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Protective Ribosomal Preparation from *Shigella sonnei* as a Parenteral Candidate Vaccine

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A parenteral *Shigella* ribosomal vaccine (SRV) was investigated in animals for safety, antibody-inducing capacity, and protective activity. Ribosomal preparations from a *Shigella sonnei* phase I avirulent strain were obtained and shown to possess chemical, sedimentation, and other properties typical of bacterial ribosomes. No endotoxin contamination was revealed by a ketodeoxyoctonate assay, although the presence of some kind of O antigen was evidenced by serological findings and the high activity of SRV in inducing the O-antibody response and immunological memory in animals. SRV was nontoxic in mice, guinea pigs, and monkeys and induced no local reactions when injected subcutaneously in reasonable doses. Significant protection against a local *Shigella* infection (Sereny test) was seen in guinea pigs injected with SRV (efficiency index, about 60%) and the specificity of the protection was evident from cross-challenge experiments. The protective efficiency of SRV was especially high in rhesus monkeys challenged orally with virulent *Shigella* cells (89%, as calculated from the summarized data of several experiments in 71 animals). Protection in monkeys was long lasting and could be demonstrated several months after injection of SRV. An inexpensive technique can be used for the production of SRV on a large scale. The high immunogenicity of SRV is discussed in terms of the amplifying effect of the ribosome, which serves as a delivery system for polysaccharide O antigen. Further study of SRV as a candidate vaccine for humans seems justified by the data obtained.

Different attempts to create an effective *Shigella* vaccine have been made since the end of 19th century. Parenteral vaccines were reported to be effective in the past, when *Shigella dysenteriae* predominated and the crude endotoxin from *S. flexneri* was included in the parenteral vaccine, which was extensively used in the Red Army during the Second World War. The protective capability of this polyvaccine was not confirmed, however, by a subsequent controlled study (60). Another approach to developing a *Shigella* vaccine is based on Bezredka's idea (9a) of local (intestinal) immunity and on the assumption that this immunity is best induced by presenting the antigen directly to intestinal mucosa, i.e., by oral vaccination. However, killed bacteria and their products were not effective as oral vaccines (61), and the original idea was supplemented by specific requirements for the antigen delivery system. It was assumed that a prolonged action of the antigen was necessary to induce protective immunity in intestinal lymphoid tissue and that a live vaccine strain which could survive in the intestine without invading epithelial cells would be most appropriate (21, 22, 30, 41, 48). Many mutant and recombinant strains were developed and tested as vehicles for the O antigen, which proved to be of primary importance in natural immunity against shigellosis, and significant progress was achieved in determining the genetics and virulence factors of *Shigella* cells. However, the practical problem of developing an effective *Shigella* vaccine was not solved unambiguously in these studies, and the medical use of a live vaccine against dysentery is limited to one country (47). The most recent version of the live-vaccine concept requires that the antigen be delivered directly to mucosal lymphoid cells by a live vector strain which is capable of invading the epithelial cell layer without spreading in the intestinal mucosa (28). Several candidate vaccines of this type were recently proposed (28, 45, 49). It is unclear, however, whether an antigen delivered by a viable carrier which translocates through the epithelial cell barrier can be reproduced or replaced by the parenteral antigen presented in a suitable form. The possible use of polysaccharide-protein conjugates as parenteral vaccines was recently discussed (55).

In this study, an attempt was made to obtain a parenteral *Shigella* vaccine with ribosomal preparations from *S. sonnei*. This type of vaccine was first described in 1965 by Youmans and Youmans (65) and was intensively investigated for the next 15 years in many laboratories. The results of these studies, reviewed in references 9, 13, 18, and 26, showed that ribosomal vaccines prepared from various bacterial, fungal, or protozoal pathogens are highly protective against the respective infection in experimental animals. The long list of effective ribosomal vaccines includes those for infections with quite different types of pathogenesis and various protection mechanisms. They were also reported to be protective against local infections caused by *Streptococcus mutans* (27) or *Candida albicans* (42, 43). These results indicate that ribosomal vaccines are capable of stimulating not only systemic immunity but also local immunity. Although the mechanisms of protection and the nature of the protective moiety of bacterial ribosomes are disputable (9, 17, 18, 51), the very fact that ribosomal vaccines are highly protective can be regarded as a general principle which cannot be ignored, however unclear their molecular mechanisms are. It also seems reasonable to apply this principle to

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Shigella infections and to test the respective ribosomal preparations as new candidate vaccines.

MATERIALS AND METHODS

Bacteria. Avirulent *S. sonnei* 9090 phase I was cultured in a 40-liter fermentor with casein broth (pH 8.2) at 37°C for 6 to 8 h. Avirulent *S. flexneri* 2b strain 516 M was obtained from J. A. Belaja (Moscow, USSR) and cultivated in the same way. Virulent *S. sonnei* 1041 and *S. flexneri* 2b strain 660 for challenge experiments were cultured on casein agar plates.

Ribosomal preparations. Bacterial cells were centrifuged and washed three times with 10^{-4} M phosphate buffer (pH 7.2). A ca. 30% suspension in 10^{-2} M phosphate buffer (pH 7.2) supplemented with 30 mM $MgCl_2$, 0.44 M sucrose, and 0.5% sodium dodecyl sulfate (SDS) was sonicated for 3 min (MSE-150 apparatus; medium probe; amplitude, 16 to 18 μm), and large particles were centrifuged at 13,000 rpm for 15 min. A Janetzky (Leipzig, Germany) K-24 centrifuge was used for high-speed centrifugation throughout. Two techniques were used for further fractionation. (i) In accordance with the modified procedure of Youmans and Youmans (65), crude ribosomes were precipitated by ultracentrifugation at 225,000 × *g* for 2 h (Beckman-Spinco L-2 65B apparatus; rotor, 50 Ti). The pellet was resuspended in 0.25% SDS–30 mM $MgCl_2$–10^{-2} M phosphate buffer (pH 7.2) and incubated at 22°C for 30 min. After clarification (15,000 rpm, 20 min), the supernatant was ultracentrifugated as described above and the clear yellow pellet was resuspended in 10^{-4} M phosphate buffer (pH 7.2) containing 30 mM $MgCl_2$. (ii) A two-step polyethylene glycol (PEG) precipitation technique was developed (38) on the basis of the data of Expert-Bezançon et al. (19). Dry PEG 6000 (Serva) was added to the supernatant to yield a 10% (wt/wt) final concentration, and the suspension was incubated on the bench for 20 min. Crude ribosomes were pelleted at 13,000 rpm for 20 min and resuspended in magnesium phosphate buffer. After clarification (10,000 rpm, 10 min), PEG 6000 was added to a 5% (wt/wt) final concentration and the suspension was incubated again at 22°C for 20 min. The purified ribosomes were pelleted at 13,000 rpm and resuspended as after ultracentrifugation. All operations were performed at 4°C when other conditions are not indicated. The final product was lyophilized and stored at 4°C for several months without a loss of activity. The ribosomal preparations obtained by the two methods were carefully compared and found to be quite similar in their chemical compositions and biological properties (38, 39). Therefore, they are referred to as a *Shigella* ribosomal vaccine (SRV) independently of the method of preparation. The slight differences in extinction coefficients and yields of the preparations are noted below. The most important characteristics of protective activity will be illustrated by comparative data.

Endotoxin and LPS preparations. A trichloroacetic acid extract of *S. sonnei* 9090 was prepared as described by Staub (59) and purified by ultracentrifugation for 3 h at 105,000 × *g*. It contained 8% protein and less than 1% RNA and is referred as endotoxin. Lipopolysaccharide (LPS) was obtained by phenol-water extraction and purified by ultracentrifugation (63). Protein and RNA contents were less than 1%. Both preparations were lyophilized and stored at 4°C.

Chemical and physical analyses. Protein content was determined by the Lowry method, total sugars were determined by the phenol-sulfate method, RNA was determined by the orcinol reaction, hexoses were determined by the anthrone method, and ketodeoxyoctanate (KDO) was determined by the thiobarbiturate method (53). A reaction with carbocyanine dye (Stains all; Serva) was performed as described previously (15) to detect lipid A by measuring the characteristic shift in the absorption spectrum of the dye (31).

Visible and UV spectra were scanned with a Pye-Unicam SP-30 UV spectrophotometer supplemented with a drive. Sedimentation properties were studied by 10 to 40% linear sucrose gradient centrifugation at 50,000 rpm for 3 h (rotor, SW 50.1; 4°C). After the run, the gradient was withdrawn by puncturing of the tube. The A_{254} was monitored with a Uvicord C (LKB, Uppsala, Sweden).

Serological reactions. O antibodies were determined by a passive hemagglutination (PHA) test with Formalin-treated sheep erythrocytes coated with heat-activated LPS. Serologically active O antigen was assayed in the O-PHA inhibition test; twofold dilutions of a sample were mixed with the proper dilution of standard hyperimmune rabbit O antiserum containing 1 or 2 hemagglutinating units of O antibodies, and O erythrocytes were added. The activity of the O antigen in the sample was characterized by the minimal concentration that inhibited hemagglutination under standard conditions (the reference LPS preparation was used throughout).

IEP. For immunoelectrophoresis (IEP), the method of Scheidegger (56) was used with 0.5% agar plates and barbital buffer (pH 8.2; ionicity, 0.1). Hyperimmune rabbit antisera for IEP were prepared by multiple injections of SRV or LPS from *S. sonnei* or *S. flexneri*.

Experiments in mice. Male random-bred or F_1 (CBA × C57BL/6) animals weighing 16 to 18 g were used throughout. In the acute toxicity test, mice were injected intraperitoneally (i.p.) or intravenously (i.v.) with 0.5 ml of the preparations in phosphate-buffered saline (PBS) and mortality was recorded for 3 days. The antibody response was determined in mice injected i.p. or i.v. with graded doses of SRV, LPS, or endotoxin. Blood was collected from the retro-orbital sinus on day 7 after immunization. Protection experiments were performed with mice immunized subcutaneously (s.c.) and challenged on day 7 by an i.p. injection of 75×10^6 virulent *S. sonnei* 1041 cells. Twofold dilutions of the culture were tested in groups of control mice to determine the 50% lethal dose. Mortality was determined on day 3 after challenge.

Experiments in guinea pigs. The keratoconjunctival model of a local *Shigella* infection (57) was used to determine the protective activity of the vaccine. Random-bred animals of both sexes and weighing 160 to 240 g were immunized s.c. with 0.5 ml of vaccine and challenged 2 weeks later with 5×10^6 virulent *S. sonnei* 1041 cells in 5 μl of saline. The challenge dose was introduced into the conjunctival sac with a microsyringe, and light massage was applied to the closed lids. The results of the challenge were scored on day 6 and expressed as the percentage of resistant eyes in each group. The efficiency of vaccination (EV) was calculated as $EV = [(C - V)/C] \times 100$, where C and V are the percentages of affected eyes in the control and vaccinated groups, respectively.

Experiments in monkeys. Adult *Macaca mulatta* animals of both sexes, from 2.5 to 7 years of age, and weighing 3.5 to 6.0 kg were maintained at the Sukhumi Institute of Experimental Pathology and Therapy. The ribosomal vaccine was injected s.c. in one or two doses into the subscapular region, which was depilated in advance when local reactions to the vaccine were evaluated. Blood samples were collected from the cubital vein before and several times after immunization. Immunized and control animals were challenged orally with

TABLE 1. Chemical composition and UV absorption characteristics of SRV

Component or characteristic	Mean	Range
RNA	54.6	47–63
Protein	36.9	31–46
Hexoses	8.6	3–10
DNA		0–0.8
KDO	0.1	0.1
$E_{260}^{0.1\%,\ 10\ mm}$		
Ribosomes prepared by ultracentrifugation	12.1	11.1–12.5
Ribosomes prepared by PEG precipitation	8.1	7.1–9.1
A_{260}/A_{280}		
Ribosomes prepared by ultracentrifugation	1.9	1.7–2.0
Ribosomes prepared by PEG precipitation	1.7	1.6–1.9

75×10^9 *S. sonnei* 1041 cells in 10 ml of nutrient broth. The results were scored according to clinical symptoms. Pathogen excretion was determined by bacteriological analyses performed several times before the experiment, every day during the first 2 weeks after challenge, and twice each week during the next 2 months.

Statistical methods. Means and standard errors of the means were calculated by routine procedures. Student's *t* test was used to compare means. In protection experiments, the statistical significance of the difference between vaccinated and control groups was evaluated by Fisher's exact method (62).

RESULTS

Chemical data. As shown in Table 1, the main constituents of SRV were RNA and protein, while carbohydrate contamination varied between 3 and 10%. KDO, a characteristic part of LPS, was not detected. The SRV preparations did not induce the metachromatic shift in the spectrum of carbocyanine dye with a new absorption maximum at 460 nm, which is typical of LPS and lipid A preparations (15, 31). The yields of ribosomes averaged 2.7% of the dry weight of bacterial cells for the ultracentrifugation method and 5 to 6% for the PEG fractionation method.

Spectrophotometry and sedimentation properties. UV absorption spectra were typical of bacterial ribosomes (Fig. 1); there was one absorption maximum at 260 nm, and the A_{260}/A_{280} ratio varied from 1.6 to 2.0. The extinction coefficient was lower in the preparations obtained by PEG precipitation than in those obtained by ultracentrifugation.

One peak was revealed in the A_{254} distribution curve after sucrose density gradient centrifugation of ribosomal preparations in presence of 100 mM $MgCl_2$, while a decrease in the salt concentration to 1 mM resulted in a partial dissociation of ribosomal particles into subunits (Fig. 2).

Determination of serologically active O antigen. The presence of O antigen in the ribosomal preparations was revealed by an O-PHA inhibition test. The active concentration of SRV was 50 to 200 μg/ml, the geometric mean being $97 \times\!\div 1.8$ μg/ml. At the same time, the minimal active concentrations of several LPS and endotoxin preparations from *S. sonnei* ranged from 0.1 to 0.6 μg/ml, the mean being 0.22×1.9 μg/ml. It can be concluded that whatever the molecular nature of ribosomal O antigen, its content in SRV is equivalent to about 0.2% LPS by weight when the two preparations are compared by O-antibody-neutralizing capacity.

IEP. The best rabbit antisera obtained by hyperimmunization with ribosomes revealed multiple (six to eight) anti-

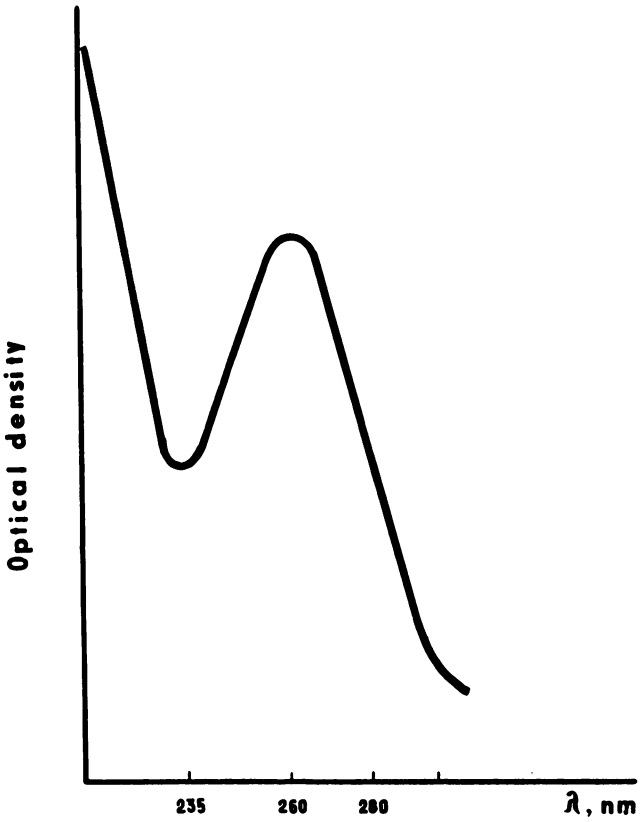

FIG. 1. UV absorption spectrum of the ribosomal preparation from *S. sonnei*.

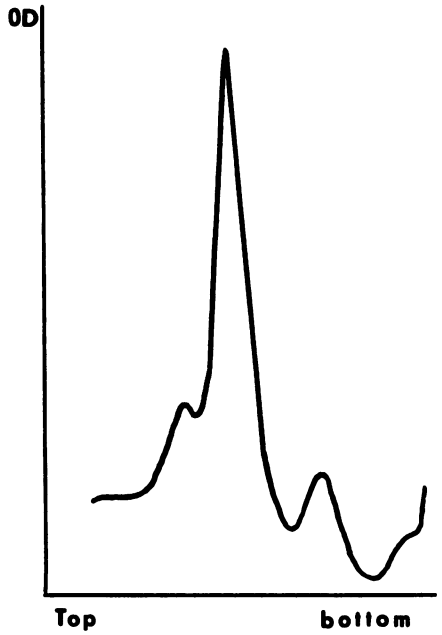

FIG. 2. Sedimentation profile of *S. sonnei* ribosomes in a linear density gradient of sucrose. Ultracentrifugation was performed in a buffer containing 0.1 M $MgCl_2$ at 50,000 rpm in an SW-65 rotor. OD, optical density.

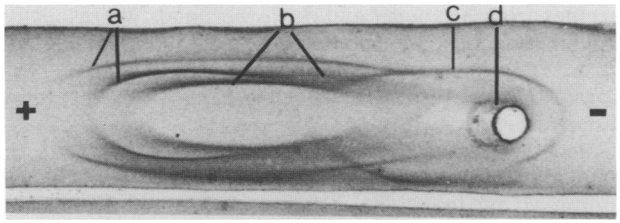

FIG. 3. IEP of *Shigella* ribosomes. A ribosomal preparation was applied to the central well, and the troughs were filled with rabbit hyperimmune antiribosomal serum. Some additional lines were sometimes visible in the intermediate region of the immunoelectropherogram. See the text for an explanation of a, b, c, and d.

genic components of different mobilities in SRV (Fig. 3). Two long arcs extending to the far anodic region (a) disappeared after digestion with trypsin (crystalline trypsin; Spofa, Prague, Czechoslovakia; enzyme/substrate ratio, 1:10; 37°C; 3 h in the presence of 1 mg of polyvinylsulfate per ml) or RNase (Reanal, Budapest, Hungary; enzyme/substrate ratio, 1:10; 37°C; 30 min). Probably these fast-migrating components were the RNA-associated proteins. The components of intermediate mobility (b) were seemingly protein in nature; they disappeared after trypsin digestion and were resistant to RNase digestion. The last two lines (c and d) were located near the start and were not sensitive to either enzyme. The outer one could be identified as corresponding to the ribosomal O antigen. It was revealed with hyperimmune O antiserum and disappeared when ribosomal antiserum or O antiserum was absorbed with homologous LPS. An additional arc was seen in the intermediate zone of IEP after treatment of SRV with trypsin.

Ribosomes from *S. flexneri* 2a were isolated, and hyperimmune rabbit antiserum against them was raised to investigate the cross-reactivity of ribosomes from two *Shigella* species in IEP. All of the recognized components of SRV, except for O antigen, appeared to be identical to those in *S. flexneri* ribosomes in ordinary IEP as well as in comparative IEP (54).

TABLE 2. Active protection against systemic *Shigella* infection induced by s.c. immunization of mice with SRV

Vaccine	No. of survivors/total no. immunized with the following dose (μg) of vaccine[a]		
	10^{-3} to 10^{-2}	4×10^{-2} to 4×10^{-1}	1.0 to 10.0
Ribosomal vaccine	9/20	30/31	38/39
Endotoxin	6/10	29/31	21/21

[a] Data from three independent experiments are summarized. The challenge dose (75×10^6 microbial cells) caused 100% lethality in nonvaccinated mice, amounting to 5.6, 8.0, and 11.0 50% lethal doses in different experiments.

Acute toxicity. All mice survived the i.p. or i.v. injection of ribosomes in doses of up to 2,000 μg, while the 50% lethal doses for several endotoxin and LPS preparations varied from 50 to 320 μg. The same results were obtained in random-bred rats and guinea pigs after s.c. injection of SRV in doses of up to 5,000 μg (the effect of higher doses was not determined).

Protection in mice. The mouse model of *Shigella* infection has little in common with human dysentery. Nevertheless, it can be of some value when properly interpreted. The summarized data for three experiments of this type are presented in Table 2.

Evidently, SRV was highly active in these experiments; less than 1 μg of ribosomes was enough to protect almost all of the animals against a massive challenge, which caused 100% mortality in control mice, and even several nanograms provided about 50% protection. The protective activity of SRV was similar to that of endotoxin, while the content of serologically active O antigen in these lots of SRV was about 200 times lower than that in endotoxin.

Protection against a local *Shigella* infection in guinea pigs. In accordance with our previous results (39, 40), the standard experiment in guinea pigs consisted of one s.c. injection of 200 μg of ribosomal preparation followed by intraconjunctival challenge 2 weeks later. The results of several experiments, summarized in Fig. 4, showed that most animals

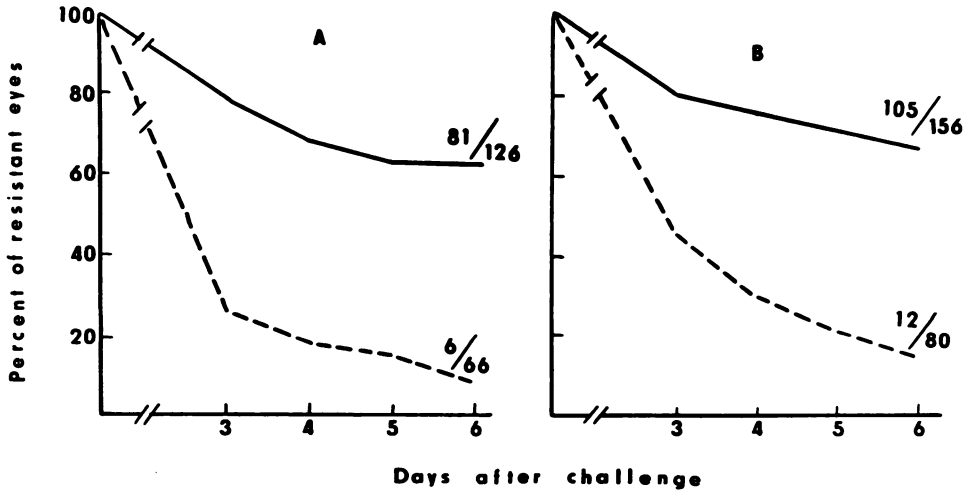

FIG. 4. Protective activity of *S. sonnei* ribosomes against a local *Shigella* infection in guinea pigs (keratoconjunctival model). Experiments were performed with SRV preparations obtained by ultracentrifugation (A) or PEG fractionation (B). The resistance level (percentage of unaffected eyes) is shown for immunized (solid line) and control (broken line) animals. The ratio of resistant to challenged eyes is shown for each group.

TABLE 3. Specificity of the protective action of ribosomal vaccines from *S. sonnei* and *S. flexneri* against a local *Shigella* infection in guinea pigs

Vaccine preparation[a]	% of resistant eyes (no. resistant/no. challenged) after challenge with:	
	S. sonnei	*S. flexneri*
SRV from *S. sonnei*	77 (46/60)	17 (5/30)
SRV from *S. flexneri*	22 (13/58)	57 (16/28)
None (control group)	25 (15/60)	16 (5/32)

[a] One s.c. injection of 200 μg.

injected with SRV became resistant to the challenge, which affected 80 to 90% of eyes in control animals. The difference between vaccinated and control groups was highly significant, as determined by the chi-square test ($P < 0.001$). The ribosomal vaccine prepared by ultracentrifugation had the same protective activity as did that prepared by PEG fractionation, the efficiencies of vaccination being 58 and 61%, respectively. The same efficiencies were observed in experiments with higher doses of SRV (1,000 μg) or when 200 μg was injected twice, with a 1-week interval.

The specificity of protective immunity induced by SRV in guinea pigs was investigated in two sets of experiments. In the first one, different groups of guinea pigs were immunized with ribosomal preparations obtained from *Escherichia coli* (strain MRE-600) or *Salmonella minnesota* (Re mutant, strain 595) or with SRV from *S. sonnei*. A standard immunization protocol (one injection of 200 μg) was used, and all animals were challenged 2 weeks later with the virulent *S. sonnei* strain. Only SRV was protective in this experiment; ribosomes of the other origins had no protective activity at all. The second set of data was obtained with ribosomal vaccines from *S. sonnei* and *S. flexneri* and two respective virulent strains used for homologous and heterologous challenges. The summarized data for these experiments (Table 3) showed that the protective effect of each SRV preparation was species specific.

Safety and protection experiments in monkeys. At the beginning of these experiments, the safety of SRV was determined by injection of different doses of the vaccine (Table 4, experiments 1 to 3). A mild local reaction (induration of less than 1.6 cm in diameter) was seen in only 1 of 29 monkeys injected with 600 μg of SRV, while very high doses (3,000 to 5,000 μg) caused more intense reactions (induration, 1.6 to 2.5 cm) in 3 of 4 animals. No differences were found in leucocyte count, erythrocyte sedimentation rate, or body temperature when the individual levels before and after vaccination were compared. It must be noted, however, that all of these characteristics were highly variable in monkeys.

The results of protection experiments are presented in Table 4. It is evident that the challenge dose was high enough to evoke a clinical infection in all control monkeys. The typical symptoms included watery diarrhea and general intoxication, while typical dysentery with blood or mucus in stools was observed only in several cases. As a rule, the illness continued for 2 to 3 days only, and therapy was needed only in a few cases. Several conclusions could be drawn from the data presented: (i) SRV was highly protective in all experiments, (ii) the effect of 600 μg was the same as that of 3,000 to 5,000 μg, (iii) no difference was found between preparations obtained by ultracentrifugation or by PEG fractionation, (iv) one injection of SRV was enough to induce resistance to *Shigella* infection in monkeys, and (v) the protective effect of vaccination was quite evident 23 weeks after two injections or 13 weeks after one injection of SRV (observation times). If the results of all monkey exper-

TABLE 4. Protective activity of SRV in monkeys

Expt	Immunization[a]			Challenge		Results of challenge[c]			
	Vaccine	Dose (mg)	No. of injections	Time (wk)[b]	No. of monkeys	Without symptoms	Intestinal reaction	Disease	
								Diarrhea	Dysentery
1	SRV-U	600–1,000	2	4	5	5			
	SRV-U	3,000–5,000	2	4	4	4			
	None				2			2	
2	SRV-U	600	2	4	5	4		1	
	SRV-U	600	2	13	5	4		1	
	SRV-U	600	2	22	5	3	1	1	
	None				4			4	
3	SRV-PEG	600	2	5	5	5			
	SRV-PEG	600	2	16	5	5			
	None				5			5	
4	SRV-PEG	600	1	4	4	1	2	1	
	SRV-PEG	600	2	4	4		2	2	
	None				5			3	2
5	SRV-PEG	600	1	4	5	5			
	SRV-PEG	600	1	13	6	4	2		
	None				5			3	2

[a] SRV was prepared either by ultracentrifugation (SRV-U) or by PEG fractionation (SRV-PEG). In experiment 1, the dose of vaccine was 200 or 1,000 μg/kg of body weight. In other experiments, the fixed dose was injected into all animals.

[b] Interval between the last injection and challenge.

[c] Intestinal reaction: 1 or 2 loose stools only on the first day after challenge. Disease: watery stools often with mucus and without blood (diarrhea) or with blood (dysentery).

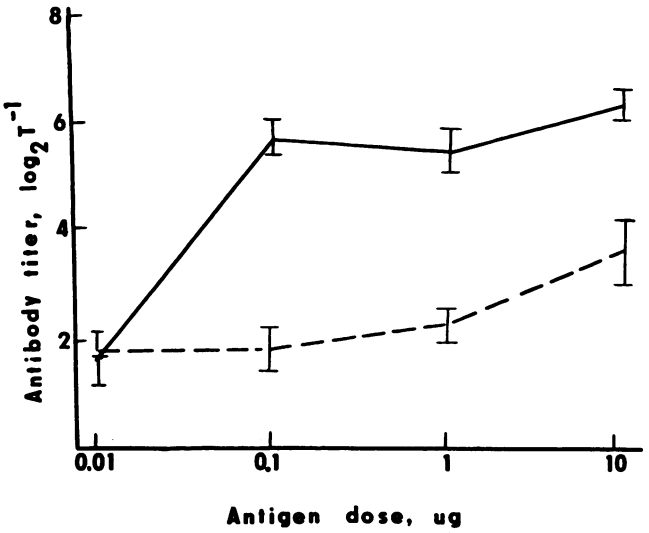

FIG. 5. Serum O-antibody response in mice injected with different doses of SRV (solid line) or endotoxin (broken line). The results of two independent experiments are summarized. Antibody titers in individual sera were determined by a PHA test on the seventh day after i.p. injection of vaccines. Seven to 10 mice were used for each antigen dose. Standard errors of the mean are shown by vertical bars. T, titer.

iments are summarized irrespective of vaccine dose, production method, immunization schedule, and other experimental details, the total morbidity is 6 of 53 vaccinated monkeys (11%), in contrast to 100% morbidity in 21 control monkeys (efficiency of vaccination, 89%).

Bacteriological analyses were made daily during the 5 weeks after challenge, and an animal was defined as a pathogen excretor if at least one coproculture was positive during a week. The numbers of excretors on weeks 1, 2, and 5 were constant in 21 control monkeys, 21 (100%), 18 (86%), and 19 (90%), but diminished in 53 vaccinated monkeys, 53 (100%), 34 (64%), and 7 (13%). The same acceleration of pathogen clearance in immunized monkeys was evident when the percentages of positive cultures were calculated. At the end of the observation period, these indexes amounted to 18% in vaccinated animals and 81% in control animals ($P < 0.001$).

Systemic O-antibody response to SRV. Injections of SRV induced in mice a primary O-antibody response which was much more intense than that against endotoxin (Fig. 5). The difference was not as significant when secondary responses were compared.

In guinea pigs, one s.c. injection of SRV induced a rather high level of serum O antibodies: titers of 1:80 and higher were found in 50% of animals 1 week after immunization with 50 to 200 μg of ribosomes. In most cases, the O-antibody titers declined slowly during the next weeks but remained elevated, even at the fifth week postvaccination. No correlation was seen between the individual O-antibody titers in sera and the resistance to challenge at 2 weeks after immunization.

SRV was much more active in inducing a primary O-antibody response than was endotoxin injected in the same doses (Table 5). This result is in contrast to the comparative activities of these preparations in the O PHA inhibition test, as shown above, SRV was 200 to 1,000 times less active than was endotoxin.

TABLE 5. Serum O-antibody response in guinea pigs injected parenterally with SRV or endotoxin

Vaccine[a]	No. of animals	% of animals with an O-antibody titer of:				Mean antibody level[b]
		<10	20–40	80–160	>320	
SRV	78	13	36	46	5	1:52 (5.7 ± 0.2)
Endotoxin	17	59	35	6	0	1:11 (3.4 ± 0.2)
None	12	67	33	0	0	1:14 (3.5 ± 0.2)

[a] Both vaccine preparations were injected s.c. in a single dose of 50 to 200 μg. The serum O-antibody titer was determined on the seventh day.

[b] Geometric mean titer ($\log_2$ of the reciprocal titer ± standard error of the mean).

In monkeys, 600 μg of SRV injected s.c. led to an increase in the mean O-antibody titer in the serum from below 1:10 to 1:32 or 1:550 (in different experiments) on the seventh day, with a subsequent decrease to 1:56 or 1:40 at the sixth week postvaccination.

DISCUSSION

The properties of the ribosomal preparations used in this study comply with the known properties of bacterial ribosomes (58) in terms of RNA/protein ratios, UV absorption spectra, sedimentation characteristics, and extinction coefficients. The carbohydrate contamination detected by chemical analysis represented from 3 to 10% of the preparations by weight.

The possible contamination of SRV with LPS is especially important in the context of the discussion of the role of LPS in the protective effect of different ribosomal vaccines (9, 17, 32, 50, 51). The presence of whole LPS molecules cannot be proved without a chemical analysis for the core or lipid A component, and the sensitive carbocyanine dye assay (15, 31) seems to be especially useful in the study of different ribosomal vaccines (14, 24). However, the negative results of this assay with our ribosomal preparations turned out to be not reliable. In model experiments done by one of us (14a), both RNA and ribosomes interfered with the reaction between LPS and carbocyanine dye, preventing the metachromatic shift that is characteristic of the lipid A portion of LPS. The negative results of the KDO determination are much more reliable evidence against the significant contamination of SRV with LPS. However, the ribosomes were capable of neutralizing O-antibodies in a PHA inhibition test, induced O-antibody formation in animals, and reacted with O antiserum in IEP. The results of all of these tests can be easily explained if the ribosomal O-specific component is not composed of whole LPS molecules but is a polysaccharide moiety which has O-antigen specificity and which is similar to the cytoplasmic precursor of LPS composed of polymerized O side chains (37). An O antigen of this type has been described in many bacterial species (4, 6, 44), particularly in *S. sonnei* (33). It was also detected in the strain used in this study to produce ribosomal preparations (16).

The toxicity of SRV in mice is extremely low; doses of up to 2,000 and even 5,000 μg caused no deaths when injected i.p. or i.v. No toxic effect of SRV was seen after parenteral injection of several milligrams into monkeys, as well as rats and guinea pigs (data not shown). Local reactions in monkeys were moderate or absent. It can be concluded that SRV is well tolerated by experimental animals and can be injected parenterally without any appreciable risk of adverse reac-

tions. Such a low toxicity is typical of most ribosomal vaccines (18, 26).

The protective activity of SRV in mice is very high, since several nanograms of ribosomes produce a significant effect. However, it is difficult to relate these data to human dysentery, since the crucial step in the pathogenesis of the latter—epithelial cell invasion (21, 34)—is not reproduced in parenterally challenged mice. The mouse model probably can characterize the endotoxic component of shigellosis, an important determinant in the progress of infection (10).

The guinea pig model of shigellosis seems to be very valuable for testing *Shigella* vaccines because of its similarity to human shigellosis in terms of basic pathogenic mechanisms and virulence factors. It is especially useful in the case of parenteral vaccines which present no problems with antigen delivery and dosage. Numerous experiments with different batches of SRV led to the following conclusions. (i) Parenteral SRV provides highly significant ($P < 0.001$) protection of guinea pigs against local *Shigella* infection. (ii) SRV is effective when given in a single injection without any adjuvant. (iii) The protective effect of SRV is species specific, as neither ribosomes from *E. coli* and *S. minnesota* nor ribosomes from *S. flexneri* protected against challenge with *S. sonnei* and no protection was seen when animals were immunized with *S. sonnei* ribosomes and challenged with virulent *S. flexneri*. (iv) SRV prepared by PEG fractionation had the same activity as that prepared by ultracentrifugation. Some comments on these results are needed.

The fact that parenteral vaccination is capable of inducing effective protection against local infection caused by *S. sonnei* is not a trivial one. Recently, the protective effect of parenteral immunization with surface proteins of *S. flexneri* was reported (1, 52). Ribosomes of *S. flexneri* were also protective in guinea pigs (7).

The specificity of the protective action of SRV is evident from the cross-challenge experiments. Ribosomes from *S. sonnei* and *S. flexneri* protected guinea pigs only in the case of a homologous challenge. The most probably interpretation of these data is that the O antigen, which is the main species-specific antigen of *Shigella* species, must in some way be involved in the protection induced by SRV.

In monkeys, usually regarded as the most reliable model of human dysentery, the protective effect of SRV was highly evident. The high virulence of the strain used for challenge is evident from the fact that watery diarrhea was seen in almost all unimmunized monkeys, and in some of them, true dysentery developed. Even under these conditions of challenge, SRV provided long-lasting protection, the efficiency of vaccination being 89% (as calculated from the summarized data of all the experiments).

A noteworthy detail of the monkey protection experiments is that a clear-cut protective effect of SRV was not accompanied by an immediate elimination of pathogens; the accelerated clearance of pathogens in immunized monkeys became evident only at 5 to 7 weeks after challenge. A similar observation was made recently with an effective live vaccine (2). It is possible that in both cases the defense mechanism is directed primarily at the epithelial cell invasion step and has little influence on the initial colonization of the intestinal lumen of challenged monkeys. Evidently, pathogen excretion in monkeys challenged with many billions of *Shigella* cells has little in common with that in human shigellosis, which can be elicited by several microorganisms.

The successful vaccination of monkeys with SRV is in evident contrast with early experiments of parenteral immunization of monkeys with viable bacteria, which was found to be unsuccessful (23). The absence of a protective effect of such a vaccine is often interpreted as evidence of the ineffectiveness of parenteral vaccination against shigellosis in general (20, 25, 28, 64). The significant effect of SRV in monkey protection experiments suggests that local protection against shigellosis is actually attainable by parenteral immunization, provided that an antigen is presented in suitable form. Being completely safe and highly protective in the doses used, SRV seems to meet these most important requirements for a candidate vaccine.

Ribosomes obtained by PEG fractionation had the same activity in monkey protection experiments as did SRV prepared by ultracentrifugation. This result is in agreement with those from experiments with guinea pigs and seems to be promising as the basis for the large-scale production of an inexpensive ribosomal vaccine, if required. Precipitation of ribosomes by PEG was studied in detail by Expert-Bezançon et al. (19) as a way to obtain synthetically active preparations. However, such ribosomes are heavily contaminated with endotoxin and therefore cannot be used as a vaccine. In the technique used in our study, SDS was included in buffer solutions and an additional purification step was introduced to remove endotoxin and to make the procedure suitable for vaccine production.

SRV is a very potent inducer of the systemic O-antibody response in all animal species studied. When compared with endotoxin on the basis of O-antigen content (as determined by the O PHA inhibition test), ribosome-associated O-antigen will have antibody-inducing activity 2 to 3 orders of magnitude higher than that of endotoxin, which is recognized as the most immunogenic molecular form of O-antigen (29).

The role of serum O antibodies in immunity to *Shigella* cells is unclear. Recently, some correlation was found between the level of these antibodies and resistance to *Shigella* infection in humans (11, 12). It is possible that they can be of value as an antiendotoxic component of the immune defense against *Shigella* infection or in preventing the spread of pathogens. Parenteral SRV is also capable of inducing a very intensive immunoglobulin A O-antibody response of the mucosal immune system in guinea pigs, rats, and monkeys; these data were analyzed in separate studies (36, 46).

The unusually high protective activity of SRV and its capacity to induce an intense mucosal and systemic O-antibody response could be explained on the basis of the concept of a vaccine complex which consists of two parts with quite different functions (3). The haptenic O polysaccharide represents an antigen-specific (epitope-bearing) part which is a very weak antigen when used alone (35), while the ribosomal particle represents an important auxiliary part which is responsible for the effective targeting and presentation of O antigen to the cells of local and systemic immune systems. Vaccine components of this type are well known under the names delivery system, vehicle, and carrier, etc., without clear-cut differences between the terms. Taking into account the fact that the protective immunity induced by the ribosomal vaccine is species specific, the main function of the ribosomal vector could be related to O polysaccharide, the immunogenicity of which is amplified by this association. Some protein antigens can also be involved in the immunogenic effect of SRV. The existence of a species-specific protein of ribosomal origin in *Shigella* cells was assumed on the basis of immunochemical studies (5), while the significance of an extrinsic protein in the immunogenicity of a ribosomal vaccine from *Salmonella* cells was evidenced by

Johnson and coworkers (32, 51). Recently, the polypeptides related to lipid A-associated protein were detected in SRV by an immunoblot technique (34a).

The role of the ribosomal particle as a highly efficient delivery system for weak antigens is in line with the hypothesis of a superantigen which has been proposed by Berry et al. (9) to explain the high immunogenicity of *Salmonella* ribosomes and which can be applied to the recent data on the importance of different delivery systems for the immunogenic effects of antigens. Most of the results obtained with other ribosomal vaccines can be understood on the basis of this concept. It can also explain the early observations of Belozerski and Gekker, who reported in 1948 on the enhanced immunogenicity of a *Shigella* somatic antigen associated with the nucleoprotein fraction of the cell (8). It is probable that the formation of immunogenic antigen-ribosome complexes is not a unique feature of ribosomal vaccines but is a usual way of antigen presentation in numerous situations when the lysis of bacterial or animal cells occurs in vivo and ribosomes are released from cells. Immunogenic complexes of ribosomes with antigens of different origins (viral, bacterial, or tissue) would be of considerable importance as amplification mechanisms which increase the efficiency of antigen presentation and intensity of the immune response.

An intensive study of ribosomal vaccines in the 1970s led to the conclusion that their effect is due to the effective presentation of antigen rather than to some exotic mechanism of reverse transcription. In the 1980s the interest of immunologists switched to modern approaches like synthetic, conjugate, and recombinant vaccines, and investigations of ribosomal vaccines almost stopped. Although most ribosomal preparations were proved to meet the two main requirements for a vaccine—efficiency and safety—this field of vaccinology has now been almost abandoned and, accordingly, the overall situation does not seem favorable for SRV. Nevertheless, the results of experimental studies make this preparation a promising candidate vaccine that needs to be evaluated in clinical experiments and field trials.

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