

In Vitro and In Vivo Studies of Streptomycin-Dependent Cholera Vibrios

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Streptomycin-dependent cholera vibrio strains were derived from Inaba, Ogawa, and NAG vibrios by the method of Mel. These phenotypes grew more slowly and attacked fermentable substances after a longer period of time than the streptomycin-sensitive parent strains. Rabbits injected with streptomycin-sensitive strains and their streptomycin-dependent forms showed homologous agglutinin production. Patas monkeys fed with 10^9 streptomycin-dependent strains shed them for 1 to 2 days without ill effect, whereas the same number of streptomycin-independent organisms caused disease. The possibility of the application of multiple doses of streptomycin-dependent organisms in oral immunization against cholera was considered.

Interest in streptomycin-dependent (SmD) enteropathogenic organisms has been stimulated by the use of such strains as oral immunizing agents against *Shigella* (13), *Salmonella enteritidis* (17), and *Salmonella typhosa* (16) infections. Streptomycin-resistant cholera vibrios have been isolated frequently, but only one SmD strain of the Ogawa serotype of this organism has been studied from the point of view of the functions of its ribosomal particles (7). It was considered desirable to investigate further SmD *Vibrio* phenotypes and to compare their metabolic activities and immunological characteristics with those of the streptomycin-sensitive (SmS) strains from which they were derived, in preparation to the possible use of SmD mutants as oral immunizing agents against cholera.

MATERIALS AND METHODS

Cultures. The organisms selected for this experiment were *Vibrio cholerae* Inaba no. 569B (In1) received from the Haffkine Institute in Bombay, *V. cholerae* Inaba no. 204 (In2) and no. 350 (In3) cultured in Calcutta in 1963, *V. cholerae* Ogawa no. 11 (Og1) isolated in Thailand in 1959, and "non-cholera" vibrios no. 280 (NAG1), 281, and 282 isolated in the Mediterranean area from patients with diarrhea. The last group of vibrios was not agglutinable with Inaba and Ogawa sera.

Preparation of SmD strains. The "one-stage" method of Mel et al. (13; *personal communication*) was employed. Twenty-four hour cultures of strains In1, Og1, and NAG1, which were susceptible to 12.5

to 25 μ g of streptomycin (Sm) in the tube test, were streaked to Brain-Heart-Infusion (BHI) agar plates (pH 7.6 to 7.8) containing 40 mg of Sm per 100 ml. After 48 hr of incubation at 37 C, the growth was restreaked to BHI agar without and with the same amount of Sm. Daily transfers of colonies from one BHI-agar-Sm plate to another was continued until a clone was isolated that did not grow on Sm-free BHIA medium seeded with 1.2×10^{10} to 1.4×10^{10} organisms. This was accomplished with strain In1 after 32 transfers, with strain Og1 after 49, and with strain NAG1 after 64 transfers. Strain 282 has not developed an SmD phenotype after 180 transfers. The SmD clones have been maintained on media containing 40 mg of Sm per 100 ml and tested for reversal in vitro by plating approximately 10^9 organisms to Sm-free BHI agar once a week, as well as by inoculating 10-ml samples of BHI broth with 1.2 to 1.4×10^8 , 10^7 , 10^6 , 10^5 , and 10^2 SmD organisms, respectively, incubating the tubes for 3 days at 37 C, and streaking from them to Sm-containing and Sm-free BHI agar plates.

Strains In2, In3, and 281 formed SmD clones but showed reversal at the 10^6 to 10^8 level. They were not employed in further investigations.

Bacteriological media and methods. Optical and electron microscopes were used to observe the bacterial cells. Motility was determined in semisolid mannitol agar containing 0.4% agar (Difco), 1% mannitol, 1% Trypticase (BBL), 0.5% NaCl, 1% Andrade indicator, and 0.4% of a 0.25% solution of bromothymol blue (pH 7.2 to 7.4). Alkaline peptone broth (APB) was made with 1% Trypticase plus 1% NaCl (pH 7.6). Whenever possible, desiccated media (BBL, Difco, or Oxoid) were employed. Chemicals (CP) were purchased from J. T. Baker Chemical Co. (Phillipsburg, N.J.), Fisher Scientific Co. (Pittsburgh, Pa.), Nutritional Research Laboratories (Cleveland, Ohio), Mann Fine Chemicals, Inc. (New York, N.Y.), Miles

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Laboratories, Inc. (Elkhart, Ind.), and Sigma Chemical Co. (St. Louis, Mo.).

The growth curves of the SmS strains and their SmD derivatives were determined by inoculating 90 to 100 organisms in the growth phase into tubes containing 10 ml of APB, by plating 0.1-ml samples of the growth at hourly intervals to duplicate BHI agar, and by counting the number of colonies after 3 days of incubation at 37 C.

Carbohydrate fermentation was determined in Phenol Red Broth (BBL) to which 1% of the respective carbohydrate was added. Growth in the presence of NaCl was tested in 1% aqueous Trypticase solution with 3, 5, 7, and 10% NaCl, respectively, at pH 7.6.

Forty milligrams of Sm (Eli Lilly & Co., Indianapolis, Ind.) per 100 ml was added to all media in which SmD strains were tested, if not stated otherwise.

The inocula consisted of approximately 300 vibrios from a 4-hr-old BHI broth culture. Growth was observed for 2 to 8 days, according to the respective test.

Catalase was tested by the H₂O₂ method, indole formation was tested with the Kovacs reagent, utilization of citrate was tested in Koser's medium, and the Voges-Proskauer (O'Meara) and the methyl red tests in MR-VP Broth (BBL) after incubation at 35 C. Details of these tests were described by Ewing et al. (4), Edwards and Ewing (3), and Gradwohl (10). Alkaline phosphatase and β -galactosidase were determined by the procedure recommended by Brunschede and Bremer (1).

Agglutination of chicken red blood cells was performed by the method of Finkelstein and Mukerjee (8), and hemolysis by the method of Feeley and Pittman (5). Susceptibility to cholera phage was determined by the technique of Mukerjee (14). Polyvalent cholera phage was obtained through the courtesy of R. M. Sayamov of the Research Institute in Rostov-on-Don.

Susceptibility to antibiotics was estimated with the aid of Dispens-O-Discs (Difco) on BHI agar and quantitated by the standard tube-dilution method.

All tests were carried out in duplicate.

Serological methods. Sera against the vibrios employed in this experiment were prepared by injecting groups of three rabbits intravenously at 4- to 5-day intervals with 0.2, 0.2, 0.5, 1.0, and 2.0 ml of heat-killed (100 C for 1 hr) organisms and then with 0.2, 0.5, and 1.0 ml of live organisms grown on BHI agar for 14 to 16 hr at 37 C, harvested with 0.15 M NaCl and adjusted to contain 1.1×10^8 to 1.4×10^8 vibrios per ml. The rabbits were bled 5 to 7 days after the last injection and the homologous sera were pooled and stored at -20 C without a preservative.

Agglutination tests were carried out with twofold dilutions of inactivated (56 C for 30 min) sera, adding to each 0.5-ml dilution 1.2×10^6 to 1.5×10^6 live organisms suspended in 0.5 ml of 0.15 M NaCl, and incubating for 2 hr at 50 C and then overnight at 2 C. Only 3+ and 4+ reactions were considered positive.

Chemical composition. The vibrios were disintegrated by grinding according to Gilbert (9).

Protein was determined by the method of Lowry et al. (12) and N by the Kjeldahl method.

Ribosomal protein was estimated by determining

the total N, deducting the RNA N, and multiplying the result by 6.25 as recommended by Petermann (14a).

Deoxyribonucleic acid (DNA) was assayed by the diphenylamine method, reading the results in the Coleman no. 9 nephelocolorimeter at 590 nm.

Ribonucleic acid (RNA) was extracted and estimated by the procedure of Ramming and Pilch (15). The absorbance for 1 mg of RNA at 260 nm was considered equivalent to 25 optical density (OD). If necessary, corrections were made for DNA determinable by the diphenylamine reaction.

Ribosomes. The method of Tissières et al. (18) was employed, by using the second procedure [10 mM phosphate buffer (pH 7.0) with 1 mM Mg²⁺] for the separation of the 30S and 50S particles. The yield was about 10 mg of 70S per g of vibrios. The purity of the 30S and 50S ribosomal particles was approximately 80 and 90%, respectively.

Experiments in patas monkeys. Groups of four patas monkeys (*Erythrocebus patas*), weighing 4.2 to 6.1 kg, of either sex were fed with 1.2×10^9 to 1.4×10^9 vibrios through the duodenal sound after food, but not water, was withheld for 24 hr. Stool specimens were collected daily and inoculated into APB. After 4 and 24 hr of incubation BHIA, Cholera Medium (Oxoid) and McConkey plates were streaked, and the growth was examined for vibrios and other intestinal pathogenic bacteria by routine procedures (6). Both Sm-free media and media containing 100 μ g of Sm per ml were employed for the examination of each specimen. Blood was drawn before, and 10 and 30 days after, infection.

RESULTS

Whether the organisms were susceptible to or dependent on Sm, they were gram-negative, comma-shaped, and motile with one polar flagellum. Electron microscopy revealed small and elongated forms, but their proportion did not differ significantly when counted on photographs taken with the electron microscope.

Colony appearance did not permit differentiation between SmS and SmD phenotypes. SmD strains grew more slowly than the SmS phenotype, and the colonies were smaller. The growth curves of SmS strains in Sm-free APB and of SmD organisms in APB-Sm are compared in Table 1. SmS organisms did not multiply in the Sm-containing medium, and vice versa, during the period of observation. Table 1 was composed of the data obtained from experiments with the In1, Og1, and NAG1 vibrios.

The following carbohydrates were fermented by the In1, Og1, and NAG1 strains and their SmD mutants without gas formation in 1 to 2 days: dextrose, D-fructose, levulose, D-galactose, D-mannitol, D-mannose, and sucrose. L-Arabinose was attacked only by Og1SmS and Og1SmD. Cellobiose was slowly decomposed by In1 and Og1

TABLE 1. Number of colonies of streptomycin-sensitive and streptomycin-dependent vibrios in streptomycin-free and streptomycin-containing alkaline peptone broth

Time	SmS ^a organisms, Sm ^c free broth		SmD ^b organisms, broth with Sm (40 mg/100 ml)
hr			
0	90 ±	7 ^d	95 ± 3
1	179 ±	11	98 ± 9
2	1,273 ±	101	179 ± 12
3	3,789 ±	211	327 ± 21
4	11,830 ±	520	1,894 ± 60
5	102,277 ±	1,930	7,340 ± 357
6	569,310 ±	3,231	11,980 ± 325
7	711,038 ±	4,246	83,006 ± 498
8	956,342 ±	5,356	201,459 ± 879

^a Streptomycin-sensitive.

^b Streptomycin-dependent.

^c Streptomycin.

^d Mean and SD.

but not by NAG1. Maltose was fermented within 1 to 2 days by Og1 and NAG1 but in 3 to 4 days by In1. D-Sorbitol was not attacked by In1 but was split in 3 to 4 days by Og1 and NAG1. Trehalose fermentation took 3 to 4 days. None of the tested strains fermented adonitol, dulcitol, L-inositol, inulin, lactose, melezitose, melibiose, raffinose, rhamnose, salicin, or xylose within 5 days. The SmD mutant usually took 1 day longer to change the color of the indicator in the media than the SmS phenotype in spite of the presence of Sm in the broth used for the study of the SmD vibrios.

All SmS and SmD organisms were indole, methyl red, catalase, and lipase positive. Glucose was utilized both oxidatively and fermentatively in Hugh-Leifson medium. Strong acetoin formation was present only in NAG1 cultures. Citrate was utilized. Indophenol oxidase was present in all strains. Gelatin was liquefied by the SmS strains in 3 to 4 days and by the SmD vibrios in 4 to 8 days. Malonate was utilized only by both phenotypes of NAG1. NO₃ was reduced to NO₂ without gas formation by all. L-Arginine dihydrolase could not be demonstrated, but L-lysine and L-ornithine decarboxylases were produced. Phenylalanine deaminase formation, growth in the presence of KCN and in 6 to 10% NaCl, and H₂S formation were not observed. SmS and SmD NAG1 were hemolytic and agglutinated chicken red blood cells.

Alkaline phosphatase produced 0.6 ± 0.03 mmole of *o*-nitrophenol from 1.8 × 10⁸ SmS and 0.4 ± 0.05 millimole from SmD cells, whereas the β-galactoside test with 3.5 × 10⁸ vibrios resulted in the release of 20 ± 3 nmoles

of *o*-nitrophenol by SmS, and 11 ± 2 nmoles of *o*-nitrophenol by SmD strains. These numbers represent the means and SD values of six parallel determinations in each vibrio extract.

In1SmS was susceptible to the polyvalent cholera phage in 1:300, and In1SmD was susceptible in 1:50 dilution, whereas Og1SmS showed plaque formation with 1:1,000 and Og1SmD with 1:300 dilution. The NAG organisms (including no. 281 and 282) were not susceptible to this phage in 1:50 dilution.

Susceptibility to antibiotics is shown in Table 2. There was no significant difference in the sensitivity of SmS and SmD vibrios. NAG1 was resistant to polymyxin B, colistin, and novobiocin, whereas In1 and Og1 were not.

Protein, RNA, and DNA composition of the vibrios. The results of these determinations in In1SmS and In1SmD vibrios are shown in Table 3. The per cent protein values of all the cells were Og1SmS, 61.1 ± 0.6; Og1SmD, 62.1 ± 0.7; NAG1SmS, 59.3 ± 0.4; and NAG1SmD, 60.3 ± 0.6; whereas the per cent RNA values of the same cells were Og1SmS, 4.0 ± 0.4; Og1SmD, 4.2 ± 0.4; NAG1SmS, 3.8 ± 0.3; and NAG1SmD, 4.0 ± 0.4. The DNA content values were Og1SmS, 11.6 ± 0.5; Og1SmD, 12.2 ± 0.4; NAG1SmS, 13 ± 0.4; NAG1SmD, 12.6 ± 3.

The ratio of per cent protein to per cent RNA (Pr/R) was relatively constant in the various fractions. This ratio differed by more than 10% only, when the results of the analysis of 70S ribosomes derived from SmS and SmD organisms were compared. Similar differences of the Pr/R were found in the 70S ribosomes isolated from Og1SmS and Og1SmD strains, 0.61 and 0.79, and in NAG1SmS and NAG1SmD organisms, 0.67 and 0.88, respectively. The results represent the means of six determinations.

Rabbit sera. Rabbit sera were tested against homologous and heterologous organisms, before and after absorption with the other strains employed in the experiment. There was no demonstrable reaction between NAG antigens and anti-Inaba and Ogawa sera, nor did Inaba and Ogawa organisms absorb agglutinins from the anti-NAG sera. The agglutination pattern of In1 and Og1 antigens and antisera is shown in Table 4. There was a cross-reaction between Og1 and In1, due to the common A antigen. Perhaps there was also an additional component common to SmD organisms. SmS antigens appeared to produce higher agglutinin titers than did SmD organisms in the rabbits.

Responses of patas monkeys. NAG1SmS vibrios did not cause disease in four patas after the oral administration of as much as 1.3 × 10¹⁰ to 1.5 × 10¹⁰ organisms. They did not stimulate the

TABLE 2. Minimal concentrations of some antibacterial agents inhibitory for streptomycin-sensitive and streptomycin-dependent vibrios

Antibacterial agent	Inhibition ^a					
	In1SmS ^b	In1SmD ^c	Og1SmS	Og1SmD	NAG1SmS	NAG1SmD
Cephalothin.....	12.5 ^d	6.3 ^a	3.1	4.7	3.2	1.6
Chloramphenicol.....	1.2	1.2	2.3	1.2	4.7	4.7
Colistin (Colymycin).....	3.1	2.3	1.6	1.6	>50	>50
Erythromycin.....	0.8	0.8	1.2	1.2	4.7	4.7
Furadolin.....	18.7	18.7	12.5	9.4	25	12.5
Neomycin.....	9.6	4.7	6.3	3.2	4.7	3.1
Novobiocin (Albamyacin).....	4.7	3.1	2.3	3.2	>50	>50
Penicillin G.....	>100	>100	>100	>100	50	100
Polymyxin B.....	0.8	1.2	1.2	0.8	>50	>50
Streptomycin.....	12.5	>400	25	>400	18.7	>400
Sulfadiazine.....	100	150	50	25	200	200
Tetracycline.....	3.2	4.7	1.6	0.8	6.3	6.3

^a Concentration (micrograms or, in the case of penicillin G and polymyxin B, units per ml) was adjusted to nearest 0.1.

^b Streptomycin-sensitive.

^c Streptomycin-dependent.

^d Averages of two determinations, using concentrations of the antibacterial agents decreasing by one-half in consecutive tubes, to 0.781 μ g/ml.

TABLE 3. Protein, RNA, and DNA composition of *Inaba vibrio* particles

Fraction	Phenotype	Per cent protein	Per cent RNA	Protein-RNA ratio	Per cent DNA
Entire cells	SmS ^a	62.7 \pm 0.9 ^c	4.1 \pm 0.5	15.3	12.3 \pm 0.5
	SmD ^b	63.2 \pm 1.1	4.5 \pm 0.4	14	11.8 \pm 0.3
Total ribosomes	SmS	43.2 \pm 0.5	56.1 \pm 0.5	0.77	<1
	SmD	45.2 \pm 0.4	54.5 \pm 0.2	0.83	<1
70S	SmS	44.7 \pm 0.6	55.3 \pm 0.5	0.64	<1
	SmD	43.2 \pm 0.4	56.0 \pm 0.6	0.77	<1
50S	SmS	47.4 \pm 0.7	52.2 \pm 0.2	0.91	<1
	SmD	45.8 \pm 0.3	53.9 \pm 0.5	0.85	<1
30S	SmS	45.1 \pm 0.4	54.1 \pm 0.4	0.83	<1
	SmD	43.9 \pm 0.3	56.2 \pm 0.5	0.78	<1

^a Streptomycin-sensitive.

^b Streptomycin-dependent.

^c Means and sd of six determinations.

formation of circulating agglutinins. All original NAG strains (including no. 281 and 282) were then tested by W. Burrows at the University of Chicago for cholera toxin formation in the rabbit ligated intestinal loop, with negative results (*personal communication*).

A single oral administration of 1.2×10^9 to 1.4×10^9 In1SmD or Og1SmD organisms gave the same results as the NAG vibrios. The period of excretion of NAG, In1SmD, and Og1SmD was short, usually less than 2 days (Table 5), whereas In1SmS and Og1SmS were passed with the stools for several days. The SmS phenotypes caused some diarrhea and a significant increase (>1:40) of the homologous serum agglutinin titers.

All successfully infected animals recovered without ill effect within 2 to 3 days after cessation of the diarrhea.

DISCUSSION

Streptomycin-dependent (SmD) phenotypes of vibrios were isolated by the "short" method of Mel et al. (13; *personal communication*). Such mutants could not be derived from all seven strains tested. It is not yet known if this failure is related to the Sm-susceptibility of some organisms.

The SmD phenotypes did not show significant deviations from the usually observed biochemical pattern of cholera vibrios as described by R.

TABLE 4. Agglutination tests with sera against streptomycin-susceptible and streptomycin-dependent *Inaba* and *Ogawa* vibrios

Serum against	Absorbed with	Reciprocal agglutination titer with antigen			
		In1SmS ^a	In1SmD ^b	Og1SmS	Og1SmD
In1SmS	0	1,280 ^c	960	240	240
	In1SmS	0 ^d	0	0	0
	In1SmD	80	0	0	0
	Og1SmS	640	640	0	40
	Og1SmD	480	480	0	0
In1SmD	0	320	480	60	60
	In1SmS	0	40	0	0
	In1SmD	0	0	0	0
	Og1SmS	160	160	0	40
	Og1SmD	240	120	0	0
Og1SmS	0	120	120	960	640
	In1SmS	0	0	480	480
	In1SmD	0	0	640	480
	Og1SmS	0	0	0	0
	Og1SmD	0	0	0	0
Og1SmD	0	80	80	240	320
	In1SmS	0	0	120	240
	In1SmD	0	0	120	120
	Og1SmS	0	40	0	40
	Og1SmD	0	0	0	0

^a Streptomycin-sensitive.^b Streptomycin-dependent.^c Means of two determinations.^d Less than 40.

TABLE 5. Response of patas to orally administered streptomycin-sensitive and streptomycin-dependent vibrios

Observation	In1		Og1		NAG1	
	SmS ^a	SmD ^b	SmS	SmD	SmS	SmD
Diarrhea (days)	1.5 ^c (1-2)	0	1.8 (0-3)	0	0	0
Excretion of vibrios (days)	4.3 (2-7)	1.3 (1-2)	4 (2-6)	1.3 (0-2)	1.3 (0-2)	1.3 (1-2)
Serum agglutinin titer ^d	400	0 ^e	380	0	0	0
	(160-640)		(80-1280)			
30 days	140	0	110	0	0	0
	(80-320)		(40-320)			

^a Streptomycin-sensitive.^b Streptomycin-dependent.^c Means to nearest 0.1 and (range), groups of 4 animals.^d Reciprocal titer, mean to nearest 5 and (range).^e Twenty or less.

Hugh (Bacteriol. Proc., p. 52, 1962) and Ewing et al. (4). The NAG strains resembled Heiberg group I *V. cholerae* biotype El Tor. SmD strains grew more slowly, which may explain their tardy action on fermentable substances. The signifi-

cantly lower alkaline phosphatase and β -galactosidase activity of SmD vibrios, however, could be ascribed also to retarded or unusual protein formation rather than merely to their slower multiplication because SmS and SmD organisms

used in the experiments with these enzymes were equal in number, and the preparation time was too short to influence the results of the tests carried out with the respective vibrio extracts.

The decreased susceptibility of SmD strains to cholera phage requires further investigation.

Sm apparently prevents the attachment of protein to RNA in the ribosomes in vibrios, and the functional ribosome form appears to be the 70S (30S + 50S) particle (7). In the present studies, the Pr/R ratio of SmS and SmD vibrios did not differ significantly, with the exception of 70S ribosomes in which less material reacting in the Lowry et al. (12) test as protein appeared to be present in proportion to the per cent of RNA. This may be a reflection of the influence of Sm on the protein-synthesizing apparatus at the functional ribosome in SmD organisms.

In spite of some differing characteristics of the SmD phenotypes, their parenteral administration to rabbits caused significant agglutinin production but at a somewhat lower level than the parent SmS strains. It has to be seen whether this observation was fortuitous or whether it holds true also with other strains. We recorded similar results with SmS and SmD shigellae (D. Mel, O. Felsenfeld, and B. Cvjetanović, *Bull. World Health Organ.*, *in press*).

Cholera is a disease in which the organisms do not circulate in the bloodstream and, as a rule, do not enter the intestinal mucosa. The illness is caused by vibrio toxins liberated on the epithelial surface of the gut. Antitoxic immunity is essential to prevent the development of disease, but additional means may have to be sought to further antibacterial local (intestinal) defense that is not necessarily reflected in the appearance of circulating antibodies, the former being primarily a function of the intestinal immunocyte system [reviewed by Burrows (2) and Heremans (11)].

The SmD organisms appeared to be of low pathogenicity. One single oral administration of 10^9 live SmD Inaba and Ogawa organisms did not cause disease, nor the appearance of agglutinins in the sera of patas monkeys, whereas the same number of SmS vibrios of the parent strains was followed by diarrhea and the development of circulating antibodies. This phenomenon closely resembles the lack of symptoms and demonstrable serum antibodies when man or monkeys were given one single dose of live SmD *Shigella* (13; D. Mel, O. Felsenfeld, and B. Cvjetanović, *Bull. World Health Organ.*, *in press*) or SmD *S. typhosa* (B. Cvjetanović, D. Mel, and O. Felsenfeld, *Bull. World Health Organ.*, *in press*) per os, whereas 3 to 5 oral immunizations with these organisms were necessary to convey a significant degree of immunity. Therefore, the

administration of multiple doses of SmD cholera vibrios had to be considered. Experiments are under way to investigate whether repeated oral administrations of SmD vibrios are as feasible for eliciting homologous local (intestinal) immunological responses as are multiple doses of SmD shigellae and typhoid bacilli. Preliminary investigations showed that the oral administration of 5 doses of 1.2×10^9 to 1.5×10^9 Og1SmD organisms each, within 10 days, protected patas against a challenge with 1.1×10^8 to 1.3×10^8 Og1SmS organisms, but further elaboration and quantitation of this vaccination procedure are necessary before this method can be standardized and, eventually, applied to man.

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