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Conjugates of Synthetic Cyclic Peptides Elicit Bactericidal Antibodies against a Conformational Epitope on a Class 1 Outer Membrane Protein of *Neisseria meningitidis*

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Bactericidal antibodies directed against surface loops of class 1 outer membrane proteins play a crucial role in protection against meningitis and sepsis caused by *Neisseria meningitidis*. So far, all efforts to obtain protective antibodies against these apparently conformational epitopes by using linear peptide analogs have been in vain. In this study, conjugates of head-to-tail cyclic peptides encompassing the predicted top of a protective surface loop were used for immunization. A series of 18 cyclic peptides with a ring size ranging from 7 to 17 residues, conjugated to tetanus toxoid, was investigated. Antipeptide and anti-whole-cell immunoglobulin G (IgG) titers elicited by the conjugates were determined. Conjugates of three peptides, containing 14, 15, and 17 amino acid residues (peptides 7, 12, and 13, respectively), induced an anti-whole-cell titer when Quillaja saponin A was used as the adjuvant. When alum was used as the adjuvant, the conjugate of peptide 12 did not elicit an anti-whole-cell response. From the Quillaja saponin A group, some of the sera obtained with conjugates of peptides 7 and 12 and all sera obtained with the peptide 13 conjugate were bactericidal in vitro. None of the sera evoked with alum as the adjuvant showed bactericidal activity. Nonbactericidal sera contained IgG1 primarily, whereas bactericidal sera showed significant titers of IgG2a and IgG2b. Class 1 protein-derived synthetic cyclic peptides which are capable of eliciting bactericidal antibodies, such as peptide 13 derived from meningococcal strain H44/76, represent potential candidates for a (semi)synthetic vaccine against meningococcal disease.

Meningitis and sepsis caused by serogroup B meningococci are life-threatening diseases for which introduction of an effective vaccine is required. Bactericidal antibodies against the class 1 outer membrane protein (OMP) play a major role in protection against meningococcal disease (13). A correlation between the bactericidal activity of human immune sera and the levels of class 1 protein-specific antibody titers has been observed (17, 18). Therefore, the class 1 proteins can be regarded as promising vaccine candidates.

During the past decade, several vaccines against meningococcal disease that contain OMPs as major protective components have been developed. However, clinical trials conducted with these experimental vaccine preparations revealed a degree of protection that was too low to justify public vaccination programs (5, 8, 10, 14, 21). These observations emphasize the need for vaccines that selectively direct the immune response towards the protective epitopes.

A two-dimensional model of the class 1 OMP predicts that the protein possesses eight cell surface-exposed loops (16). The bactericidal antibodies are directed against the predicted apices of loops 1 and 4 (11, 16). These apices demonstrate interstrain variation and determine the sero-subtype of meningococci. The heterogeneity of the class 1 protein is limited; seven sero-subtypes cover more than 80% of a worldwide collection of group B meningococcal isolates (2).

Several attempts to elicit bactericidal antibodies by using linear synthetic peptides derived from meningococcal OMP surface loops have been made. Antibodies against the peptides and the denatured protein were obtained. Unfortunately, the response against the OMP in its native conformation was usually very low (16). As a consequence, the antisera did not have any bactericidal activity.

For a number of peptides, for example, peptides derived from lysozyme (3) and influenza virus hemagglutinin (12), improvement of antigenic or immunogenic properties has been obtained by (disulfide) cyclization. Likewise, it seems necessary to decrease the conformational freedom of meningococcal peptides by cyclization in order to mimic the protein surface loop to a greater degree. It was recently reported that immunization of rabbits with a cyclic disulfide 36-residue peptide derived from loop 4 of a meningococcal class 1 OMP induces antibodies with antibactericidal activity (9). We have been investigating the possibility of using smaller, well-defined peptides to raise bactericidal antibodies. Since the three-dimensional structure of meningococcal class 1 OMPs is unknown, the optimal ring size of a cyclic peptide is difficult to predict. Therefore, we decided to prepare a series of cyclic peptides of various ring sizes in an attempt to mimic the protein surface loop with one or more peptides. In this paper we report an investigation of the relation between immunological properties and ring size of a series of 18 head-to-tail lactam-cyclic peptides.

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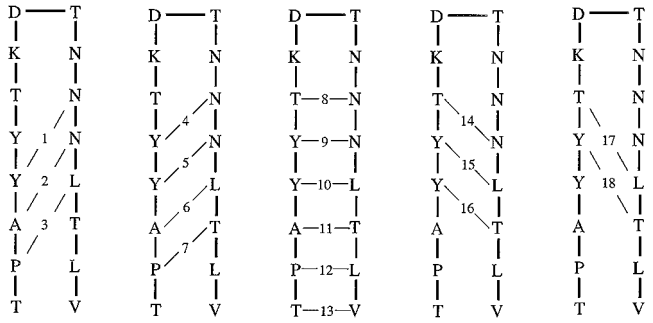

FIG. 1. Synthetic cyclic peptides derived from surface loop 4 of the P1.7,16 class 1 protein of reference strain H44/76. For conjugation purposes, a Lys-(SAMA) residue was incorporated into the cyclic peptides between the residues that are connected by the line containing the peptide number.

MATERIALS AND METHODS

Peptides 1 to 11 and 14 to 18 (Fig. 1) were prepared by solid-phase cyclization as described earlier (6). Peptides 3, 7, 12, and 13 were prepared by cyclization of side chain-protected precursors in solution as described below (i.e., for chemical comparison peptides 3 and 7 were prepared by both methods; peptides 3 and 7 from the second synthesis were used in the immunological experiments). Tetanus toxoid was obtained from the Laboratory of Vaccine Production of our institute. This material meets the requirements of the World Health Organization for human administration.

Synthesis of peptides 3, 7, 12, and 13. Starting from *N*-fluorenylmethoxycarbonyl–Pro-2–chlorotrityl resin (4), *N*-fluorenylmethoxycarbonyl- and side chain-protected peptides were assembled on a 10-μmol scale with an automated multiple-peptide synthesizer equipped with a 48-column reaction block (AMS 422; ABIMED Analysen-Technik Gmbh, Langenfeld, Germany) as described earlier (6). The following side chain protecting groups were used: *tert*-butyl for Thr and Tyr, *tert*-butyloxycarbonyl for Lys-181, 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-ethyl (7) for Lys-0 (an added residue for conjugation purposes), and triphenylmethyl for Asn.

After assembly of the sequences, cleavage of the N-terminal *N*-fluorenylmethoxycarbonyl groups was effected with piperidine–*N*,*N*-dimethylacetamide (DMA) (2:8, vol/vol). The peptide resins were washed with DMA (six times with 0.5 ml and three times with 3 ml), *tert*-amyl alcohol (three times with 0.5 ml), and diethyl ether (three times with 3 ml), successively. The reaction columns were then removed from the synthesizer for cleaving of the side chain-protected peptides from the polymer. The peptide resins were treated with 200 μl of acetic acid–2,2,2-trifluoroethanol–dichloromethane (1:1:8, vol/vol/vol) at 0, 5, 10, 15, 20, 25, and 30 min. After each addition, the fluid was allowed to drain from the columns under the force of gravity. The collected filtrates were concentrated and lyophilized (three times) from dioxane.

The cyclization was performed with approximately 10 μmol of lyophilized side chain-protected peptide. The crude product was dissolved in DMA (2 ml) containing 30 μmol of benzotriazolyloxy-tris-[*N*-pyrrolidino]phosphonium hexafluorophosphate and 60 μmol of *N*-methylmorpholine. The mixture was left to stand overnight at room temperature. Next, 20 μl of hydrazine hydrate was added to the reaction mixture. After the mixture stood for 30 min at room temperature, chloroform (5 ml) was added. The organic layer was washed with water (four times with 5 ml) and dried with anhydrous magnesium sulfate. The magnesium sulfate was removed by centrifugation (5 min, 4°C, 6,000 × *g*), and the organic layer was separated and concentrated to obtain the Lys-0 side chain-deprotected peptide.

Each peptide was then incubated for 2 h with a solution of 25 μmol of pentafluorophenyl *S*-acetylmercaptoacetate, 25 μmol of 1-hydroxybenzotriazole, and 25 μmol of *N*-methylmorpholine in 1 ml of DMA. Next, chloroform (5 ml) was added, and the solution was washed with water (four times with 5 ml). After the organic layer was concentrated, 1 ml of acetic acid-water (9:1, vol/vol) was added and all peptides were lyophilized. Side chain deprotection of the peptides [except of the N^{ε}-(*S*-acetylmercaptoacetyl)lysyl, Lys(SAMA) residue] was performed with trifluoroacetic acid-water (95:5, vol/vol) as described elsewhere (6).

The peptides were purified by semipreparative high-performance liquid chromatography (HPLC) as described earlier (6) and obtained in a yield of about 30%. The structural integrity of each peptide was confirmed by fast atom bombardment mass spectrometry (6). Mono-isotopic value of MH^+ found/calculated: peptide 3, 1,639.6/1,639.7; peptide 7, 1,741.0/1,740.8; peptide 12, 1,854.1/1,853.9; and peptide 13, 2,054.1/2,054.0.

Cyclic peptide-tetanus toxoid conjugates. Each purified cyclic *S*-acetylmercaptoacetyl peptide (~1 μmol) was dissolved in 50 μl of 10% sodium dodecyl sulfate in water and diluted with 200 μl of water. A solution of 4.2 mg (~28 nmol of protein, corresponding to ~1.1 μmol of bromoacetyl functions) of bromoacetylated tetanus toxoid (16) in 2.0 ml of 0.1 M sodium phosphate containing 5 mM EDTA, pH 6.0 (conjugation buffer), was added, followed by 25 μl of 2 M NH_2OH dissolved in the same buffer to effect *S*-deacetylation of the peptide. After 48 h, 150 μl of 2-aminoethanethiol solution (3.0 mg/ml in the conjugation buffer) was added. After a further period of 16 h, the conjugates obtained were purified by gel filtration using PD-10 columns (Pharmacia), equilibrated in phosphate-buffered (pH 7.2) saline.

Immunization experiments. On days 0, 14, and 42, groups of five BALB/c mice (20 to 24 g) were immunized with the preparations indicated in the legends to the figures. The final concentration of Quillaja saponin A (Quil A) in the vaccine was 40 μg/ml. $AlPO_4$ was used at a final concentration of 1 mg/ml. A volume of 0.5 ml containing 50 μg of peptide conjugate was injected subcutaneously (each groin, 250 μl). Groups 1 to 18 are numbered according to Fig. 1. Groups 3, 7, 12, and 13 were immunized with conjugates prepared from peptides synthesized as described in this paper, and remaining groups were immunized with conjugates prepared from peptides synthesized as described previously (6).

ELISA. The whole-cell enzyme-linked immunosorbent assay (ELISA) was performed as described elsewhere (1). In order to quantify antibodies recognizing a linear synthetic peptide, ELISA plates were incubated with an N^{α}-acetylated peptide amide (residues 177 to 196), derived from meningococcal strain H44/76 class 1 OMP (19), at a concentration of 20 μg/ml in a 0.04 M carbonate buffer at 37°C for 2 h. Immunoglobulin G (IgG) antibodies were detected as described previously (16).

Serum bactericidal assay. *Neisseria meningitidis* serogroup B strain H44/76 (B:15:P1.7,16:L3,8) and H44/76-derived mutant strain HI-5, lacking the class 1 OMP (15), were used in the serum bactericidal assay. The lyophilized strains were rehydrated with Mueller-Hinton broth (MHB), and a small sample of this suspension was inoculated onto several gonococcus-type (GC) agar plates containing 1% IsoVitaleX. After incubation overnight at 37°C in 5% CO_2, the colonies were harvested with a sterile polyester swab and suspended in MHB with 15% glycerol. Working seed lots of 1.5 ml were stored at −70°C. A sample of this working seed lot was tested for surface expression of class 1 OMP, lipo-oligosaccharide, and capsular polysaccharide by colony blotting. To eliminate phase variation, a fresh aliquot was plated on GC agar plates with IsoVitaleX for each day's experiment. A scrape was taken from the frozen seed lot and streaked onto a GC agar plate with IsoVitaleX. After incubation overnight at 37°C in 5% CO_2, colonies were harvested in 2 ml of sterile MHB. Approximately 100 to 200 μl of the stock bacterial cell suspension was added to 20 ml of sterile MHB preequilibrated at room temperature to yield an A_{620} of between 0.07 and 0.10. The culture flask was then incubated for approximately 1.5 h at 37°C with 160-rpm shaking until the A_{620} was between 0.23 and 0.29. This yielded approximately 10^9 CFU/ml. The bacterial cells were diluted in sterile 50 mM phosphate buffer (pH 7.2) containing 10 mM $MgCl_2$, 10 mM $CaCl_2$, and 0.5% (wt/vol) bovine serum albumin BSA (assay buffer) until a concentration of 10^5 CFU/ml was reached (bacterial working concentration).

All sera of mice to be tested were heat inactivated for 30 min at 56°C. Pooled sterile baby rabbit (3 to 4 weeks old; lot 11814; Pel Freeze Clinical Systems, Brown Deer, Wis.) serum, with no bactericidal activity against the strains to be tested, was used as a source of complement in this assay. A sterile polystyrene U-bottom 96-well microtiter plate was used for the serum bactericidal assay. The total volume in each well of the plate was 50 μl: 25 μl of serially diluted serum in assay buffer (starting dilution, 1:5), 12.5 μl of bacterial suspension, and 12.5 μl of complement (final concentration, 25%, vol/vol, in assay buffer). Controls samples were (i) buffer, bacteria, and complement; (ii) buffer and bacteria; and (iii) serum and bacteria. A known positive sample, i.e., monoclonal antibody MN5C11G (anti-P1.16), was included in each assay. After all components were added to each well of the plate, a 7-μl aliquot of the control samples with buffer, bacteria, and complement was plated onto a GC agar plate containing 1% IsoVitaleX. The microtiter plate was then incubated for 60 min at 37°C. The GC agar plate with 1% IsoVitaleX was incubated for 18 h at 37°C in 5% CO_2. After 1 h of incubation, a 7-μl aliquot was taken from each well of a lane by using a multichannel pipette and spotted onto a GC agar plate containing 1% IsoVitaleX. After 18 h of incubation at 37°C in 5% CO_2, the colonies on time zero and 60-min-incubation plates were counted (individual colonies could be counted up to 50 CFU per spot). The average number of CFU at time zero was used as 100%. The serum bactericidal titer is reported as the reciprocal value of the highest serum dilution which is effective for ≥50% killing.

RESULTS AND DISCUSSION

The class 1 protein of reference strain H44/76 (B:15:P1.7,16) was chosen as a model for the synthesis of cyclic peptides. The peptides are derived from the predicted apex of surface loop 4 which determines the P1.16 subtype (the P1.7 epitope is situated on loop 1). The core determinant of the P1.16 epitope comprises the residues TKDTNNN (11).

Initially, cyclic peptides of 7 to 14 amino acid residues were prepared by solid-phase head-to-tail cyclization between residues Asp-182 and Thr-183 (6). In this way peptides 1 to 11 and

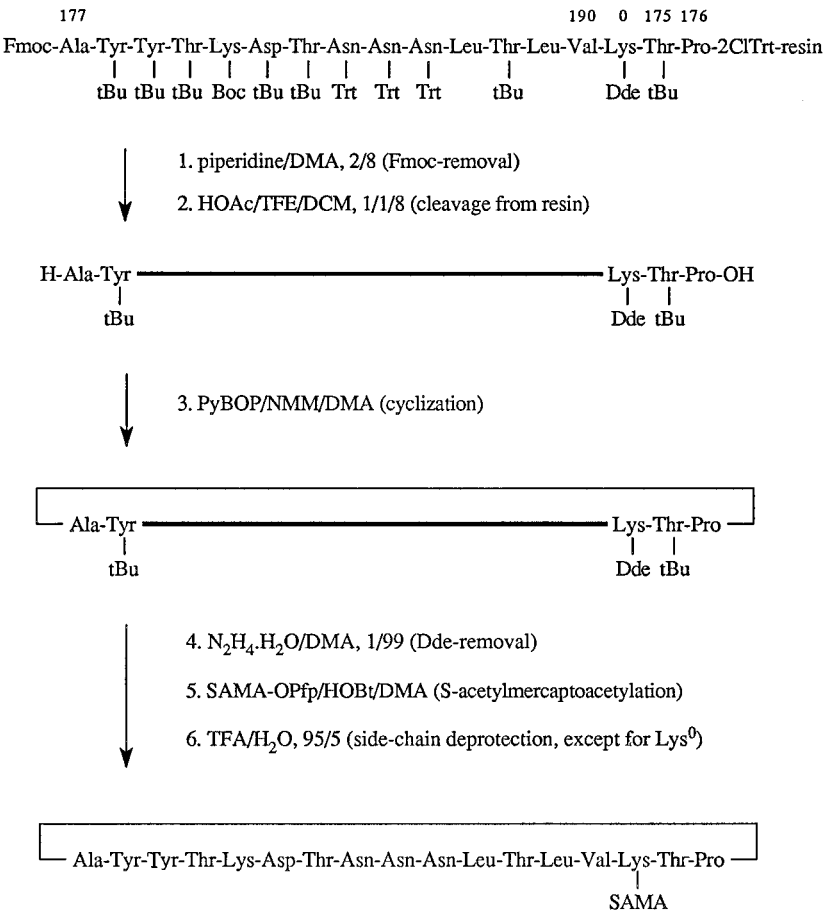

FIG. 2. Outline of the synthesis of cyclic peptide 13, cyclo[175-190–Lys-0(SAMA)]. Numbers of amino acid residues correspond to their positions in the class 1 outer membrane protein of meningococcal strain H44/76. Lys-0 is an added residue to enable selective conjugation of the cyclic peptide. Peptides 3, 7, and 12 were also prepared according to this scheme. Abbreviations: Boc, *tert*-butyloxycarbonyl; tBu, *tert*-butyl; DCM, dichloromethane; Dde, 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-ethyl; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; NMM, *N*-methylmorpholine; PyBOP, benzotriazolyloxy-tris-[*N*-pyrrolidino]phosphonium hexafluorophosphate; SAMA, *S*-acetylmercaptoacetyl; SAMA-OPfp, pentafluorophenyl *S*-acetylmercaptoacetate; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; Trt, triphenylmethyl.

14 to 18 (Fig. 1) were obtained. The peptides contain an added Lys(SAMA) residue, which enabled a selective conjugation with bromoacetylated tetanus toxoid (6) at a position located opposite the sequence which corresponds to the predicted top of the surface loop in meningococcal class 1 OMP. All peptides were purified by semipreparative HPLC and conjugated to bromoacetylated tetanus toxoid. When it became clear that the largest peptides, i.e., 3, 7, and 16, which were difficult to prepare by solid-phase cyclization, showed the most promising immunological properties (see below), a second strategy was developed for synthesis of larger cyclic peptides. In this strategy (Fig. 2), side chain-protected peptides were cleaved from a solid support; this was followed by cyclization in solution (20) between Pro-176 and Ala-177 and introduction of an *S*-acetylmercaptoacetyl group on the additional lysyl residue opposite the predicted top of the loop. Finally, the side chain protecting groups, except *S*-acetylmercaptoacetyl, were removed. Four cyclic peptides of 13 to 17 residues, i.e., 3, 7, 12, and 13 (Fig. 1), were prepared. After purification, these four cyclic peptides were also conjugated to bromoacetylated tetanus toxoid and used in immunological experiments.

Mice were immunized with the conjugates obtained by using Quil A or $AlPO_4$ as the adjuvant. Sera were collected for immunological evaluation in ELISAs and in vitro bactericidal assays. In ELISAs, titers of antipeptide antibodies or anti-whole-cell antibodies were determined. In the peptide ELISA, a linear synthetic peptide containing the P1.16 epitope sequence was used as the coating antigen. Figure 3A shows that almost all cyclic peptide conjugates, as well as outer membrane complexes (OMC) of meningococcal strain H44/76, elicited a significant IgG antipeptide titer.

In the whole-cell ELISA, strain H44/76 meningococci were used as the coating antigen. In this case it is assumed that antibodies recognizing the conformational epitope in the membrane-integrated protein will be detected predominantly. Whereas almost all cyclic peptide conjugates evoked an antipeptide titer, only a few were able to raise a significant IgG antibody titer against whole cells (Fig. 3B). With $AlPO_4$ as the adjuvant, the conjugates of peptides 7 and 13 elicited a titer against the conformational epitope. When Quil A was applied as the adjuvant, in addition marked responses to the conjugates of peptides 3, 12, and 16 were observed. High titers of conformation-directed antibodies were evoked by OMC preparations in the presence of either adjuvant. However, it should be noted that OMC contain about 10 to 20% bacterial lipopolysaccharide, which is also a potent adjuvant.

Pooled antisera elicited with the conjugate of cyclic peptides 7, 12, and 13, with Quil A as the adjuvant, induced comple-

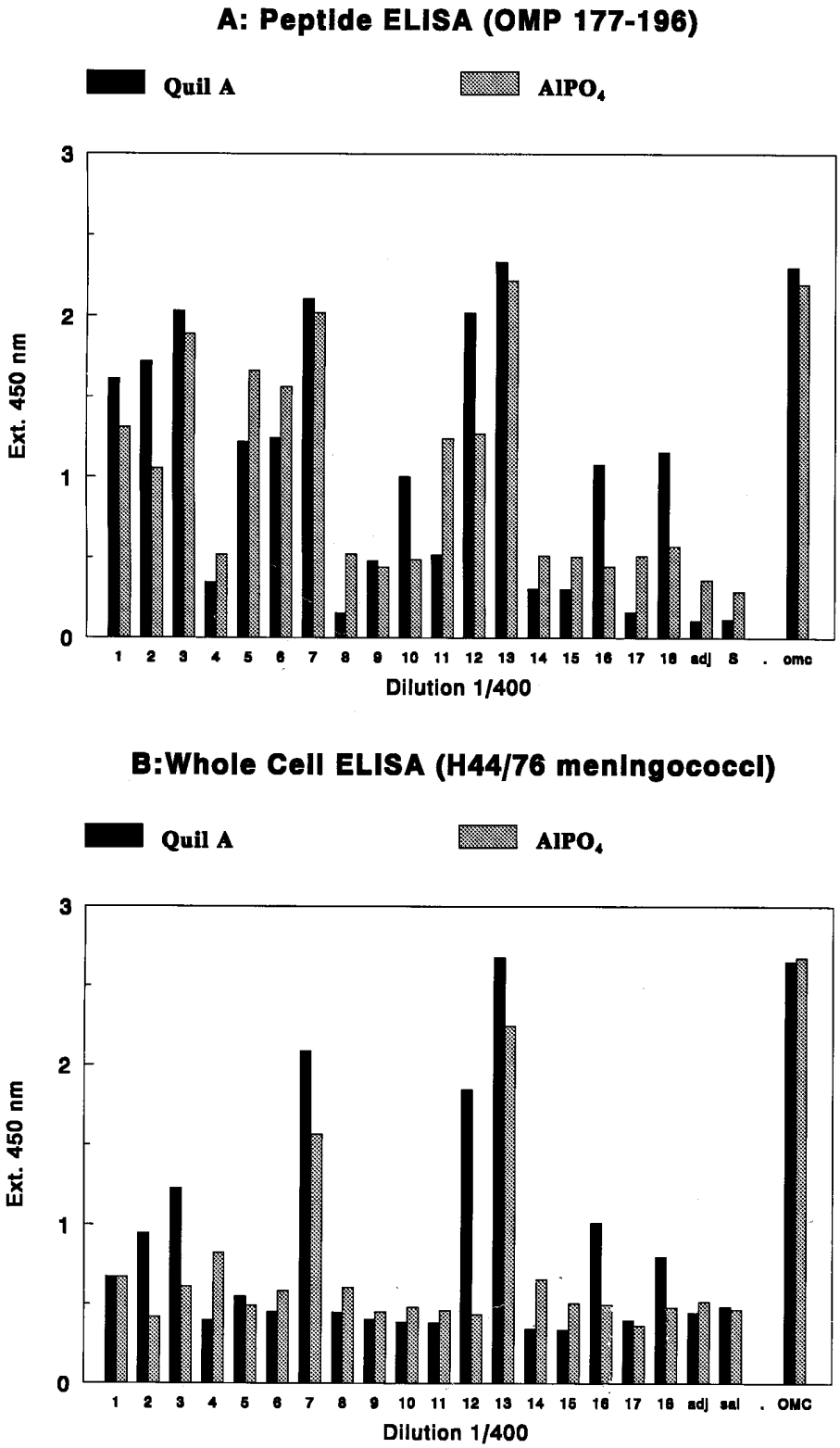

FIG. 3. IgG responses obtained with cyclic peptides derived from a meningococcal class 1 protein surface loop. BALB/c mice were immunized with the tetanus toxoid-conjugated cyclic peptides shown in Fig. 1. Negative control groups were immunized with adjuvant or saline only. A positive control group was immunized with OMC of meningococcal strain H44/76. Groups 1 to 18 are numbered according to Fig. 1. Groups 3, 7, 12, and 13 were immunized with conjugates prepared from peptides synthesized as described in this paper, and remaining groups were immunized with conjugates prepared from peptides synthesized as described previously (6). adj, adjuvant; S and sal, saline. (A) IgG responses in pooled sera obtained after immunization with the adjuvant Quil A or $AlPO_4$, as measured with the linear synthetic peptide OMP(177–196), i.e., A-Y-Y-T-K-D-T-N-N-N-L-T-L-V-P-A-V-V-G-K. The results obtained with a 400-fold dilution of the antisera are shown. (B) IgG responses in pooled sera, as measured by whole-cell ELISA with strain H44/76 meningococci (1, 16). Quil A or $AlPO_4$ was used as the adjuvant. In the antisera elicited with the cyclic peptides, no antibodies were detectable when an H44/76-derived mutant strain lacking the class 1 OMP (15) was used as the antigen in the ELISA (data not shown).

TABLE 1. Results of the bactericidal assay of sera elicited with tetanus toxoid conjugates of cyclic peptides 3, 7, 12, and 13 or with OMC[a]

Peptide conjugated with TT or OMC	Reciprocal dilution[b] of serum sample:				
	1	2	3	4	5
3	<5	<5	<5	<5	<5
7	<5	80	40	10	<5
12	<5	40	<5	<5	<5
13	640	160	320	160	80
OMC	5,120	2,560	640	2,560	1,280

[a] Peptides were obtained by the second synthetic method (see Fig. 2); OMC was from meningococcal strain H44/76.

[b] The reciprocal value of the highest serum dilution which is effective for ≥50% killing.

ment-mediated lysis of strain H44/76 meningococci in vitro. Specificity of the bactericidal activity of the antisera could be confirmed by using H44/76-derived mutant strain HI-5, lacking the class 1 OMP (15), as target cells in the bactericidal assay (data not shown). When individual sera were tested, it was found that conjugates 7 and 12 did not induce bactericidal antibodies in all mice. However, conjugate 13 consistently induced bactericidal antibodies in all immunized mice (Table 1).

The antisera elicited to conjugates 7 and 13, using $AlPO_4$ as the adjuvant, did not demonstrate bactericidal activity, although the sera reacted well with the conformational P1.16 epitope in the whole-cell ELISA. When the isotype of the anti-OMP antibodies present in these antisera was determined, it was found that the response consisted of IgG1 primarily (Fig. 4). With Quil A as the adjuvant, the same conjugates were found to elicit IgG2a and IgG2b antibodies as detected in the whole-cell ELISA. The latter sera were bactericidal in vitro. Apparently, Quil A influences the Ig isotypes that are elicited by the antigen, inducing a shift to IgG2a and IgG2b. Although this was not investigated, different patterns of cytokine induction are likely to be involved.

Differences in Ig isotype may explain, at least in part, the differences in bactericidal activity observed between sera elicited with cyclic peptides and OMC preparations. In the

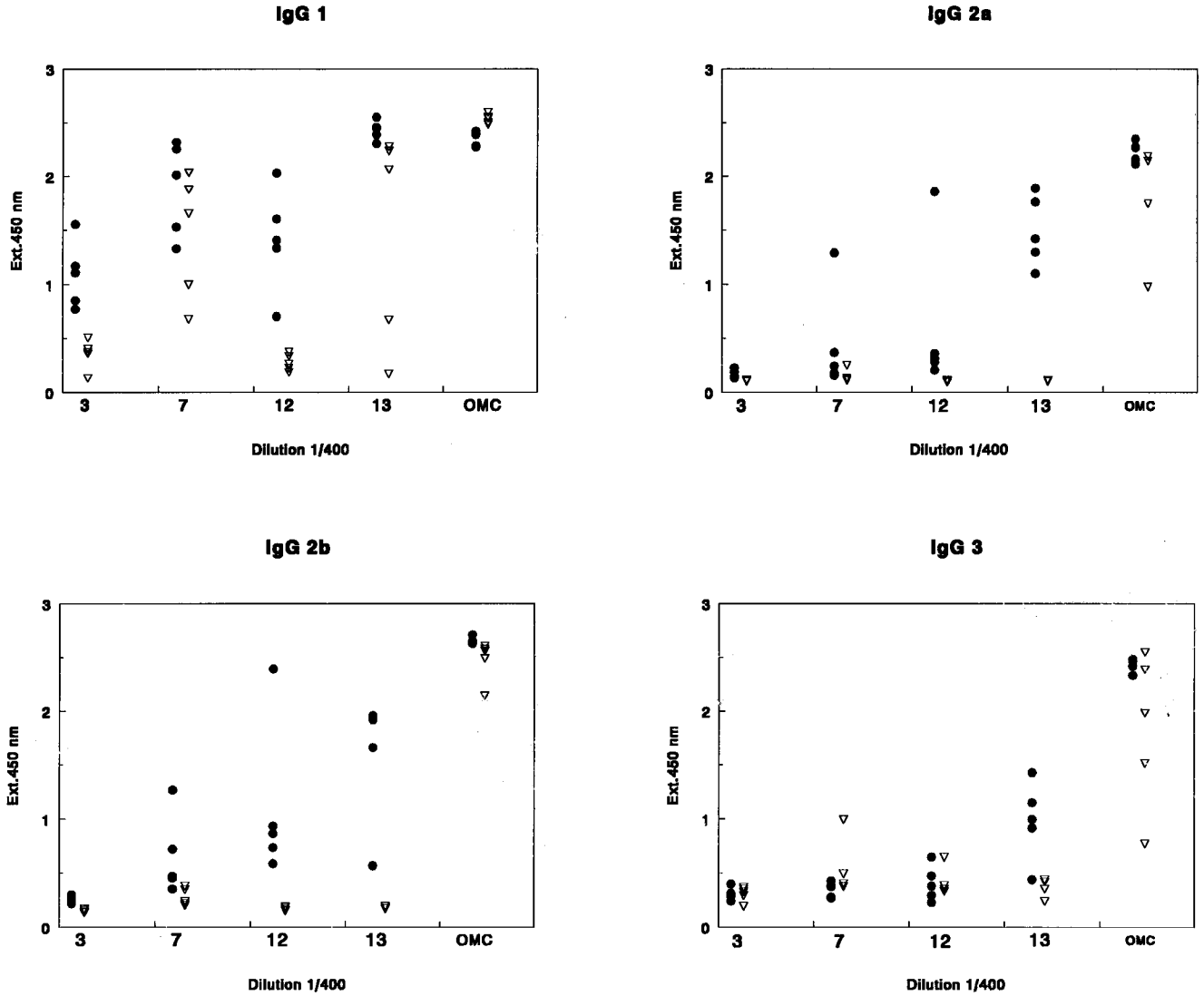

FIG. 4. IgG subclass responses in individual sera from groups 3, 7, 12, and 13, as well as the OMC group, as measured by whole-cell ELISA with strain H44/76 meningococci (1, 16). Quil A (●) or $AlPO_4$ (▽) was used as the adjuvant.

strongly bactericidal OMC antisera, a high titer of the complement-binding IgG2a and IgG2b isotypes was detected in the whole-cell ELISA. Unfortunately, the most predominant IgG isotype in mice during the humoral response to protein antigens is IgG1, which is less able to bind complement compared with other Ig subclasses. In humans, IgG1, IgG2, and IgG3 are the leading subclasses. These are the human Ig isotypes that bind complement most efficiently. Presumably, with conjugate 13, conformation-directed and bactericidal antibodies will be elicited in humans as well.

Our combined results prove that cyclic peptides containing 7 to 13 amino acid residues (i.e., peptides 1 to 6, 11, and 14 to 18) cannot mimic loop 4 of the class 1 OMP of meningococcal strain H44/76. The structures of cyclic peptides 7 and 12, containing 14 and 15 residues, respectively, seem to approach the structure of the loop in the native OMP. The consistent results obtained with the conjugate of cyclic peptide 13 (17 residues) indicate that this peptide mimics loop 4 of the protein to a great extent.

Future research will focus on theoretical as well as practical aspects. From a theoretical point of view it is of interest to understand the interaction of the peptides with (Fab fragments of) monoclonal antibodies at a molecular level. This requires conformational studies of peptides and peptide-Fab complexes using techniques like nuclear magnetic resonance spectroscopy, X-ray diffraction, and molecular modeling. Vaccine development studies will include the assembly of cyclic peptides derived from surface loop 1 or 4 of other meningococcal subtypes.

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