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Immunogenicity and Efficacy of Oral or Intranasal *Shigella flexneri* 2a and *Shigella sonnei* Proteosome-Lipopolysaccharide Vaccines in Animal Models

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Immunity against shigellosis has been shown to correlate with the presence of antibodies specific for *Shigella* lipopolysaccharide (LPS). We here propose a new candidate vaccine for shigellosis composed of purified *Shigella flexneri* 2a or *Shigella sonnei* LPS hydrophobically complexed with group C type 2b *Neisseria meningitidis* outer membrane protein proteosomes. Immunization of mice either orally or intranasally with this complex induced specific homologous anti-LPS antibodies in both intestinal and respiratory secretions as well as in sera. Strong anamnestic responses were found after two or three immunizations. LPS alone, alkaline-detoxified LPS, or alkaline-detoxified LPS complexed with proteosomes was not effective. Oral or intranasal immunization of guinea pigs with two or more doses of this proteosome-LPS vaccine elicited homologous protection against *Shigella* keratoconjunctivitis (Serény test). These data demonstrate that proteosomes can be used as an effective mucosal vaccine delivery system and that orally or intranasally administered acellular vaccines can protect against *Shigella* infections.

Shigella flexneri and *Shigella sonnei* as well as other *Shigella* species represent a major cause of diarrheal diseases in developing countries (12). It has been shown that type-specific protection against shigellosis can be acquired in humans after infection with wild-type or attenuated bacteria (2, 4, 9, 13, 19). There is direct evidence that anti-type-specific lipopolysaccharide (LPS) antibodies are associated with this protection (2, 4). Although clearance of *Shigella* cells and similar mucosal pathogens from invaded tissue may involve several host defense mechanisms, it is widely recognized that local mucosal immune responses, especially secretory immunoglobulins (Igs), play a major role in protection (11, 25, 26, 28). In this regard, levels of these antibodies in serum, to the extent that they reflect local antibody production, may be viewed as measures or markers of protection, whether or not they actively contribute to bacterial clearance of such mucosal infections (4, 28).

Since the demonstrations in 1955 by Higgins et al. (10) and in 1967 by Formal et al. (6) that parenteral immunization with live or killed *Shigella* bacteria was ineffective in protecting humans or monkeys against *Shigella* infection, the major thrust of *Shigella* vaccine research has focused on the use of orally administered live genetically constructed or attenuated vaccines, and several of these approaches are particularly promising (7, 15). Nevertheless, the successful development of live *Shigella* vaccines has been difficult to accomplish, partly because of the relatively narrow window between efficacy and safety of certain vaccine candidates (7, 15).

Proteosomes are preparations of neisserial outer membrane protein vesicles that have previously been shown to enhance the parenteral immunogenicity of peptides and other antigens hydrophobically complexed to them (16, 17). Moreover, large-scale vaccine trials with such meningococcal outer membrane protein preparations noncovalently complexed to meningococcal polysaccharides have demonstrated that such vaccines are safe for human use (30, 31). In the present study, we evaluated an acellular approach to induce type-specific anti-*Shigella* immunity using purified *Shigella* LPS. In particular, we evaluated the mucosal immunogenicity and efficacy in animal models of *S. flexneri* 2a and *S. sonnei* LPS hydrophobically complexed to proteosomes (prot-LPS). These *Shigella* vaccine candidates were designed for oral or intranasal administration in order to achieve direct sensitization of targeted mucosal tissues and thereby stimulate mucosal Ig production and local immunity.

MATERIALS AND METHODS

All materials unless indicated otherwise were purchased from Sigma Chemical Co., St. Louis, Mo.

LPS preparation. LPS was extracted from single isolates of *S. flexneri* 2a or *S. sonnei* by hot phenol by established methods (29). Alkaline-detoxified LPS (LPSad) was prepared by mild alkaline treatment as previously described (23).

Proteosome preparation. Outer membrane proteins from group C serotype 2b *Neisseria meningitidis* were extracted with detergent as described previously (17).

Vaccine preparation. Purified LPS or LPSad and group C serotype 2b *N. meningitidis* outer membrane proteins were mixed at a 1:1 (wt/wt) ratio in phosphate-buffered saline (PBS) containing 1% Empigen (Albright and Wilson, Whitehaven, Cumbria, Great Britain). The final concentration of proteosomes and either LPS or LPSad was 2 mg/ml. The mixture was dialyzed across a dialysis membrane with a 1,000-molecular-weight cutoff (SpectraPor 6; Spectrum Medical Industries, Los Angeles, Calif.) against PBS at 4°C for 10 days with daily buffer exchanges. The vaccine preparations, prot-LPS or prot-LPSad, were stored at 4°C and

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diluted to the specific concentration with PBS prior to immunization.

CL-4B column. Samples of purified LPS or vaccine preparation were eluted over a CL-4B-200 column (2.5 by 40 cm), and fractions were assayed for protein by the Bradford method (3) with bovine serum albumin (BSA) as standard. The LPS level in each fraction was calculated by competition enzyme-linked immunosorbent assay (ELISA) as follows. Samples (100 μl) from each fraction were incubated with 300 μl of specific-LPS-positive guinea pig serum diluted 1:150 in ELISA blocking buffer for 1 h at 37°C. Homologous LPS samples ranging from 100 to 3.125 μg/ml were incubated as standards. The serum and samples or standards were then incubated for 2 h at 37°C in 96-well plates that had been precoated with LPS. The plates were developed as described below (see "ELISA" below). The amount of LPS in each fraction was calculated with the standard curve obtained from the LPS standards.

Immunizations. (i) Mice. BALB/c mice (7 to 10 weeks old; four to five per group) were immunized by either the intranasal or oral route. Orally, 100 μl of PBS with 0.2 M $NaHCO_3$ containing 100 μg of LPS (or LPSad) was given either alone or complexed with 100 μg of proteosomes (200 μg of prot-LPS) via a bent metal feeding tube. For intranasal immunization, following light ether anesthesia, 25 μl of PBS containing 10 μg of LPS (or LPSad) either alone or complexed with 10 μg of proteosomes (20 μg of prot-LPS) was slowly placed via micropipette in one or both of the nares. A control group in each experiment received diluent without antigen.

(ii) Guinea pigs. DH guinea pigs (2 to 3 months old) were anesthetized intramuscularly with 5 mg of ketamine · HCl plus 1.17 mg of xylazine · HCl and immunized with the prot-LPS complex via either the oral or intranasal route. Orally, 200 μl of PBS with 0.2 M $NaHCO_3$ containing 200 μg of the prot-LPS complex was used to immunize; intranasally, 50 μl of PBS with 40 μg of the prot-LPS complex was used.

Collection of mucosal secretions. (i) Murine lung lavage. Nine to 11 days after the last immunization, mice were sacrificed by CO_2 suffocation and the lungs were exposed. A cannula was inserted into the trachea, and using a three-way stopcock, the lungs were lavaged twice with 1 ml of PBS containing 0.1% BSA and the two lavage fluids were combined.

(ii) Murine intestinal lavage. Following the lung lavage, 20 to 25 cm from the small intestine was removed, and 2 ml of PBS containing 0.1% BSA, 50 mM EDTA, and 0.1 mg of soybean trypsin inhibitor per ml was passed through and collected. Phenylmethylsulfonyl fluoride was added to the intestinal wash after collection (1 mM, final concentration). Both lung and intestinal washes were vortexed vigorously and centrifuged at 1,000 × *g* for 20 min to remove cells and debris, the supernatants were collected, NaN_3 (0.1%, final concentration) was added, and the supernatents were stored at −20°C until assayed.

Serum preparation. Blood from mice was collected after they were sacrificed, and sera were stored at −20°C until assayed.

ELISA. The antibody level in the sera and in the intestinal and lung lavage fluids was determined as described previously (4). Briefly, 96-well flat-bottom high-binding plates (Costar, Cambridge, Mass.) were precoated with the specific LPS, blocked with BSA-casein, and washed three times. Serially double-diluted samples in blocking solution were incubated in the plates, and after the incubation period, the plates were washed three times and alkaline phosphatase-conjugated anti-mouse IgG or IgA diluted 1:1,000 was added. The plates were then washed three times, *p*-nitrophenylphosphate was added, and the A_{405} was measured. The antibody titer noted is expressed as the geometric mean of the maximal dilution which elicited an optical density equal or above the cutoff value at the specific processing period. The processing periods and cutoff values are mentioned in the relevant figure legends.

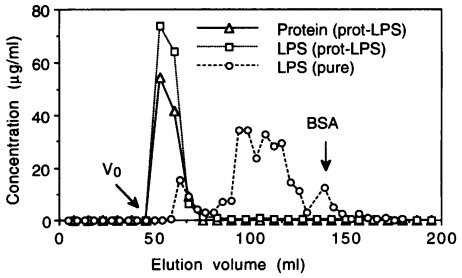

FIG. 1. Protein and LPS levels in fractions eluted from a CL-4B column after application of the prot-*S. flexneri* 2a LPS complex and LPS levels in column fractions after application of *S. flexneri* 2a LPS alone.

Challenge in guinea pigs (Serény test). Based on the standard challenge assay for the pathogenesis of shigellosis (22), the conjunctival sac of one eye of the animal was inoculated with 30 μl of a suspension containing an estimated 10^8 homologous bacteria (*S. flexneri* 2a or *S. sonnei*), and the eyelids were lightly massaged. All experiments were performed with the same stock strains, and for *S. sonnei*, only Congo red-positive colonies were picked for culturing for the assay. By using this method, 70 to 80% Congo red-positive colonies were identified in the suspension of *S. sonnei* used for inoculation in the Serény tests. An accurate determination of the inoculum was determined by plating on blood agar. Two to 3 days after inoculation, the eyes were blindly observed for the development of keratoconjunctivitis. The degree of severity of keratoconjunctivitis was defined as follows: no infection, no signs of irritation or keratoconjunctivitis; mild infection, any signs of irritation or keratoconjunctivitis but not purulent; severe infection, fully developed keratoconjunctivitis with purulence. Protection was calculated according to the following formula: 1 − (% immunized infected/% control infected), where infected represents either severe infection or any infection.

Statistics. Data from the ELISA results were analyzed by Student's *t* test (see Table 1) or the general linear models test (see Fig. 3 and 4). Data from guinea pig challenge experiments were analyzed by the two-tailed Fisher exact test.

RESULTS

Complex efficiency. Figure 1 shows the protein and *S. flexneri* 2a LPS elution patterns as measured by gel filtration column chromatography with CL-4B. When the prot-LPS complex was passed over the column, LPS and proteosomes were detected in the same fractions at the void volume of the column (Fig. 1, dotted and solid lines, respectively). In contrast, LPS alone eluted later (Fig. 1, dashed line) and was thus easily differentiated from LPS complexed to proteosomes.

Immunogenicity in mice. Initial experiments were performed by immunizing twice with a 3-week interval between

TABLE 1. Anti-LPS IgG in mouse serum after two doses of vaccine 3 weeks apart

Vaccine	Anti-LPS IgG			
	Intranasal immunization		Oral immunization	
	GMT[a]	Range[b]	GMT[a]	Range[b]
S. flexneri 2a				
LPSad	<6		<6[c]	
Prot-LPSad	<6		14[c]	9–21
LPS	<6		<6	
Prot-LPS	1,838	1,227–2,735	528	408–683
S. sonnei Prot-LPS	4,222	3,200–5,571	113	27–467

[a] Geometric mean of the maximal reciprocal dilution elicited an optical density greater than 0.5 after 1 h of incubation with substrate.
[b] GMT ± 1 standard error of mean.
[c] Three doses of vaccine at weeks 0, 1, and 4.

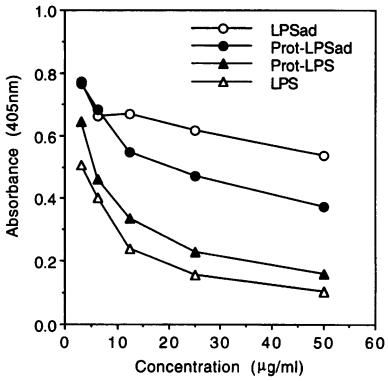

FIG. 2. Inhibition of specific antibody binding to solid-phase *S. flexneri* 2a LPS in ELISA. LPSad, prot-LPSad, LPS, or prot-LPS was incubated with LPS-positive guinea pig serum prior to its application to an ELISA.

doses. Nine to 11 days after the last immunization, sera and secretions from intestines and lungs were collected, and the specific anti-LPS antibody levels were measured by ELISA. LPS or LPSad (three doses) prepared from *S. flexneri* 2a could not induce anti-LPS antibody in sera when given alone (Table 1). Interestingly, prot-LPSad preparation elicited only minimal levels of serum antibody even after three doses (Table 1). Neither LPS, LPSad, nor prot-LPSad induced any detectable IgA in intestinal or lung secretions (data not shown). In marked contrast, the prot-LPS complex with *S. flexneri* 2a LPS was remarkably effective in inducing homologous anti-LPS IgG production in sera (Table 1). In this case, intranasal immunization was more effective than oral immunization ($P = 0.02$), although 10-fold less vaccine was given. The strong immunogenicity of the prot-LPS vaccine was confirmed with *S. sonnei* LPS (Table 1), but in this case, the advantage of the intranasal route was less significant ($P = 0.07$). No heterologous anti-LPS antibodies were detected in the sera (data not shown).

Since prot-LPSad was not effective in inducing anti-LPS antibodies, we investigated the idea that alkaline detoxification may affect the antigenicity of LPS. When LPSad was used as the detecting solid-phase antigen on the ELISA plate, results with natural or postimmunization antisera were comparable to those obtained with native LPS (data not shown). This indicated that the antigenicity of LPSad was equivalent to that of native LPS. Nevertheless, an inhibition ELISA (described in Materials and Methods) showed that LPSad and prot-LPSad were markedly less effective than native LPS or prot-LPS in inhibiting binding of natural anti-LPS antibodies to native LPS (Fig. 2). These data demonstrate that alkaline detoxification altered the antigenicity that LPS expresses in solution.

To determine the optimal immunization regimen for prot-LPS vaccines, mice were immunized either orally or intranasally with proteosomes complexed with *S. flexneri* 2a LPS by four different protocols: (i) one dose, (ii) two doses 1 week apart, (iii) two doses 3 weeks apart, and (iv) three doses 1 and 3 weeks apart. The LPS-specific serum antibody titers of mice immunized with two or three doses of vaccine (Fig. 3) showed that the highest levels of both IgG and IgA were achieved when one booster dose was given 3 weeks after priming with one (Fig. 3b) or two (Fig. 3c) doses ($P < 0.001$). IgA production was measurably improved by a third dose ($P < 0.001$), while for IgG, two doses of vaccine elicited antibody levels comparable to those elicited by the three-dose regimen. One dose of vaccine was insufficient to elicit any detectable serum antibody levels by the standard assay; extension of the sensitivity of the assay (by overnight incubation with substrate) revealed low levels of antibody even after one dose of prot-LPS (data not shown). The combined analysis of the different regimens elicited that both oral and intranasal routes of administration induced comparable levels of IgG ($P = 0.26$) and IgA ($P = 0.56$) in serum even though the amount of antigen administered intranasally was 10-fold less than that given orally (Fig. 3).

The induction of specific anti-*S. flexneri* 2a LPS IgA levels in murine intestine lavage fluids by prot-LPS was similar to the serum responses in that protocols iii and iv were the most effective ($P = 0.02$) (Fig. 4) and one dose of vaccine failed to elicit detectable antibody levels. Intranasal immunization was effective in induction of both intestinal and lung IgA. Induction of higher levels of anti-LPS IgA in lung lavage fluids, however, was far more easily attained by intranasal than by oral immunization ($P < 0.001$), while oral immunization was more effective then intranasal immunization in inducing intestinal IgA ($P = 0.03$) (Fig. 4).

Challenge in guinea pigs. Guinea pigs were immunized

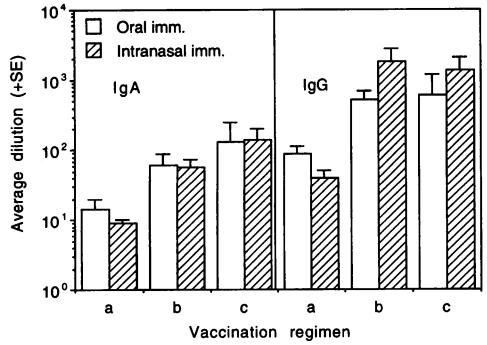

FIG. 3. Anti-LPS IgG and IgA in sera of mice immunized either orally or intranasally with prot-LPS complex. Four or five animals were immunized either with two doses at 0 and 1 weeks (a), with two doses at 0 and 3 weeks (b), or with three doses at 0, 1, and 4 weeks (c). The results are expressed as the geometric mean of the maximal dilution elicited an optical density greater than 0.5 after 1 h of incubation with substrate.

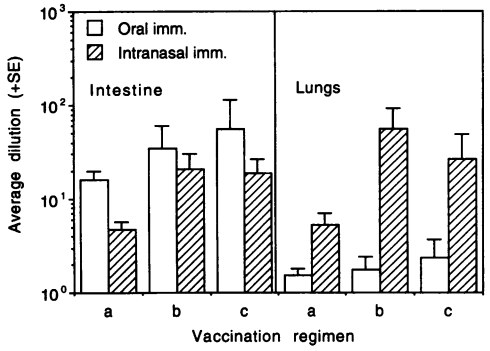

FIG. 4. Anti-LPS IgA in intestines and lungs of mice immunized either orally or intranasally with prot-LPS complex as described in the legend to Fig. 3. The results are expressed as the geometric mean of the maximal dilution elicited an optical density greater than 0.5 (intestines) or 0.2 (lungs) after 2 h of incubation with substrate.

with prot-LPS vaccines containing either *S. flexneri* or *S. sonnei* LPS for experimental challenge studies with the guinea pig keratoconjunctivitis model of mucosal shigellosis (Serény test). Three experiments were performed with prot-*S. flexneri* 2a LPS and two with prot-*S. sonnei* LPS with different immunization regimens. Two weeks after the last immunization, the animals were challenged with homologous bacteria. Two to 3 days later, the animals were examined for keratoconjunctivitis and were divided into three clearly identifiable categories as follows: no infection, mild to moderate infection, and severe infection.

The results of these Serény tests (Tables 2 and 3) show that the prot-LPS complex vaccines elicited in vivo protection against homologous bacteria infecting a distal mucosal site. In the *S. flexneri* experiments (Table 2), all animals in control groups (16 of 16) were severely infected. After intranasal or oral immunization, 14 of 19 (74%, $P < 0.001$) and 11 of 16 (69%, $P < 0.001$), respectively, of the animals were protected from severe infection (Table 2). In addition, after oral immunization, 9 of 16 animals (56%, $P < 0.001$)

TABLE 2. Homologous challenge (Serény test) in guinea pigs after immunization with prot-*S. flexneri* 2a LPS

Group	Route of immunization	No. infected with the following type of infection/total:			% Calculated protection	
		None	Mild	Severe	Any infection	Severe infection
1[a]	Intranasal	0/6	1/6	5/6	0	17
	Oral	4/6	0/6	2/6	67	67
	Control	0/7	0/7	7/7		
2[b]	Intranasal	2/4	2/4	0/4	50	100
	Oral	4/4	0/4	0/4	100	100
	Control	0/4	0/4	4/4		
3[c]	Intranasal	2/9	7/9	0/9	22	100
	Oral	1/6	2/6	3/6	17	50
	Control	0/5	0/5	5/5		
1 + 2 + 3	Intranasal	4/19	10/19	5/19	21	74
	Oral	9/16	2/16	5/16	56	69
	Control	0/16	0/16	16/16		

[a] Immunization at 0 and 3 weeks; challenge with 7.6×10^7 bacteria per eye.
[b] Immunization at 0, 1, and 3 weeks; challenge with 1.0×10^8 bacteria per eye.
[c] Immunization at 0, 1, 3, and 8 weeks; challenge with 2.6×10^8 bacteria per eye.

TABLE 3. Homologous challenge (Serény test) in guinea pigs after immunization with prot-*S. sonnei* LPS

Group	Route of immunization	No. infected with the following infection/total:			% Calculated protection	
		None	Mild	Severe	Any infection	Severe infection
1[a]	Intranasal	9/12	3/12	0/12	71	100
	Oral	6/6	0/6	0/6	100	100
	Control	1/8	3/8	4/8		
2[b]	Intranasal	8/10	2/10	0/10	75	100
	Control	3/16	10/16	3/16		
1 + 2	Intranasal	17/22	5/22	0/22	73	100
	Control	4/24	13/24	7/24		

[a] Immunization at 0, 3, and 4 weeks; challenge with 3.1×10^8 bacteria per eye.
[b] Immunization at 0 and 3 weeks; challenge with 4.3×10^8 bacteria per eye.

were protected against any infection (Table 2). In the *S. sonnei* experiments (Table 3), 17 of 22 intranasally immunized animals were protected against any infection compared with only 4 of 24 animals in the control groups resulting in 73% protection ($P < 0.001$). Following oral immunization, 6 of 6 animals were completely protected compared with only 1 of 8 in the control group (100% protection, $P = 0.005$).

DISCUSSION

In this study, the prot-LPS complexes were found to be highly effective acellular vaccines against *S. flexneri* 2a and *S. sonnei*. As demonstrated by size exclusion chromatography with the CL-4B column, the *Shigella* LPS was completely complexed with the *N. meningitidis* outer membrane proteins. In addition, inhibition ELISAs with prot-LPS indicated that the complex was formed in a configuration that maintained the native orientation and exposure of the polysaccharide antigenic determinants. In contrast, immunization with LPS alone or prot-LPSad was ineffective. That LPS alone was not immunogenic is not particularly surprising since the induction of both IgG and IgA in serum and secretions are known to be T-cell dependent (18) and LPS is a well-established T-cell independent antigen (24). The success of induction of anti-LPS antibodies by mucosal immunization with the prot-LPS vaccine implies that the proteosome proteins are able to act in this context as T-cell-stimulating carrier proteins even though they are bound to the LPS hydrophobically and not covalently. This concept was previously indicated by the ability of proteosomes to induce anti-peptide IgG when hydrophobically complexed to small peptides that, like haptens, were not immunogenic alone (16, 17).

The inability of the LPSad to be immunogenic even with proteosomes requires further explanation. The data indicate that LPSad does not maintain the same antigenic structure as native LPS. Specifically, we found that although LPSad was as effective as native LPS as a solid-phase plate antigen in a standard ELISA, in solution, LPSad was defective compared with native LPS. This was shown by its reduced ability to recognize natural anti-*S. flexneri* LPS antibodies, resulting in reduced inhibition of binding of such antibodies to native LPS. This altered antigenicity of soluble LPSad may have been a critical factor responsible for its poor immunogenicity even with proteosomes. Although previous investigators using base-hydrolyzed LPS found that it was as

antigenic as native LPS in their assays, *S. flexneri* LPS may be more sensitive to such treatment than the *Escherichia coli* LPS they used (21, 23). It is also possible that the adjuvant property of lipid A in LPS may act in synergism with proteosomes in the prot-LPS vaccine and that this is abrogated when ester-linked fatty acids are removed by alkaline treatment (20, 23, 27). This alteration in lipid A can lead also to the inability of LPSad to associate properly with proteosomes, thus reducing its immunogenicity. Accordingly, since both the gastrointestinal tract and the nasopharynx are replete with commensal bacteria containing LPS, it is unlikely that alkaline treatment of LPS would be recommended for human vaccine development of these mucosal vaccines.

The mechanism by which the prot-LPS complex reacts with the immune system to induce the responses demonstrated here warrants elucidation. Aizpurua and Russel-Jones (1) have noted that proteins with lectin or lectin-like binding activity are good mucosal immunogens, whereas proteins lacking such activity are ineffective and may be suppressive to serum responses. The B subunit of cholera toxin which binds to G_{M1} ganglioside is a good example of this type of antigen. Mucosal adjuvant activity of liposomes (14) and microspheres (5) appears to depend on their physical size and structure that promotes enhanced mucosal uptake and processing. Both of these concepts may be relevant to proteosomes because of their vesicular nature and ability to activate lymphocytes, suggesting lectin-like binding properties.

The data presented here indicate that proteosome-based vaccines designed to protect against intestinal pathogens such as *Shigella* species would be effective by either the oral or intranasal route. Potential advantages of intranasal immunization include lower dosages, perhaps due to more efficient vaccine uptake by avoiding intragastric and intestinal degradation. In addition, intranasal immunization induced a greater amount of a specific IgA in murine lungs than did oral immunization. These differences strongly suggest that a substantial portion of the intranasal vaccine was absorbed in the respiratory tract and not swallowed. The data also imply that intranasal proteosome vaccines may be preferable for induction of immunity to protect against pathogens that invade the respiratory tract, especially when antibodies to LPS or polysaccharides confer immunity, such as pneumonia caused by gram-negative organisms.

The results of the Serény tests in guinea pigs demonstrate that both oral and intranasal prot-LPS vaccines can protect in vivo against mucosal infection with homologous bacteria. The inoculation of ca. 10^8 bacteria in the Serény test as performed in this study is comparable to the challenge inoculum given in Serény tests used to evaluate live vaccines including the T32-Istrati strain which was reported to be protective in humans (19) and two attenuated vaccine strains which were protective in monkeys (8). Further evaluation of the relative efficacy of oral and intranasal immunization can be performed with subhuman primates even though, as in the Serény test, many more *Shigella* organisms are needed to infect animals than to infect humans. These results demonstrate that proteosome vaccines are potent mucosal immunogens that can stimulate the common mucosal immune system to induce antibody production and protection against pathogenic challenge even at locations distal to the immunizing site. The data in this report suggest that such studies are warranted in preparation for development of such vaccines as candidates for immunogenicity and efficacy trials in humans. The results presented here are encouraging for the concept that safe acellular oral or intranasal vaccines that protect against shigellosis and other mucosal pathogens may soon be possible.

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