Fig. 2, study 1, G5). Similarly, in study 2, mice immunized with 2  $\mu\text{g}/\text{mouse}$  of PS-TT in weeks 0, 2, 4, and 6 responded with titers against MDWNMHAA-BSA ( $P = 0.03$ , weeks 6 and 8) (Fig. 2, study 2, G5, week 8); these titers appeared higher than those obtained in study 1 but were not significantly different from those corresponding to a high dose of TT (100  $\mu\text{g}/\text{mouse}$ ) (Fig. 2, study 2, G6, week 8) (corrected  $P = 0.45$ ; week 8). However, these titers were correlated with anti-LPS titers that were greater than those from G6 (TT) in week 8 (corrected  $P = 0.03$ ) (Fig. 3, study 2, G5 and G6, week 8).

**Specificities of the polyclonal antibodies. (i) Study 1.** In order to investigate the epitope specificities of the polyclonal antibodies, competitive-inhibition ELISAs were performed. The antisera with the highest endpoint titers, obtained at week 8 for one mouse from each group, G1, G2, and G3, immunized three or four times (Table 1), were used in these experiments. Titration of the antisera was performed for each experiment, and the dilution used corresponded to an  $A_{405}$  of  $\sim 0.5$  after 30 min (see Fig. S1A in the supplemental material). *S. flexneri* Y LPS was used as the solid-phase antigen.

Competitive-inhibition ELISA with *S. flexneri* Y LPS, PS (Fig. 1, compound 1), free MDWNMHAA (Fig. 1, compound 2), and MDWNMHAA-BSA (Fig. 1, compound 3) as inhibitors was performed with *S. flexneri* Y LPS as the solid-phase antigen. All of the inhibitors inhibited the binding of the anti-MDWNMHAA polyclonal antibodies from mouse no. 1 serum from G1 (IgG) (see Fig. S1B in the supplemental material), with the greatest inhibitory activity at about 30% at a concentration of 100  $\mu\text{g}/\text{ml}$  for LPS, PS, and MDWNMHAA-BSA. A lower inhibition value (20%) was obtained with the free peptide under the same conditions. These data confirm the binding of the anti-peptide polyclonal antibodies with LPS and PS.

The best inhibitors of the binding of polyclonal antibodies from mouse no. 1 from G2 (IgG) (see Fig. S1C in the supplemental material) were LPS and MDWNMHAA-BSA, with the

greatest inhibitory activity at 25 to 33% at a concentration of 100  $\mu\text{g}/\text{ml}$ . A lower inhibition value was obtained with the PS and the free peptide under the same conditions (7% and 4%, respectively).

The best inhibitors of the binding of polyclonal antibodies from mouse no. 1 from G3 (IgG) (see Fig. S1D in the supplemental material) were LPS, PS, and MDWNMHAA-BSA, with the greatest inhibitory activity at 20 to 30% at a concentration of 100  $\mu\text{g}/\text{ml}$ . A lower inhibition value was obtained with the free peptide under the same conditions (10%).

**(ii) Study 2.** The antisera with highest endpoint titers, obtained at week 8 for one mouse from each group, G1, G2, G3, and G4, immunized three or four times (Table 2), were used in these experiments. Titration of antisera was performed for each experiment, and the dilution used corresponded to an  $A_{405}$  of  $\sim 0.6$  after 30 min (see Fig. S2A in the supplemental material). *S. flexneri* Y LPS was used as the solid-phase antigen.

All the inhibitors inhibited the binding of the anti-MDWNMHAA polyclonal antibodies from mouse no. 3 serum from G1 (IgG) (see Fig. S2B-G1 in the supplemental material), with the greatest inhibitory activity at about 50% at a concentration of 100  $\mu\text{g}/\text{ml}$  for MDWNMHAA-BSA. A lower inhibition value (40%) was obtained with the LPS and the free peptide under the same conditions. The isolated PS was the poorest inhibitor (10%) at 100  $\mu\text{g}/\text{ml}$ . These data confirm the binding of the anti-peptide polyclonal antibodies with LPS and PS.

The best inhibitors of the binding of polyclonal antibodies from mouse no. 4 from G2 (IgG) (see Fig. S2B-G2 in the supplemental material) were LPS and MDWNMHAA-BSA, with the greatest inhibitory activity at 35 to 40% at a concentration of 100  $\mu\text{g}/\text{ml}$ . A lower inhibition value (10%) was obtained with PS. No inhibition of the binding of polyclonal antibodies to LPS with the free peptide was observed under the same conditions.

The best inhibitors of the binding of polyclonal antibodies from mouse no. 3 from G3 (IgG, see Fig. S2B-G3 in the supplemental material) were LPS, PS, and MDWNMHAA-BSA, with the greatest inhibitory activity at 20 to 25% at a concentration of 100  $\mu\text{g}/\text{ml}$ . A lower inhibition value (7%) was obtained with the free peptide under the same conditions.

The best inhibitors of the binding of polyclonal antibodies from mouse no. 2 from G4 (IgG) (see Fig. S2B-G4 in the supplemental material) were LPS, PS, and MDWNMHAA-BSA, with the greatest inhibitory activity at 30 to 70% at a concentration of 100  $\mu\text{g}/\text{ml}$ . A lower inhibition value (22%) was obtained with the free peptide under the same conditions.

## DISCUSSION

This study illustrates the concept of peptide-carbohydrate mimicry with an *S. flexneri* Y O-PS-mimetic peptide using an immunological approach. Thus, an MDWNMHAA-TT conjugate used in immunizations of BALB/c mice elicited antibodies directed against the O PS of the *S. flexneri* Y LPS. Conversely, antisera elicited by immunizations of mice with an *S. flexneri* Y O-PS-TT conjugate cross-reacted with MDWNMHAA-BSA, as probed by ELISA at different time points.

The immunogenicity of MDWNMHAA-TT was manifested in primary and secondary antibody responses (immunological

memory), with MDWNMHAA titers that increased after the three booster immunizations given to mice in both of the studies (Fig. 3, G1). However, these titers were much lower than those observed in a similar immunological study (6) with a PS-mimetic peptide, DRPVY, of the *Streptococcus* group A cell wall PS (15). Interestingly, DRPVY adopts secondary (turn) structures in the VPY region in both the free and antibody-bound forms (22), in contrast to MDWNMHAA, which lacks defined structure in the free peptide (21). The display of peptide secondary structures (turn conformations) was also observed with another immunogenic *Streptococcus* group B carbohydrate-mimetic peptide (18). This led us to postulate that defined turn structures in carbohydrate-mimetic peptides are immunodominant elements (epitopes) and thus associated with immunogenicity (20), a hypothesis that has been reinforced in this study. Similar results were also observed by Dyson et al. (13) and Craig et al. (12) for peptides derived from proteins.

The results also demonstrate that the peptide MDWNMHAA can act as an antigenic mimic when attached to a carrier protein and can induce anti-carbohydrate antibodies. However, in study 1, high titers against the O-PS region of the *S. flexneri* Y LPS were evident only after an 8-week period in which a high dose of the peptide conjugate had been administered to mice four times (G1). This result indicates that the kinetics of the immune response are slow and that several homologous immunizations are required for memory to develop and to raise high antibody titers. This time point (week 8) also corresponds to the highest anti-peptide titer obtained, perhaps a prerequisite for the presence of cross-reactive antibodies, as observed earlier (6). These anti-LPS titers, despite the fact that they were 0.5-fold lower than those against the peptide, were specific to carbohydrates and of similar or higher magnitude than those reported previously (2, 6, 14, 25). Antigenic cross-reactivities between the peptide and the O PS were demonstrated by the LPS- and PS-specific inhibition of the anti-peptide polyclonal antibodies binding to LPS with serum obtained after the fourth immunization (G1). The fact that MDWNMHAA and PS were poorer inhibitors than MDWNMHAA-BSA and LPS, respectively, is likely due to the presentation of multiple copies of the critical epitopes.

However, when a conjugate containing 30% more copies of peptide per TT was administered at the same doses to mice (G1 and G2, study 2), IgG-anti-LPS titers were observed 2 weeks earlier than in study 1, corresponding to high anti-peptide titers. This result suggests that (MDWNMHAA)<sub>50</sub>-TT is a more immunogenic conjugate than (MDWNMHAA)<sub>32</sub>-TT. However, anti-LPS titers obtained with (MDWNMHAA)<sub>50</sub>-TT appeared lower than with (MDWNMHAA)<sub>32</sub>-TT in week 8, despite the higher anti-peptide titers (G1, week 8). This may be the result of a carrier-induced suppression effect, which can decrease the production of antigen-specific antibodies, as observed earlier in a similar immunological study with a strongly immunogenic PS-mimetic peptide conjugate [(DRPVY)<sub>39</sub>-TT] (6). No clear difference in the LPS-cross-reactive antibody response was observed when (MDWNMHAA)<sub>50</sub>-TT was administered to mice three times (G2) instead of four (G1), suggesting that when a more immunogenic mimetic peptide conjugate is used, fewer injections are required. These anti-LPS titers were also specific to carbohydrate (G1 and G2).

The combined results led us to conclude that (i) MDWN MHAA is an antigenic mimic of the O PS of *S. flexneri* Y, since high titers of cross-reactive antibodies were obtained after immunizations of mice with MDWNMHAA-TT; (ii) MDWN MHAA is a poorly immunogenic peptide, suggesting the importance of predisposed secondary structures for immunogenicity; and (iii) the anti-peptide antibodies were specific for the PS of *S. flexneri* Y, since this compound inhibits the binding of antibodies to LPS. Nevertheless, the results reported here indicate that the lack of predisposition of the  $\alpha$ -helix turn in the free-peptide conformational ensemble does not preempt a cross-reactive immune response. In addition, functional mimicry, as opposed to structural mimicry, of the PS by the peptide is also clearly sufficient to elicit a cross-reactive immune response.

The facts outlined above led us to implement a heterologous prime/boost immunization strategy in order to improve the immune response to the MDWNMHAA peptide. These strategies have recently shown promising results, enhancing both cellular and humoral immune responses against a variety of viral and bacterial pathogens (27, 37). Accordingly, we altered the immunization schedule (Table 1) with a heterologous boost in week 2. The following strategies were attempted in study 1: (i) priming with (MDWNMHAA)<sub>32</sub>-TT and boosting with PS-TT (G2) and (ii) priming with PS-TT and boosting with (MDWNMHAA)<sub>32</sub>-TT (G3).

Increases in the anti-MDWNMHAA titers were seen after the heterologous booster in mice and were stronger after boosting with PS-TT (study 1, G2, weeks 4 and 6), indicating immunological memory and antigenic mimicry between the immunogens. In contrast, boosting with (MDWNMHAA)<sub>32</sub>-TT (study 1, G3, weeks 4 and 6) led to a response that more closely resembled the primary response observed for G1 in week 2 after priming with peptide conjugate. Comparison of the results of priming with (MDWNMHAA)<sub>32</sub>-TT and PS-TT suggests that activation of naïve B cells, and possibly T helper cells, is enhanced when (MDWNMHAA)<sub>32</sub>-TT is given as a primer vaccine. On the other hand, PS-TT appears to be a more effective booster vaccine. This may be due to a different mechanism of processing and presentation of the antigens, considering the different chemical natures of the immunogens, and also due to the antigen availability, since very different doses of immunogens were required. In any event, no cross-reactive anti-LPS (IgG) titers were observed in week 4 or 6 in spite of a small increase in the anti-LPS IgM response in week 4 (not shown).

Remarkably, a last homologous boost (Table 1) resulted in few or no significant increases in titers against MDWNMHAA but very high anti-LPS (IgG) titers (study 1, G2 and G3, week 8), evidence of a booster effect leading to a fast, strong response with high IgG titers and clearly confirming the presence of memory B cells and plasma cells that had been effectively restimulated. These anti-LPS antibodies were also specific to carbohydrate. The inhibition results also indicate the effect of multivalent presentation and differences in the polyclonal specificities, and perhaps avidities, among G1, G2, and G3 polyclonal antibodies, as expected, since they were developed after different immunization strategies.

The implementation of extended heterologous prime/boost strategies, with an additional heterologous boost to focus the response, was performed in study 2. This strategy had been

successfully applied in the case of a *Neisseria meningitidis* outer membrane vesicle vaccine (24). The following immunization schedules were attempted in study 2: (i) priming with (MDWNMHAA)<sub>50</sub>-TT and boosting twice with PS-TT (G3) and (ii) priming with PS-TT and boosting twice with (MDWNMHAA)<sub>50</sub>-TT (G4).

An increase in the anti-MDWNMHAA titers was seen after the first heterologous booster was given to the mice (study 2, G3 and G4, weeks 4 and 6) with similar titers, indicating immunological memory and antigenic mimicry between the immunogens. Cross-reactive anti-LPS (IgG) titers were observed. Increases in titers against MDWNMHAA after a last heterologous boost (Table 2, G3 and G4, week 8) confirmed the presence of memory B cells and plasma cells that had been effectively restimulated. These antibodies bound specifically to LPS. The effect of multivalent presentation and the differences in the polyclonal specificities among G3 and G4 polyclonal antibodies were evident, as expected, since they were developed after different immunization strategies. In G3, the anti-PS response seemed to dominate, while in G4, the anti-peptide response appeared to be the dominant response (G3 and G4).

The existence of immunological carbohydrate-peptide mimicry was further demonstrated by the increase in IgG titers to MDWNMHAA elicited by repeated immunization with PS-TT (G4, study 1, and G5, study 2, weeks 6 and 8), evidence of the booster effect. However, only IgM anti-LPS titers were observed when a low dose of PS-TT was given four times (not shown); presumably, IgG titers would have risen at a later time. When the dose of PS-TT was 2  $\mu$ g/mouse (study 2, G5, week 8), IgG anti-LPS titers were observed in week 8.

In conclusion, a peptide mimic of *S. flexneri* Y O PS, when used as an immunogen, elicited good titers of mature antibody of isotype IgG, with immunological memory. The antibodies generated were cross-reactive with the O PS, as evaluated by an LPS ELISA binding study, and this interaction was inhibited by LPS and PS, leading us to conclude that the peptide MDWNMHAA is an antigenic mimic of the *S. flexneri* Y O PS. However, several immunizations were necessary in order to achieve a cross-reactive response, an indication of the low immunogenicity of the peptide. Taken together, previous results (6, 18) and those reported here suggest that predisposition of antibody-bound epitopes in the free-peptide conformational ensemble leads to a more rapid cross-reactive anti-PS response. Nonetheless, a cross-reactive response does develop when these epitopes are not present, suggesting that amplification of the B-cell clones requires longer immunization with multiple boosts. In addition, functional, as opposed to structural, mimicry of the PS by the peptide does not appear to compromise the ability of the peptide to elicit a cross-reactive response. Conversely, immunizations with PS-TT resulted in a cross-reactive response against MDWNMHAA, also confirming the antigenic mimicry. Our goal was to test whether prime-boost strategies would have any competitive advantage here. Extended heterologous and heterologous-homologous prime/boost strategies provided some advantage, since they led to high anti-peptide titers and high cross-reactive responses after only three injections, suggesting that these schedules might provide an effective approach to increase the immunogenicity of a carbohydrate-mimetic peptide. The present work is significant because it clearly demonstrates such cross-reactivity in an

immunological sense and sets the stage for the next phase of the study aimed at probing the protective response, and thus for vaccine development. The data presented address our initial questions and, in combination with other information on peptide-carbohydrate mimicry in the literature, lend credence to the use of carbohydrate-mimetic peptides as vaccine candidates for *Shigella*, as well as for other pathogens.

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