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Mucosal Immunogenicity of Polysaccharides Conjugated to a Peptide or Multiple-Antigen Peptide Containing T- and B-Cell Epitopes

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In this study we investigated the mucosal and systemic responses to two T-cell-independent polysaccharides, a serogroup f polysaccharide (formed of rhamnose glucose polymers [RGPs]) from *Streptococcus mutans* OMZ 175 and a mannan from *Saccharomyces cerevisiae*, covalently conjugated either to a linear peptide (peptide 3) or to a multiple-antigen peptide (MAP), both derived from *S. mutans* protein SR, an adhesin of the I/II protein antigen family of oral streptococci. Peptide 3 and MAP, which contained at least one B- and one T-cell epitope, were tested as carriers for the polysaccharides and as protective immunogens. Intragastric intubation of rats with the conjugates (RGPs-peptide 3, RGPs-MAP, mannan-peptide 3, and mannan-MAP) associated with liposomes produced salivary immunoglobulin A (IgA) antibodies which reacted with RGPs or mannan, peptide 3 or MAP, protein SR, and *S. mutans* or *S. cerevisiae* cells. Administration of conjugate boosters to the animals showed that both carriers conjugated to the polysaccharides were able to induce, in immunized animals, a salivary antipolysaccharide IgA memory. In contrast, animals primed and challenged with unconjugated polysaccharide showed no anamnestic response. Rats orally immunized with the conjugates also developed systemic primary antipolysaccharide and antipeptide IgM antibody responses which were characterized by a switch from IgM to IgG during the course of the secondary response. Data presented here demonstrated that both peptide 3 and the MAP construct can act as good carriers for orally administered polysaccharides. Unexpectedly, the use of a MAP did not further improve the immunogenicity of polysaccharides at the mucosal level; nevertheless, such a construct should be of great interest in overcoming the problem of genetic restriction induced by linear peptides.

There is at present much interest in the development of effective polysaccharidic vaccines against numerous invasive pathogens, since the major problems which have hindered their extensive development may now be bypassed. Indeed, polysaccharides are known to be poor immunogens, i.e., they induce a weak and short protection on account of their T-cell independence and consequently their failure to induce a memory. A successful way to improve the immunogenicity of polysaccharides is to conjugate them to proteins (23), and different carriers, such as tetanus toxoid, diphtheria toxoid, and diphtheria cross-reacting material 197 are presently being used in humans (2, 3, 11). These proteins are usually chosen because they have been employed for many years without untoward side effects. However, recent studies by Peeters et al. (21) and Barington et al. (4, 5) have focused attention on a potential negative effect of prior exposure to the carrier protein which could suppress antibody response to the polysaccharide administered in a polysaccharide-protein conjugate. Although this problem remains controversial (4) and needs further study, one way of circumventing the induction of this epitopic suppression could be to use a protein derived from the same microorganism to serve in the dual roles of carrier for the polysaccharide and protective immunogen. This was demonstrated by Wachsmann et al. (29) in earlier studies in which conjugation of a serotype polysaccharide to a protein (SR), both of which originate from *Streptococcus mutans* OMZ 175 and are implicated in the bacterial virulence, induced an anamnestic response against the polysaccharide and the protein; these results have recently been confirmed by Madoff et al. (16). Furthermore, as within protein SR, irrelevant determinants giving rise to autoantibodies (1) hindered the use of the whole protein; an SR-related immunogenic peptide, peptide 3 (13), containing B- and T-cell epitopes (14) was used instead of the SR protein and was successful in improving an anamnestic antipolysaccharide and antipeptide immune response (15). In the latter experiments, conjugates were administered by the parenteral route and primarily elicited systemic immunity.

Little information has been obtained about the mucosal immune response and immunological memory against polysaccharides conjugated to peptides. Most studies on the immunogenicity of polysaccharides at the mucosal level were performed with protein carriers, and our previous experiments (8) using such a conjugate demonstrated the efficacy of liposome-associated polysaccharide-protein conjugate to induce a local antipolysaccharide and antiprotein immunoglobulin A (IgA) memory after oral immunization.

The aim of this study was to evaluate, at the mucosal level, the immunogenicity and the carrier properties of SR-derived peptides devoid of epitopes that elicited autoimmune response. In these experiments, the previously used serotype polysaccharide from *S. mutans* OMZ 175 (22) was conjugated either to the linear peptide 3 or to a multiple-antigen peptide (MAP) construct containing eight copies of an SR-derived peptide. The MAP system (18, 25, 27), which was based on macromolecular structures composed of branched peptides

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supported by a minor oligomeric inert lysine core, may present advantages, mainly because of the signal amplification resulting from the multiple peptide copies. Moreover, the efficacy of peptides as carriers was tested by using mannan from *Saccharomyces cerevisiae* as an alternative polysaccharide. Local and systemic responses were analyzed after oral administration of the different conjugates associated with liposomes.

MATERIALS AND METHODS

Animals. Male randomly outbred Wistar rats (8 to 12 weeks old) bred in our own facilities were used for B- and T-cell epitope determination and for immunization studies with glycopeptides.

Bacterial strains and antigen preparation. *S. mutans* OMZ 175 (serogroup f) cells were grown in the D1-20 synthetic medium of Carlsson (10). The polysaccharide antigen of *S. mutans* OMZ 175, formed of rhamnose glucose polymers (RGPs), has already been described (26) and was purified according to the method of Benabdelmoumene et al. (6). Mannan from *S. cerevisiae* was obtained from Sigma Chemical Co. (St. Louis, Mo.) and treated as previously described (15). Recombinant protein SR (rSR) was purified from pHB*sr*-1-transformed *Escherichia coli* cell extract (20) as previously described (1). Peptide 3 (YEKEPTPPTRTPDQ), which was derived from the sequence of the *sr* gene from *S. mutans* OMZ 175 and which corresponded to the center of each proline-rich repeat (amino acids 865 to 878, 904 to 917, and 943 to 956), was already described by Gangloff et al. (13). The MAP construct contained a polylysine core matrix bearing eight radially branched copies of peptide VAPNYEKEPT corresponding to amino acids 861 to 870 of the first proline-rich repeat of protein SR and was organized as follows: $(VAPNYEKEPT)_8$-$(Lys)_4$-$(Lys)_2$-$CONH_2$. Free peptide 3, peptide 3 conjugated to ovalbumin (peptide 3-ovalbumin), and MAP were synthesized and purified by Neosystem (Strasbourg, France).

Antiserum production. Rabbit anti-peptide 3-ovalbumin IgG and anti-MAP IgG and rat antisera against rSR, peptide 3, MAP, RGPs, and mannan were prepared as previously described (13, 15). Sera were tested for the presence of antibodies by an indirect enzyme-linked immunosorbent assay (ELISA) by using plates coated with the appropriate antigens. Alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG and AP-conjugated goat anti-rat Ig, IgA, IgG, and IgM were purchased from Immunotech (Marseille, France).

Determination of T-cell epitope(s). The proliferative T-cell response to MAP was studied in rats immunized with either MAP (200 μg) or rSR (100 μg), as previously described for peptide 3 by Lett et al. (15). Briefly, 50 μl of inguinal lymph node cells from immunized animals was suspended in 96-well plates (4×10^5 cells per well) and mixed with 50 μl of RPMI 1640 supplemented with 4 mM L-glutamine, penicillin (200 IU/ml), and streptomycin (200 μg/ml) in the presence of final concentrations of MAP ranging from 0.005 to 50 μM. After 24 h at 37°C under 5% CO_2, 100 μl of RPMI 1640 supplemented with 1% normal rat serum was added to the wells. The cultures were incubated again for 48 h under the same conditions and then pulsed with 1 μCi (1 mCi = 37 MBq; specific activity, 28 Ci/mmol) of [^{3}H]thymidine (Amersham, Les Ulis, France). Titrations were performed in triplicate, and the results were expressed as the stimulation index, which corresponds to the ratio of counts per minute of cells with MAP to counts per minute of cells without MAP.

Conjugation of polysaccharides with peptide 3 or MAP. RGPs-peptide 3, RGPs-MAP, mannan-peptide 3, and mannan-MAP conjugates were prepared as previously described by Lett et al. (15) according to the method of Sanderson and Wilson (24). Briefly, 50 mg of $NaIO_4$-oxidized RGPs and mannan were covalently coupled through reductive amination to 10 mg of either peptide 3 or MAP. Conjugates were separated from free peptide by dialysis and from unconjugated polysaccharides by chromatography on DEAE Trisacryl M. On the basis of the results of hexose and peptide determinations, and assuming molecular sizes of 60,000 Da for RGPs and 600,000 Da for mannan (15), the molecular ratios of RGPs to peptide 3 and to MAP in the glycopeptides were 1/5 and 1/25, respectively. For the mannan-peptide 3 and mannan-MAP conjugates, the ratios were 1/50 and 1/30, respectively.

Immunization schedule. The methods used to prepare antigen-associated liposomes and to evaluate the amount of antigen associated with liposomes have been previously described (28). Eight groups of six rats were immunized by gastric intubation on four successive days with 0.5 ml of the appropriate immunogen diluted in phosphate-buffered saline (PBS) as follows: RGPs-peptide 3 (68.5 and 80 μg, respectively) plus liposomes, RGPs-MAP (165 and 80 μg, respectively) plus liposomes, mannan-peptide 3 (190 and 80 μg, respectively) plus liposomes, mannan-MAP (85 and 80 μg, respectively) plus liposomes, RGPs (165 μg) plus liposomes, mannan (190 μg) plus liposomes, peptide 3 (80 μg) plus liposomes, and MAP (80 μg) plus liposomes. Saliva and blood samples were harvested every 3 days from day 7 to day 25 and every 5 days from day 25 to day 55. Following the first round of intubations, rats were given boosters on days 55 and 56 with the same amount of antigen, and blood and saliva samples were harvested every 3 days from day 58 to day 70 and every 5 days from day 70 to day 90. Saliva samples were collected after the injection of 1 mg of pilocarpine. Blood samples were obtained by retroorbital punction. All the samples were individually tested for the presence of antipolysaccharide, antipeptide, anti-MAP, anti-rSR, and anti-whole-cell antibodies.

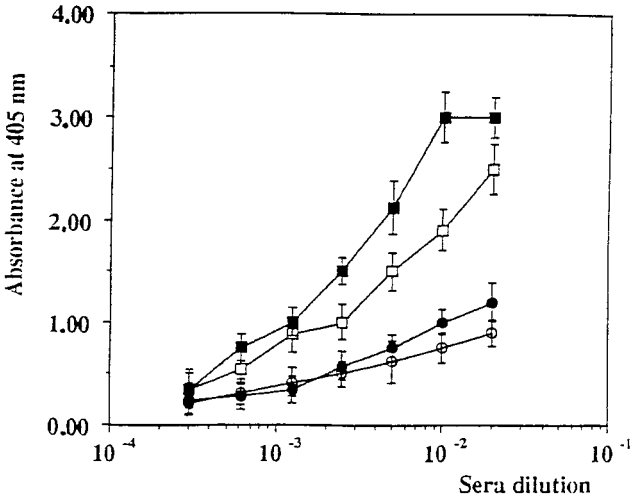

FIG. 1. Reactivity of anti-rSR antibodies with rSR (■) and MAP (●) and reactivity of anti-MAP antibodies with MAP (□) and rSR (○). The binding activity is expressed as the arithmetic mean of A_{405} of triplicate determinations.

ELISA procedures. The presence of IgA, IgG, and IgM antibodies to RGPs, mannan, peptide 3, MAP, protein rSR, and whole *S. mutans* and *S. cerevisiae* cells in saliva and sera was determined by an indirect ELISA as previously described (15). Briefly, microtiter plates (Nunclon; Poly Labo, Strasbourg, France) were coated with 50 μl of either RGPs (100 μg/ml), mannan (50 μg/ml), peptide 3 (1 μg/ml), MAP (1 μg/ml), protein rSR (1 μg/ml), *S. mutans* whole cells (10^9 bacteria per ml), or *S. cerevisiae* whole cells (10^9 yeast cells per ml) in 0.1 M carbonate buffer (pH 9.6). After overnight incubation at 4°C, the plates were blocked with PBS containing 0.05% Tween 20 (PBST) and 0.5% gelatin. Fifty microliters of serial dilutions of each individual serum or saliva sample was added to the wells (1 h, 37°C). After a washing with PBST, antibody binding was detected with 50 μl (1/5,000) of either AP-conjugated goat anti-rat IgM, AP-conjugated goat anti-rat IgG, or AP-conjugated goat anti-rat IgA (1 h, 37°C). After incubation (1 h, 37°C) with 50 μl of AP substrate (*p*-nitrophenylphosphate), the A_{405} was read with an Anthos Labtec spectrophotometer. Each assay was run in triplicate simultaneously with preimmune saliva and preimmune sera. Antibody titers were expressed as the reciprocal (in $\log_2$ units) of the highest saliva or serum dilution which gave an absorbance twice that of the preimmune control.

For the determination of the B-cell epitope(s), the presence of anti-MAP antibodies in the sera of rats immunized with either rSR or MAP was assessed as described above, by using plates coated with MAP. Covalent linkage between RGPs or mannan and peptide 3 or MAP was checked by a heterologous two-site sandwich ELISA procedure as previously described by Wachsmann et al. (29).

Analytical methods. The peptide content of each conjugate was determined by the method of Micro BCA (Pierce, Rockford, Ill.) with bovine serum albumin as the standard. Total hexose was assayed by the resorcinol-sulfuric acid method of Monsigny et al. (17) with glucose or mannose as the standard.

RESULTS

Determination of B- and T-cell epitopes on MAP. In a previous study (15), we demonstrated that peptide 3, which was derived from the sequence of SR protein, possessed at least one B-cell epitope and one T-cell epitope. By using PEP scan ELISA, it was shown that the B-cell epitope was represented by the sequence YEKE (unpublished data). We then confirmed (Fig. 1) that the MAP construct, which contained the same B-cell epitope as peptide 3, was able to induce, in rats, specific antibodies which reacted in ELISA with both MAP and native rSR antigen but did not recognize the small lysine core matrix or unrelated MAPs (data not shown). Figure 1 also shows that rat anti-rSR antibodies reacted with the peptide-dense MAP, demonstrating that the protein sequence used in this construct is accessible on the SR protein. From these results we can conclude that MAP, like peptide 3, is capable of

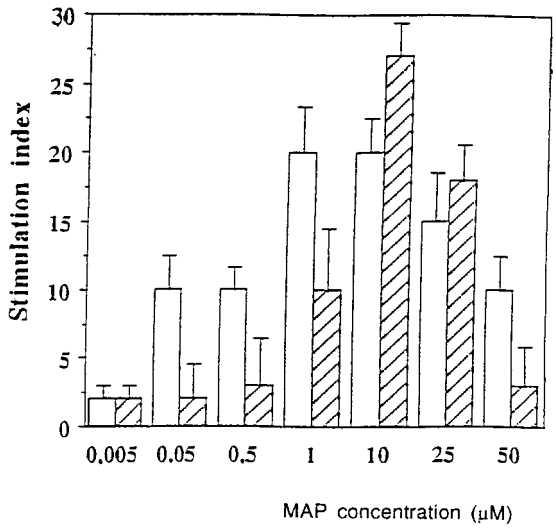

FIG. 2. T-cell-proliferative response to MAP of rats primed and challenged with MAP (□) or primed with rSR and challenged with MAP (▨). The T-cell response to MAP was assessed by [^{3}H]thymidine incorporation. Results are stimulation index values and correspond to the means (plus standard deviations [error bars]) of triplicate determinations from three individual animals. Medium background for each group of immunized animals ranged from 350 to 450 cpm and was subtracted.

inducing a peptide-specific antibody response indicating the presence of B- and probably T-cell epitopes. Furthermore, the presence of the T-cell epitope(s) was confirmed by the fact that MAP was able to induce in vitro proliferation of lymph node cells from rats primed with either MAP or rSR. The results in Fig. 2 show that significant proliferation could be induced with MAP at doses above 0.005 μM, peaking at 1 μM, and that challenge with MAP after priming with rSR induced a similar proliferative response which peaked at 10 μM, corroborating the presence of a T-cell epitope(s) in MAP. Lymph node cells from rats immunized with PBS and incomplete Freund adjuvant showed no proliferative response above the background level (data not shown), indicating that MAP does not activate rat T cells polyclonally. Taken together, these data indicate that MAP, like peptide 3, elicits a T-cell response, suggesting that MAP corresponds to a T-cell recognition site which is also recognized on the full-length rSR molecule.

Primary salivary antibody response to immunization with polysaccharide-peptide conjugates. Rats were immunized by gastric intubation with the appropriate antigens (RGPs-peptide 3, RGPs-MAP, mannan-peptide 3, mannan-MAP, RGPs, mannan, peptide 3, or MAP) associated with liposomes, and saliva samples from individual animals were screened periodically by ELISA from day 7 to day 55 for the isotype distribution of antibodies reactive against (i) RGPs or mannan, (ii) peptide 3 or MAP, (iii) rSR, and (iv) whole *S. mutans* or *S. cerevisiae* cells. Figure 3 shows the kinetics of the IgA antibody response in the saliva of rats primed with the two RGP conjugates (RGPs-peptide 3 and RGPs-MAP). Both conjugates induced a similar strong anti-RGP IgA response in saliva (Fig. 3A) superior to the response obtained after priming with unconjugated RGPs (Fig. 3A). Rats immunized with the two conjugates also exhibited a strong IgA response in saliva against peptide 3 or MAP, responses which are equivalent to those obtained with unconjugated antigen (Fig. 3B). All responses declined over a 30-day period (Fig. 3B). Moreover, saliva from rats immunized with RGPs-peptide 3 or RGPs-MAP contained antibodies which reacted with rSR (Fig. 3C) and whole *S. mutans* cells (Fig. 3D). Specific IgG and IgM antibodies were never detected in immune saliva. A similar pattern of antibody distribution and reactivity was observed in rats primed with mannan-peptide 3 or mannan-MAP (Fig. 4). These conjugates induced salivary IgA antibodies that were reactive with mannan (Fig. 4A), peptide 3, MAP (Fig. 4B), rSR (Fig. 4C), and *S. cerevisiae* cells (Fig. 4D).

Secondary salivary antibody response to immunization with polysaccharide-peptide conjugates. We then evaluated the ability of peptide 3 and MAP, conjugated to either RGPs or mannan, to induce a local secondary antibody response. The previously immunized rats were challenged by gastric intubation with the same amount of appropriate antigens on days 55 and 56. Administration of RGPs-peptide 3 or RGPs-MAP boosters to the animals (Fig. 3) showed that both peptides conjugated to this polysaccharide were able to induce, in immunized animals, a similar salivary anti-RGP (Fig. 3A) IgA secondary response, which occurred very quickly and peaked 7 days after the last booster. In contrast, animals primed and challenged with unconjugated polysaccharide showed no salivary IgA memory (Fig. 3A). Concerning the antipeptide response, we noted a similar secondary anti-peptide 3 or anti-MAP IgA response in saliva (Fig. 3B). Moreover, IgA antibodies produced after boosters were given reacted with protein rSR (Fig. 3C) and whole *S. mutans* cells (Fig. 3D). Similar results were obtained with peptide 3 and MAP conjugated to mannan (Fig. 4). Antipolysaccharide and anticarrier IgG and IgM were never detected in saliva during the secondary response.

Primary and secondary serum response to immunization with polysaccharide-peptide conjugates. Isotypes and titers of antipolysaccharide, anti-peptide 3, anti-MAP, anti-rSR, and anti-whole-cell antibodies were determined in sera from rats orally immunized with the different conjugates associated with liposomes. Oral priming of rats induced a strong anti-RGPs IgM response in serum, as shown in Table 1, with titers (expressed as $\log_2$ units) reaching 14 when either peptide 3 or MAP was used as a carrier, titers which are superior to those obtained with unconjugated RGPs. Elevated levels of anti-peptide 3 or anti-MAP IgM antibodies were also detected in sera, and unconjugated peptides gave similar responses (data not shown). Furthermore, immune sera reacted with rSR or whole *S. mutans* cells. Anti-peptide 3, anti-MAP, and anti-RGPs IgG and IgA antibodies were never detected in immune sera during the course of the primary response.

Isotypes and antibody titers of sera from animals given boosters of the same amounts of appropriate antigens are also depicted in Table 1. Challenge of rats with RGPs conjugated either to peptide 3 or to MAP induced an anamnestic response against the polysaccharide, a response which was never obtained in animals that were given boosters of unconjugated RGPs. Table 1 shows that high titers of anti-RGPs IgG and low titers of IgM antibodies were elicited. Furthermore, high levels of anti-peptide 3 and anti-MAP IgG antibodies comparable to those obtained with unconjugated peptide 3 or MAP were measured (data not shown). Antibodies produced after challenge with the conjugates also reacted with rSR and whole *S. mutans* cells. IgA antibodies were never detected in sera during the course of the secondary response. Conjugation of both peptides to mannan led to the same results (Table 1).

DISCUSSION

Following the demonstration that an immunological memory against RGPs from *S. mutans* or mannan from *S. cerevisiae* can be induced by an SR-derived peptide covalently conju-

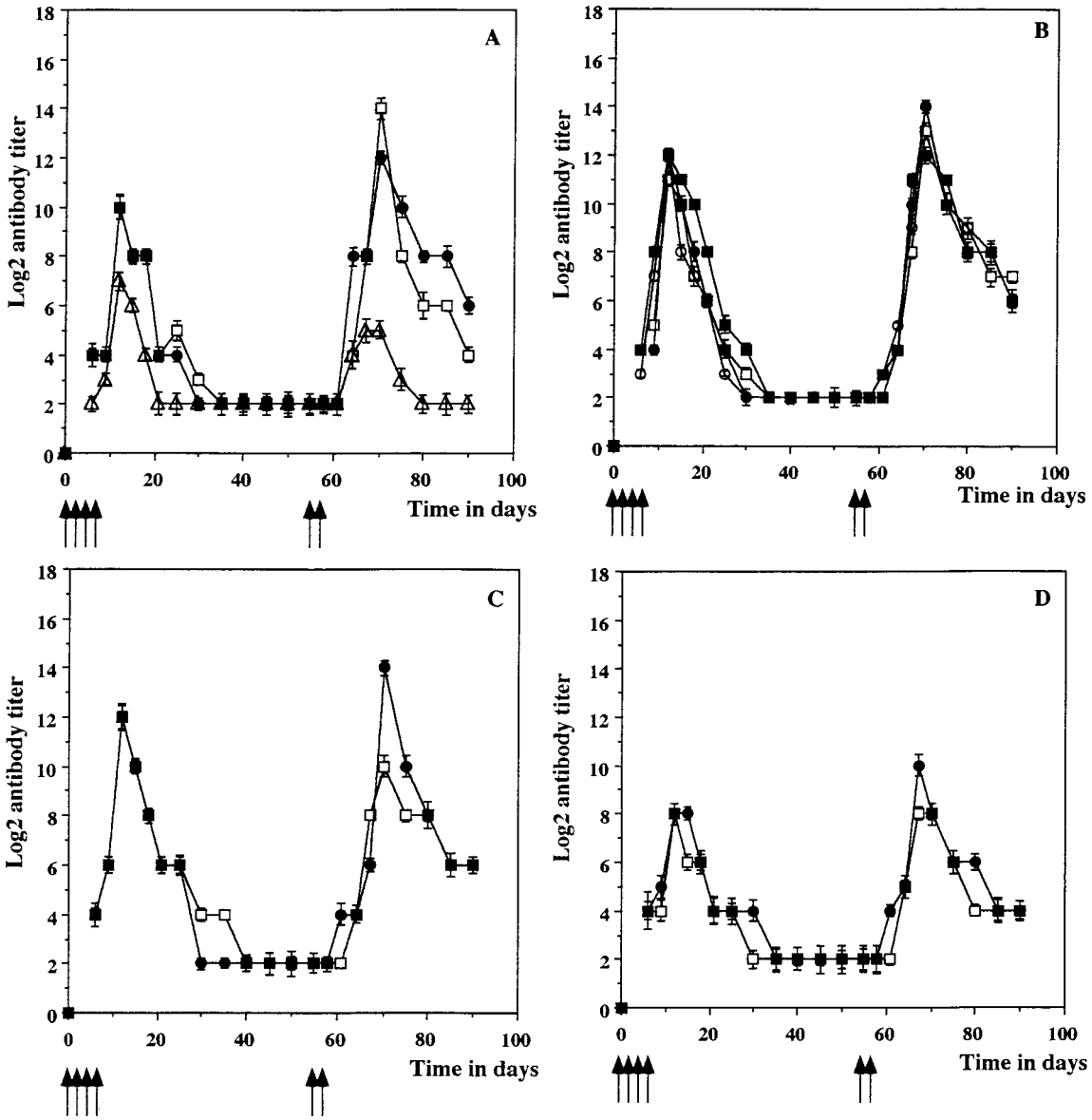

FIG. 3. Saliva IgA profiles of the antibody responses against RGPs (A), peptide 3 or MAP (B), rSR (C), and whole *S. mutans* cells (D) induced in rats after gastric intubation with either RGPs-peptide 3 (□), RGPs-MAP (●), unconjugated peptide 3 (■), unconjugated MAP (○), or unconjugated RGPs (△), all associated with liposomes. Saliva samples drawn periodically for the primary response (days 7, 10, 13, 16, 19, 22, 25, 30, 35, 40, 45, 50, and 55) and the secondary response (days 58, 61, 64, 67, 70, 75, 80, 85, and 90) were tested individually at serial dilutions for the presence of specific IgA antibodies. Antibody titers were expressed as the reciprocal (in $\log_2$ units) of the highest saliva dilution which gave an absorbance twice that of the control. The data are the means ± standard deviations (error bars) of triplicate determinations from the saliva samples of six rats.

gated to these polysaccharides, peptides were also tested as possible carrier candidates and as immunogens at the mucosal level. The present investigation was carried out with the previously used linear peptide, peptide 3, along with an octameric MAP construct including the B-cell immunodominant epitope YEKE, which is also present in peptide 3.

Earlier studies in our laboratory (15) showed that an anamnestic response against polysaccharides and peptides could be induced after immunization with a peptide containing T- and B-cell epitopes but not with a peptide containing only a B-cell epitope. Therefore, we first analyzed the MAP construct for the presence of T-cell epitope(s) by monitoring the anti-MAP humoral and cellular responses in rats. This report demonstrates that MAP is strongly immunogenic in rats and induces an in vitro proliferative response of lymph node cells from rats immunized with MAP, confirming that T cells recognize MAP. We also showed that lymphocytes primed with SR recognize the T-cell epitope(s) incorporated into MAP and that anti-SR IgG binds to MAP. These results clearly indicate that MAP contains not only a B-cell epitope(s) but also a T-cell epitope(s) present on the native protein SR.

Gastric intubation of rats with the different antigens was performed, keeping the dose of peptide constant in order to determine the conjugates' ability to induce primary and secondary antibody responses to peptide, MAP, and polysaccharides. It is generally admitted that oral challenge with soluble antigens induces tolerance rather than mucosal immunity, and many approaches have been developed for the conversion of

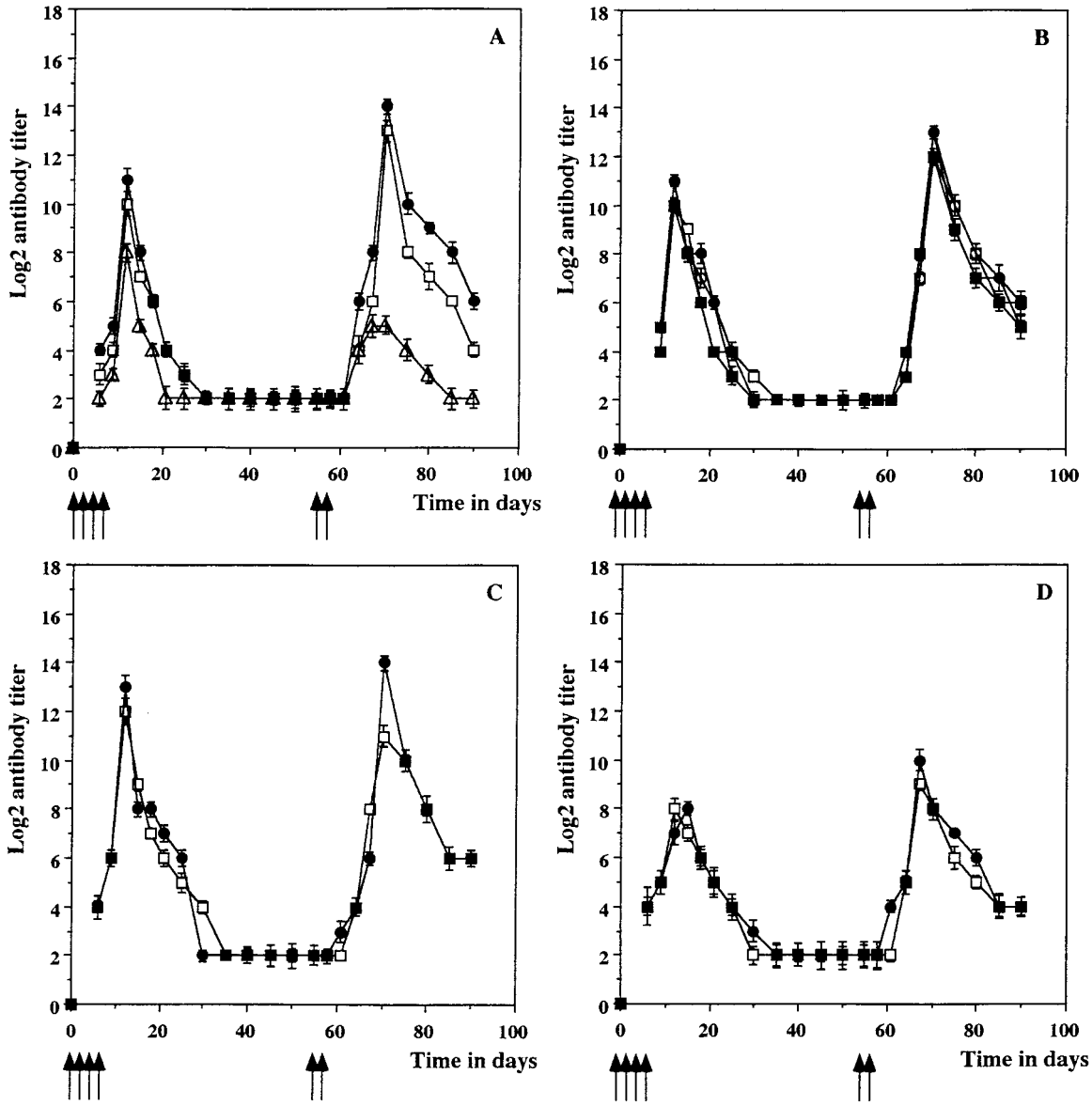

FIG. 4. Saliva IgA profiles of the antibody responses against mannan (A), peptide 3 or MAP (B), rSR (C), and whole *S. cerevisiae* cells (D) induced in rats after gastric intubation with either mannan-peptide 3 (□), mannan-MAP (●), unconjugated peptide 3 (■), unconjugated MAP (○), or unconjugated mannan (△), all associated with liposomes. Saliva samples drawn periodically for the primary response (days 7, 10, 13, 16, 19, 22, 25, 30, 35, 40, 45, 50, and 55) and the secondary response (days 58, 61, 64, 67, 70, 75, 80, 85, and 90) were tested individually at serial dilutions for the presence of specific IgA antibodies. Antibody titers were expressed as the reciprocal (in $\log_2$ units) of the highest saliva dilution which gave an absorbance twice that of the control. The data are the means ± standard deviations (error bars) of triplicate determinations from the saliva samples of six rats.

soluble antigens into particulate forms (28). As part of this study, all the antigens were associated with liposomes. Anamnestic anti-peptide 3 and anti-MAP IgA responses in saliva and IgG response in sera were demonstrated in rats orally immunized with either the conjugates or the unconjugated peptides associated with liposomes. These findings suggest that an immune response against peptide 3 or MAP at the mucosal level can be achieved with peptide 3 or MAP, either conjugated or not, associated with liposomes. Results obtained with the MAP construct are in agreement with those recently published by Nardelli et al. (19), who obtained humoral and cellular responses with an orally administered tetrameric MAP containing a gp120 peptide linked to the lipid moiety tripalmitoyl-*S*-glyceryl cysteine (P3C). Moreover, anti-MAP antibodies also bound to SR protein. This is in contrast to data obtained from other workers (7), who considered the value of MAPs for raising antipeptide antibodies that are cross-reactive with the cognate protein much more limited. However, this result could be attributed to the fact that recognition of the native protein probably depends greatly on the sequence and the stoichiometry of the MAP.

Immunization of rats with the conjugates induced a primary antipolysaccharide response higher than that obtained with unconjugated RGPs associated with liposomes. The response was principally of the IgA isotype in saliva and of the IgM isotype in sera. An important observation was that rats given boosters of glycoconjugates exhibited a strong secondary IgA response in saliva and IgG response in sera against RGPs. Rats

TABLE 1. IgM and IgG titers in antibody responses against various antigens induced in immunized rats[a]

Antibody	Immunizing antigen(s)	Titer[b] of antibody to the following coated antigen:							
		Peptides		Polysaccharides		rSR		Whole cells	
		I	II	I	II	I	II	I	II
IgM	RGPs			6.44 ± 0.44	6.50 ± 0.54			6.21 ± 0.39	5.15 ± 0.49
	RGPs-peptide 3	17.15 ± 0.61	4.50 ± 0.54	13.55 ± 0.54	8.44 ± 0.44	10.41 ± 0.34	6.21 ± 0.24	8.05 ± 0.53	6.12 ± 0.32
	RGPs-MAP	15.85 ± 0.54	4.31 ± 0.34	13.85 ± 0.25	7.54 ± 0.52	9.65 ± 0.54	4.32 ± 0.36	8.01 ± 0.54	4.12 ± 0.22
	Mannan			7.32 ± 0.47	6.99 ± 0.33			7.41 ± 0.55	6.12 ± 0.32
	Mannan-peptide 3	11.65 ± 0.75	2.16 ± 0.21	9.95 ± 0.48	6.15 ± 0.46	10.19 ± 0.61	4.06 ± 0.58	8.32 ± 0.64	6.02 ± 0.21
	Mannan-MAP	11.75 ± 0.54	2.25 ± 0.21	10.03 ± 0.42	5.50 ± 0.51	8.16 ± 0.32	2.16 ± 0.22	8.41 ± 0.42	5.99 ± 0.32
IgG	RGPs			1.82 ± 0.20	2.21 ± 0.30			2.26 ± 0.40	1.94 ± 0.43
	RGPs-peptide 3	2.10 ± 0.10	11.45 ± 0.70	2.15 ± 0.22	14.56 ± 0.53	1.95 ± 0.10	9.85 ± 0.34	2.18 ± 0.20	7.65 ± 0.53
	RGPS-MAP	2.25 ± 0.20	9.85 ± 0.80	1.95 ± 0.25	11.52 ± 0.60	1.85 ± 0.22	10.45 ± 0.70	1.85 ± 0.10	9.74 ± 0.73
	Mannan			1.98 ± 0.22	2.15 ± 0.53			1.90 ± 0.21	2.10 ± 0.53
	Mannan-peptide 3	1.89 ± 0.22	7.98 ± 0.38	2.05 ± 0.23	10.35 ± 0.61	1.94 ± 0.34	8.40 ± 0.55	2.41 ± 0.28	7.84 ± 0.54
	Mannan-MAP	1.85 ± 0.83	8.26 ± 0.43	2.31 ± 0.25	9.50 ± 0.52	2.21 ± 0.54	10.36 ± 0.71	1.60 ± 0.22	8.40 ± 0.54

[a] Rats were immunized with conjugated and single antigens, all associated with liposomes, and primary (I) and secondary (II) antibody responses were measured.
[b] Antibody titers are expressed as the reciprocal (in $\log_2$ units) of the highest serum dilution which gave an absorbance twice that of the control. The data are the means ± standard deviations of triplicate determinations from the sera of six rats.

given unconjugated polysaccharide boosters failed to develop any secondary antibody response. Taken together, these results show that peptide 3 and MAP are immunogenic and act as carriers for T-cell-independent antigens. These results were confirmed with another polysaccharide, mannan from *S. cerevisiae*, which has been implicated as a key component in many bacterial, fungal, and viral infections. Furthermore, the antibody induced by the four conjugates reacted with either *S. mutans* or *S. cerevisiae* whole cells, making such conjugates attractive for developing both antiprotein and antipolysaccharide antibodies at the mucosal level.

One of the purposes of this study was to test the efficacy of a MAP construct with regard to a linear peptide, since Francis et al. (12) showed that presentation of multiple copies on a polylysine backbone can further enhance the immune response. We found that identical levels of antipolysaccharide antibodies were induced by the two carriers, showing that the use of MAP does not seem to further improve the immunogenicity of polysaccharides at the mucosal level. Nevertheless, recent work by Calvo-Calle et al. (9) suggested that a high degree of protection against challenge with *Plasmodium berghei* sporozoites was obtained in mice by immunization with MAP containing multiple copies of the immunodominant B epitope and selected Th epitopes, which are recognized by T cells with different genetic restrictions. In this way, the MAP could be of great interest for the development of a general model of vaccine insofar as bacterially derived Th epitopes with different genetic restrictions could be introduced in the construct, overcoming the problem of genetic restriction induced by linear peptides.

Further experiments are now being done with animals of different major histocompatibility complex haplotypes to confirm the efficiency of glycopeptides with MAP as safe vaccines against several infectious diseases.

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REFERENCES

1. **Ackermans, F., A. Pini, D. Wachsmann, M. Schöller, J. A. Ogier, and J. P. Klein.** 1991. Anti-IgG antibodies in rheumatic diseases cross-react with *Streptococcus mutans* SR antigen. Clin. Exp. Immunol. **85:**265–269.
2. **Adams, W. G., K. A. Deaver, S. L. Cochi, B. D. Plikaytis, E. R. Zell, C. V. Broome, and J. D. Wenger.** 1993. Decline of childhood *Haemophilus influenzae* type b disease in the Hib vaccine era. JAMA **269:**221–226.
3. **Anderson, E. L., T. Bowers, C. M. Mink, D. J. Kennedy, R. B. Belshe, H. Harakeh, L. Pais, P. Holder, and G. M. Carlone.** 1994. Safety and immunogenicity of meningococcal A and C polysaccharide conjugate vaccine in adults. Infect. Immun. **62:**3391–3395.
4. **Barington, T., A. Gyhrs, K. Kristensen, and C. Heilmann.** 1994. Opposite effects of actively and passively acquired immunity to the carrier on responses of human infants to a *Haemophilus influenzae* type b conjugate vaccine. Infect. Immun. **62:**9–14.
5. **Barington, T., M. Skettrup, L. Juul, and C. Heilmann.** 1993. Non-epitope-specific suppression of the antibody response to *Haemophilus influenzae* type b conjugate vaccines by preimmunization with vaccine components. Infect. Immun. **61:**432–438.
6. **Benabdelmoumene, S., S. Dumont, C. Petit, P. Poindron, D. Wachsmann, and J. P. Klein.** 1991. Activation of human monocytes by *Streptococcus mutans* serotype f polysaccharide: immunoglobulin G Fc receptor expression and tumor necrosis factor and interleukin-1 production. Infect. Immun. **59:**3261–3266.
7. **Briand, J. P., C. Barin, M. H. V. Van Regenmortel, and S. Muller.** 1992. Application and limitations of the multiple antigen peptide (MAP) system in the production and evaluation of an anti-peptide and anti-protein antibodies. J. Immunol. Methods **156:**255–265.
8. **Bruyère, T., D. Wachsmann, J. P. Klein, M. Scholler, and R. M. Frank.** 1987. Local response in rat to liposome-associated *Streptococcus mutans* polysaccharide-protein conjugate. Vaccine **5:**39–42.
9. **Calvo-Calle, J. M., G. A. de Oliveira, P. Clavijo, M. Maracic, J. P. Tam, Y. A. Lu, E. H. Nardin, R. S. Nussenzweig, and A. H. Cochrane.** 1993. Immunogenicity of multiple antigen peptides containing B- and non-repeat T-cell epitopes of the circumsporozoite protein of *Plasmodium falciparum*. J. Immunol. **150:**1403–1412.
10. **Carlsson, J.** 1972. Nutritional requirements of *Streptococcus sanguis*. Arch. Oral Biol. **17:**1327–1332.
11. **Fattom, A., C. Lue, S. C. Szu, J. Mestecky, G. Schiffman, D. Bryla, W. F. Vann, D. Watson, L. M. Kimzey, J. B. Robbins, and R. Schneerson.** 1990. Serum antibody response in adult volunteers elicited by injection of *Streptococcus pneumoniae* type 12F polysaccharide alone or conjugated to diphtheria toxoid. Infect. Immun. **58:**2309–2312.
12. **Francis, M. J., G. Z. Hastings, F. Brown, J. McDermed, Y. A. Lu, and J. P. Tam.** 1991. Immunological evaluation of the multiple antigen peptide (MAP) system using the major immunogenic site of foot-and-mouth disease virus. Immunology **73:**249–254.
13. **Gangloff, S., R. M'Zoughi, E. Lett, M. Scholler, J. Baer, A. Pini, J. A. Ogier, and J. P. Klein.** 1992. Epitope mapping of *Streptococcus mutans* SR protein and human IgG cross-reactive determinants, by using recombinant proteins and synthetic peptides. J. Immunol. **148:**3249–3255.
14. **Jacobs, E., R. Röck, and L. Dalehite.** 1990. A B cell-, T cell-linked epitope located on the adhesin of *Mycoplasma pneumoniae*. Infect. Immun. **58:**2464–2469.
15. **Lett, E., S. Gangloff, M. Zimmermann, D. Wachsmann, and J.-P. Klein.** 1994. Immunogenicity of polysaccharides conjugated to peptides containing T- and B-cell epitopes. Infect. Immun. **62:**785–792.
16. **Madoff, L. C., L. C. Paoletti, J. Y. Tai, and D. L. Kasper.** 1994. Maternal

immunization of mice with group B streptococcal type III polysaccharide-beta C protein conjugate elicits protective antibody to multiple serotypes. J. Clin. Invest. **94:**286–292.

17. **Monsigny, M., C. Petit, and A. C. Roche.** 1988. Colorimetric determination of neutral sugars by a resorcinol-sulfuric acid micromethod. Anal. Biochem. **175:**525–530.
18. **Munesinghe, D. Y., P. Clavijo, M. Calvo Calle, R. S. Nussenzweig, and E. Nardin.** 1991. Immunogenicity of multiple antigen peptides (MAP) containing T- and B-cell epitopes of the repeat region of the *P. falciparum* circumsporozoite protein. Eur. J. Immunol. **21:**3015–3020.
19. **Nardelli, B., P. B. Haser, and J. P. Tam.** 1994. Oral administration of an antigenic synthetic lipopeptide (MAP-P3C) evokes salivary antibodies and systemic humoral and cellular responses. Vaccine **12:**1335–1339.
20. **Ogier, J. A., A. Pini, P. Sommer, and J. P. Klein.** 1989. Purification and characterization of the expression product of the *sr* gene of *Streptococcus mutans OMZ 175*. Microb. Pathog. **6:**175–182.
21. **Peeters, C. C. A. M., A.-M. Tenbergen-Meekes, J. T. Poolman, M. Beurret, B. J. M. Zegers, and G. T. Rijkers.** 1991. Effect of carrier priming on immunogenicity of saccharide-protein conjugate vaccines. Infect. Immun. **59:**3504–3510.
22. **Pritchard, D. G., S. M. Michalek, J. R. McGhee, and R. L. Furner.** 1987. Structure of the serotype f polysaccharide antigen of *Streptococcus mutans*. Carbohydr. Res. **166:**123–131.
23. **Robbins, J. B., and R. Schneerson.** 1990. Polysaccharide-protein conjugates: a new generation of vaccines. J. Infect. Dis. **161:**821–832.
24. **Sanderson, G. J., and D. V. Wilson.** 1971. A simple method for coupling proteins to insoluble polysaccharide. Immunology **20:**1061–1065.
25. **Smith, D. J., M. A. Taubman, C. F. Holmberg, J. Eastcott, W. F. King, and P. Ali-Salaam.** 1993. Antigenicity and immunogenicity of a synthetic peptide derived from a glucan-binding domain of mutans streptococcal glucosyltransferase. Infect. Immun. **61:**2899–2905.
26. **Soell, M., E. Lett, F. Holveck, M. Schöller, D. Wachsmann, and J.-P. Klein.** 1995. Activation of human monocytes by streptococcal rhamnose glucose polymers is mediated by CD14 antigen, and mannan binding protein inhibits TNF-α release. J. Immunol. **154:**851–860.
27. **Tam, J.** 1988. Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. Proc. Natl. Acad. Sci. USA **85:**5409–5413.
28. **Wachsmann, D., J. P. Klein, M. Schöller, and R. M. Frank.** 1985. Local and systemic immune response to orally administered liposome associated soluble *S. mutans* cell wall antigens. Immunology **54:**189–194.
29. **Wachsmann, D., J. P. Klein, M. Scholler, J. Ogier, F. Ackermans, and R. M. Frank.** 1986. Serum and salivary antibody responses in rats orally immunized with *Streptococcus mutans* carbohydrate protein conjugate associated with liposomes. Infect. Immun. **52:**408–413.