to the pathogenesis of the late phase of increased vascular permeability due to heat injury. The role of these lysosomes in other forms of vascular injury and their possible mode of action was discussed.

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Biological Properties of Freeze-Dried Attenuated Shigella Vaccines. (31724)

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We have described 2 attenuated strains of *S. flexneri* 2a which when employed as living oral vaccines rendered monkeys resistant against experimental oral challenge(1,2,3). One of these strains was a mutant of a virulent strain and was incapable of causing disease because it had lost the ability to penetrate the intestinal epithelium and reach the lamina propria(3). When fed this organism, experimental animals acted no differently from other animals fed *Escherichia coli*. Five oral doses of the mutant conferred resistance(2). The other vaccine strain was obtained by bacterial recombination of the virulent *S. flexneri* 2a with an Hfr. *E. coli* strain. The hybrid retained the capacity to reach the lamina propria but was unable to multiply to any great extent in this region(1).

Animals fed the hybrid developed an inflammatory reaction in the intestine which rapidly subsided. A single dose of the hybrid strain sufficed to confer resistance(2).

All of our previous work in attempting to confer resistance on monkeys against experimental challenge with virulent shigellae has involved the use of living oral vaccines prepared from freshly harvested bacterial cells. Should this procedure ever be considered for practical use, it is obvious that preserved preparations would be required since in most instances it would be difficult if not impossible to prepare fresh material. Furthermore, and of greater importance, it would not be possible to perform the necessary safety tests on freshly grown bacteria prior to their administration. The use of lyophilized vaccines was next considered, and this report describes some of the biological properties of freeze-dried vaccines prepared from either the mutant or the hybrid strain.

Materials and methods. Bacterial cultures. *S. flexneri* 2a mutant strain 24570 and hybrid strain X16 have been described(1,2,3). Virulent *S. flexneri* 2a strain M42-43 was was isolated from monkeys with dysentery following oral challenge with *S. flexneri* 2a strain 2457T which had been used in all of our previous work. All strains were maintained in the lyophilized state, and a new ampule was opened for each experiment.

Preparation of vaccine. A freeze-dried ampule of the stock strain was rehydrated with Brain Heart Infusion (BHI) broth (Difco) and streaked on nutrient agar plates. After incubation for 18 hours at 36°C, 6 to 10 representative colonies were emulsified into 5 ml BHI broth, and this suspension was then transferred to a seed bottle containing 600 ml BHI broth. After incubation at 36°C for 4 to 5 hours, 2 ml of the seed culture was spread over the surface of Kolle flasks which contained 60 ml BHI agar. The Kolle flasks were incubated in the inverted position for 18 hours at 36°C. Eight ml of BHI broth was then added to each flask and the bacterial growth was harvested with a rake and pooled in a single container held at 4°C. Immediately after harvesting, a 2 ml sample of the pool was removed for dilution plate counts of viable organisms and 10 ml portions of the remainder used to fill vaccine bottles whose capacity was 30 ml. Stoppers were partially inserted, and trays of bottles were placed directly on the cold shelf (—44°C) of a freeze-dryer chamber. Vacuum was applied to the chamber 3 hours later when product temperature reached —36°C as measured by an internal thermocouple. The total drying time was 42 hours including 12 hours terminal drying at +28C. The final pressure reading in the chamber as measured by a Pirani gauge was 30 μ. The stoppers were seated under vacuum in the chamber by a pressure plate and the vacuum in the chamber was then released. Aluminum seals were applied to the bottles, each bottle checked with the Tesla coil for vacuum, and the product stored at 4C.

Biological tests. Tests for the ability of various strains to invade HeLa cells, to produce keratoconjunctivitis, to cause a fatal infection in guinea pigs, and to protect monkeys against experimental challenge have been described(2,3,4,5).

Results. The vaccine prepared from cells of the mutant strain was freeze-dried on May 7, 1964. The viable count of the cell pool before drying was 4.2×10^{10} cells per ml. After drying the contents of a vaccine bottle was resuspended to its original volume with sterile distilled water; the viable count on the preparation was 5.5×10^{10} cells per ml. After storage of the dried vaccine for one month, the viable count was 2×10^{10} cells per ml and after 2 years storage (May 6, 1966) the count obtained was 2.5×10^{10} viable cells per ml. The vaccine made from cells of the hybrid strain was lyophilized on Sept. 3, 1964. The viable count before drying was 2.5×10^{10} cells per ml; 1 week after drying the viable count of the reconsituted product was 7×10^{9}. After storage for 2 months the viable count of the dried vaccine was 6×10^{9}, and the same value was recorded when counts were made from the dried product 19 months after drying.

Freshly reconstituted vaccine of either the mutant or hybrid strain and freshly harvested suspensions of 18 hr agar-grown virulent *S. flexneri* 2a were fed to guinea pigs which had been starved for 5 days. Approximately $1 \times$

10^8 viable organisms were administered and, after challenge, 1 ml tincture of opium was injected intraperitoneally. By 72 hours post challenge, 1 of 29 animals fed the mutant vaccine and 1 of 20 guinea pigs fed the hybrid vaccine had succumbed, while 20 of 31 animals which received virulent *S. flexneri* 2a had died. Histological and fluorescent antibody studies on the intestines of these animals indicated that the mutant cells failed to evoke an inflammatory reaction or to invade the lamina propria. In contrast, cells from the hybrid vaccine passed the intestinal epithelial barrier and caused an inflammatory response.

Tests for the ability to produce keratoconjunctivitis and to invade HeLa cells were next conducted. Approximately 1×10^8 viable cells from freshly rehydrated bottles of vaccine were dropped into the conjuctival sacs of guinea pigs. None of 11 animals which received the mutant cells developed keratoconjunctivitis, while all of 4 animals with the hybrid cells had positive reactions. In tissue cultures tests the mutant cells failed to invade the HeLa cells while the hybrid cells did.

The two freeze-dried preparations were tested for their capacity to protect monkeys against experimental challenge. In the case of the mutant vaccine, 5 doses of 5×10^{10} cells suspended in 20 ml BHI broth were fed at intervals of 4 days. All of the animals which received the vaccine passed the organism in their stool at one time or another during the course of feeding. Following the last vaccine dose, we failed to isolate the vaccine strain from 4 animals; 18 animals shed for no longer than 4 days; from 1 animal we isolated the vaccine strain on the fifth and from another on the seventh day after the last vaccine dose. One animal had a mild diarrhea during the course of vaccine administration. The animals were challenged with 5×10^{10} virulent *S. flexneri* 2a 10 days after the last vaccine dose was administered. This protection test was carried out 23 months after the vaccine was dried, and the results summarized in Table I indicate that a significant degree of protection was conferred. Only 1 of 24 animals which received the vaccine exhibited any signs of illness and this consisted of a mild diarrhea which subsided. On the other hand 14 of 24 control monkeys had signs of enteric disease, and 11 of these had dysentery with blood and inflammatory cells in the diarrheal stool. The animals in this experiment were not treated with antibiotics, and 7 animals in the control group died.

In the test carried out with the hybrid vaccine, 2 doses of 3.5×10^{10} cells suspended in 20 ml BHI broth were fed at an interval of 1 week and the animals challenged 14 days after the last vaccine dose. This test was performed 19 months after the product was dried. Of the 20 monkeys fed the vaccine, we failed to isolate the hybrid strain from 7 at any time. It should be noted that these animals still responded with a rise in serum antibody levels (see below). Following the last dose, we isolated the vaccine strain once from 6 animals, and the remaining 7 animals shed intermittently. Two animals had a diarrheal stool during the course of vaccine administration. Following experimental challenge, the monkeys which received the vaccine were protected (Table I). Two of 20 vaccinated animals developed mild diarrhea which subsided without treatment, while 17 of 20 control animals presented evidence of enteric disease. Of these 11 had dysentery and were treated with antibiotics when blood was observed in the diarrheal stool.

Two further protection tests were done with the lyophilized hybrid vaccine. While 2 doses of vaccine were fed, the number of organisms per dose was reduced; in one experiment the number of viable organisms per dose was 7×10^9 and in the other 1.4×10^9 bacteria per dose. The pattern of shedding in both of these experiments was similar. We failed to isolate the vaccine strain from the feces of approximately 50 % of the animals which received 2 oral doses. Animals which shed the vaccine strain after the second dose did so for no longer than 2 days with the exception of 2 monkeys from which the hybrid strain was isolated on the fifth post-vaccinal day. Three of 18 animals receiving 7×10^9 cells and 3 of 19 animals fed 1.4×10^9 hybrid bacterial had diarrheal stools during the course of vaccine administration. The animals

were challenged 11 days after the last vaccine dose. In the experiment in which the vaccine group was fed 2 doses of 7×10^9 cells, significantly fewer vaccinated animals became ill than did controls (Table I). In the experiment in which the dose was reduced to 1.4×10^9 cells, there was no significant difference in the incidence of illness between the vaccine and control groups (Table I).

TABLE I. Signs of Illness in Vaccinated and Control Monkeys Challenged 10-14 Days After the Last Vaccine Dose with Virulent *Shigella flexneri* 2a.

		Vaccine group			Control group			
Lyophilized vaccine	Dose*	No. with diarrhea	No. with dysentery†	Total ill/ Total challenged	No. with diarrhea	No. with dysentery	Total ill/ Total challenged	p, vaccine *vs* control
Mutant	50×10^9	1	0	1/24	3	11 (6 died)	14/24	$<5 \times 10^{-5}$
Hybrid	35×10^9	2	0	2/20	6	11	17/20	$<2 \times 10^{-6}$
"	7×10^9	4	5	9/20	4	13 (1 died)	17/18	$<2 \times 10^{-3}$
"	1.4×10^9	5	6	11/19	7	8	15/19	$>9 \times 10^{-2}$

* Five oral doses of the mutant vaccine given at intervals of 4 days or 2 doses of the hybrid vaccine fed at intervals of 1 week were employed.

† Dysentery-diarrhea with blood mucous and inflammatory cells in the stool.

The passive hemagglutination test was employed to determine antibody levels of prevaccination serum and of serum taken 5 to 6 days after the last vaccine dose. Similar determinations were made on serum taken at the same time from control animals represented in the 4 protection experiments. The results of this study are presented in Table II. When compared to the control group, there is a suggestion that some of the animals which received 5 doses of 5×10^{10} non-penetrating mutant cells had an antibody rise, but if this was the case, the response was not a dramatic one. On the other hand, 13 of 14 animals receiving 2 doses of 3.5×10^{10} hybrid cells had a four fold or greater rise in titer. Even those animals fed 1/25th of this dose (1.4×10^9 hybrid cells) had as good and most likely a better serum antibody response than monkeys which received the high dose of mutant cells.

Discussion. The present study summarized our preliminary efforts to characterize the biological properties of freeze-dried preparations of a mutant and a hybrid strain of *S. flexneri* 2a. We were somewhat surprised to see how well both strains withstood the freeze-drying process. After an initial drop in viable count the material remained stable on storage at 4C for up to two years. We have not tested the material beyond this point.

Freshly reconstituted dried cells of either the mutant or hybrid strain retained the biological properties of freshly grown bacteria insofar as we measured them. When tested in doses comparable to those used with freshly grown cells, the freeze-dried bacteria of the mutant strain failed to cause a fatal enteric infection or elicit significant histologic changes in starved guinea pigs, did not produce keratoconjuctivitis, and did not invade HeLa cells. Reconstituted dried cells of the hybrid strain while not causing a fatal infection when fed to starved guinea pigs did elicit an intestinal inflammation in these animals, produced keratoconjunctivitis and invaded HeLa cells.

When comparable doses of freshly rehydrated cells of either the mutant or the hybrid strains were used in monkeys as oral vaccines, they protected against experimental challenge in a manner similar to that observed when

freshly grown bacteria were employed. Signs of illness where they occurred in vaccinated animals were mild, consisting of diarrheal stools which returned to normal without resorting to treatment. On the other hand, many control animals had classical bacillary dysentery (blood, mucus, and inflammatory cells in the diarrheal stool) and some died when treatment was withheld.

While we have had prior experience in using high doses ($3.5 \times 10^{10} - 5 \times 10^{10}$) of vaccine in protection tests, the present work constitutes our first attempt to evaluate the effect of employing lower doses. When 2 doses of 7×10^9 cells were used, a significant degree of protection was observed, but more

TABLE II. Serological Response* of Monkeys Fed Lyophilized Mutant and Hybrid *Shigella flexneri* 2a Vaccines.

Vaccine	Dose	Reciprocal of pre-vaccine titer	Reciprocal of post-vaccine titer								No. of animals with		
			<15	15	30	60	120	240	480	960	1920	No rise	2-fold rise	4-fold or greater rise
Mutant	5×10^{10} (5)†	<15 (14)‡	3‡	5	5	1						10	9	5
		15 (5)		2	3									
		30 (4)		1	3									
		60 (1)				1								
Hybrid	3.5×10^{10} (2)	<15 (5)			1	1	1	1	1			0	1	13
		15 (3)					2	1						
		30 (6)				1	3	1		1				
"	7×10^9 (2)	<15 (7)			4	1		2				0	3	14
		15 (2)				1	1							
		30 (3)				1		2						
		60 (4)			1				1	1	1			
		120 (1)						1						
"	1.4×10^9 (2)	<15 (7)			1	4	1		1			4	3	11
		15 (0)												
		30 (3)						2			1			
		60 (3)					1	2						
		120 (5)						2		2	1			
Control§	None	<15 (13)	12	1								34	5	0
		15 (10)	3	3	4									
		30 (10)	2	2	4	2								
		60 (4)			2	2								
		120 (2)			1	1								

* The passive hemagglutination test was used to determine antibody levels.
† No. of doses fed.
‡ No. of animals with corresponding pre- or post-vaccine titer.
§ Control animals were bled at the same time as the vaccinated group.

vaccinated animals became ill than when the high dose of vaccine was employed. Still lower doses (1.4×10^9 viable cells) failed to confer resistance. We do not know if vaccines prepared from freshly grown cells would protect at this dosage level. It should be emphasized that these protection tests were not carried out at the same time. Thus, we are somewhat encouraged by obtaining at least a semblance of a dose-response reaction, for we can recall numerous experiments in assaying typhoid vaccine using inbred strains of mice in large numbers where acceptable dose-response data were not obtained.

Our initial experience gained from a limited number of monkeys indicated that freshly grown living cells of either the mutant or the hybrid strain failed to evoke a predictable serum antibody response when administered by the oral route(2.) In the case of the hybrid strain the observations were made on only 4 animals. Subsequent studies on larger number of monkeys have demonstrated that our preliminary assessment was incorrect in the case of hybrid bacteria, for when given by the oral route these do elicit a humoral antibody response in a large proportion of animals (4,5, Unpublished data). The results of our studies on antibody response to orally administered reconstituted freeze-dried vaccines are similar to those obtained with newly harvested bacteria. In the case of the mutant strain only 5 to 24 monkeys experienced a 4-fold or greater rise in circulatory antibody levels whereas 13 of 14 animals receiving the freeze-dried hybrid strain responded. It is important to note that animals receiving even low doses (1.4×10^9 cells) of the hybrid strain had better serological responses than monkeys receiving high doses of the mutant. Yet the former group was not protected against challenge while the latter was indicating that circulating antibody is not the decisive factor in rendering animals resistant.

Thus, the present study demonstrates that lyophilized preparations of attenuated strains of shigellae can be prepared, that after some initial drop in viable count they remain stable for periods up to 2 years at 4C and that they retain the biological activity of freshly harvested bacteria. Under these circumstances batches can be prepared on which necessary safety tests can be performed in animals prior to carrying out preliminary investigations on their efficacy in man should this be considered worthwhile.

Summary. Two *Shigella flexneri* 2a strains of reduced virulence were lyophilized and their biological properties determined. When tested 19 to 23 months after drying for their ability to cause intestinal inflammation or death in starved guinea pigs, to produce keratoconjunctivitis, and to invade HeLa cells, reconstituted freeze-dried material acted in a manner similar to that of freshly grown bacteria. Reconstituted freeze-dried cells, used as oral vaccines, protected monkeys against experimental challenge with virulent *S. flexneri* 2a. There was no correlation between the ability to produce rises in circulating antibody and the capacity to protect against illness.

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