STUDIES ON EXPERIMENTAL SHIGELLOSIS

I. Shigella Infections of Normal Mice*

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The association of members of the genus *Shigella* with diarrhea in human beings has been well established, and much is known about the epidemiology, diagnosis, and treatment of the disease; however, the pathogenesis of *Shigella* infections is not well defined. The principal obstacle to a better understanding of this aspect of shigellosis has been the lack of a suitable experimental animal. As far as is known, monkeys and higher apes are the only forms susceptible to shigellosis in nature, and there has been a notable lack of success in infecting primates experimentally without drastic manipulation (1–3). Rats, mice, and other small rodents were found to be insusceptible to ordinary challenge with *Shigella* organisms (4). By antibiotic suppression of normal intestinal flora (5, 6), or by interruption of the Krebs cycle of the host (7), it has been possible either to establish long term intestinal *Shigella* infections or to induce death of experimentally challenged mice. In view of the foregoing it appeared that mice offered promise as experimental animals for the study of shigellosis and that by further altering the organisms or the resistance of the host a recognizable infection might be produced.

The present report describes the course of experimental *Shigella* infections in normal mice and is preliminary to subsequent reports on the effect of diet, physical stress, antibiotic, and chemical treatment on experimental *Shigella* infections in mice.

Material and Methods

Animals.—

Female mice of the Naval Medical Research Institute albino strain were used throughout the study. They were held 3 to 4 weeks after weaning and weighed 12 to 20 gm. at the time of use. Mouse food consisted of "laboratory chow" manufactured by Dietrick and Gambrill, Frederick, Maryland. Water was available *ad libitum*. Normal animals were caged in groups of 10 or 20 in glass jars.

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Bacteria.—

Shigella flexneri 3, strain F-824a, was employed throughout the study. This strain, which was resistant to 200 μg. of streptomycin and 100 μg. of oxytetracycline per ml. of media, was isolated in November, 1956, from a *rhesus* monkey at the National Institutes of Health. The strain shows all the biochemical and serological characteristics of *S. flexneri* 3 and has remained smooth through all manipulations. It was maintained by daily transfers on brain-heart infusion agar slants.

Media.—

MacConkey's agar, brain-heart infusion agar (BHI), and triple sugar iron agar (TSI) were the culture media used as routine, and were prepared as needed from Difco-Bacto dehydrated products. Oxytetracycline hydrochloride (Pfizer) was added to the media, when desired, to a drug concentration of 100 μg. per ml.

Bacterial Enumeration.—

The concentration of bacteria contained in pure cultures was estimated turbidimetrically in a Coleman spectrophotometer calibrated against both Petrof-Hausser direct bacterial counts and pour plate viable bacterial counts.

Cultures.—

Isolations of *Shigella* from feces were made by collecting one or more fecal pellets, which were emulsified in sterile saline, and swabbed onto MacConkey's agar.

Quantitative blood cultures were prepared as follows—: The animal was dipped into an alcohol-iodine bath. Sterile gauze was used to dry off the tail. The tail was amputated about 2 cm. from the base, and blood was collected in a sterile 20 c.mm. hemoglobin pipette. The blood was placed into sterile water to hemolyze the erythrocytes, and aliquots of the solution were diluted in saline for plating on BHI agar.

All cultures were incubated at 37°C. Bacterial colonies appearing to be *Shigella* were transferred to TSI agar slants and finally confirmed as *Shigella flexneri* 3 by slide agglutination with type-specific antiserum.

Challenge.—

(*A*) *Oral.* By means of 1 mm. diameter polyethylene tubing attached to a syringe and needle, 0.5 ml. of culture was delivered into the stomach. Two other methods of oral challenge were used for a comparison with the routine challenge method. (1) The drinking water was adulterated with culture sufficient that 0.5 ml. of imbibed water contained the challenge dose of bacteria. (2) Food pellets were soaked in 1 ml. of bacterial culture of sufficient concentration that the ingestion of one pellet gave the animal the desired challenge dose. All three oral challenge methods produced comparable infections in the animals.

(*B*) *Rectal.* A polyethylene tube, attached to a syringe, was inserted approximately 70 mm. and 0.5 ml. of the challenge dose was delivered in the region of the transverse colon.

(*C*) *Intraperitoneal* inoculation was accomplished by injecting into the peritoneal cavity 0.5 ml. of the challenge dose.

EXPERIMENTAL AND RESULTS

LD_{50} of S. flexneri 3 for Normal Mice.—

The LD_{50} (death within 24 hours) of the strain of *S. flexneri* 3 employed was determined using the method described by Reed and Muench (8). Table I shows the LD_{50} for heat-killed and for viable *Shigella* by each route of challenge. One observes that the rectal and intraperitoneal LD_{50} doses were similar. The

number of viable organisms required to produce death in 50 per cent of the orally challenged mice was ten times the parenteral LD_{50}, and it was virtually impossible to kill mice by oral challenge with heat-killed cells.

Passage of Shigella through the Stomach.—

The magnitude of the LD_{50} dose of *Shigella* for orally challenged mice suggested that the viability of *Shigella* was markedly reduced by stomach passage.

TABLE I

LD_{50} of S. flexneri 3, Strain F-824a, by Oral, Rectal, and Intraperitoneal Challenge Routes

Challenge route	LD_{50} (death within 24 hrs.)	
	Viable cells	Heat-killed cells
Oral	4.8×10^9	$>5 \times 10^{10}$
Rectal	2.8×10^8	2.3×10^9
Intraperitoneal	4.4×10^8	5.3×10^9

TABLE II

Recovery of S. flexneri 3 from the Stomach and Intestine of Mice Following Oral Challenge

Time post challenge	Per cent of mice from which *Shigella* was isolated	
	Stomach	Jejunum
hrs.		
1	60	70
2	60	60
3	50	50
4	50	50
5	50	50

An experiment was performed to determine the effect of stomach and intestinal passage on the viability of orally administered *S. flexneri* 3. Animals were sacrificed at hourly intervals after challenge with 1×10^8 viable *S. flexneri* 3. The contents of the stomach and of the jejunum were cultured on MacConkey's terramycin agar for the presence of *Shigella*.

The results, which are presented in Table II, indicate that *Shigella* may be isolated from the stomach and from the small intestine from approximately 50 per cent of mice for 5 hours after challenge. While the number of residual bacteria in the stomach or intestine was not determined, the fact that *Shigella* was recovered from only half the animals indicated that a marked loss of viability resulted during the stomach and intestinal passage.

Persistence of Intestinal Shigella Infections.—

Mice were orally challenged with 2×10^6 *S. flexneri* 3. Feces were collected from each animal at 24 hour intervals and cultured for the presence of *Shigella*. Table III shows the

number and per cent of mice from which *Shigella* could be isolated. Quantitative counts of the number of *Shigella* excreted during the first 24 hour period were also made. Fecal pellets were collected periodically during the 24 hour period following oral challenge with the terramycin-resistant *S. flexneri* 3. Immediately after collection the feces were emulsified in saline and quantitative *Shigella* counts were made by preparing BHI agar pour plates, and MacConkey agar streak plates, each with a measured aliquot of the fecal dilution. Both media contained 100 μg. per ml. of oxytetracycline (terramycin). The average number of fecal pellets per mouse

TABLE III

Daily Recovery of Shigella from the Feces of Mice after Oral Challenge with 2 × 10^8 S. flexneri 3

Time post challenge	*Shigella*-positive mice/120 challenged	Per cent Recovery
days		
1	49	40
2	32	26
3	9	7.5
4	4	3.3
5	1	0.8
6	0	0

TABLE IV

Recovery of S. flexneri 3 from Blood of Mice Challenged with 1 × 10^8 Organisms

Time post challenge	Oral			Rectal		
	No. of positive cultures	No. of mice challenged	Per cent	No. of positive cultures	No. of mice challenged	Per cent
hrs.						
1	35	96	36	28	32	87
2	23	62	37	32	32	100
3	26	82	32	47	50	94
4	8	36	22	18	20	90
5	21	81	25	19	22	87
6	5	30	16	9	10	90
24	1	10	10	12	16	75

per 24 hour period was counted and an estimate was made of the number of *Shigella* excreted in 24 hours.

An average of 3 × 10^7 *Shigella* was present in the 24 hour fecal specimen, which indicated that the organisms reproduced during intestinal passage, and that the *Shigella* recovered from the feces was not just a reisolation of residual challenge bacteria.

Blood Phase of Shigella Infections.—

Shown in Table IV are the number and the per cent of mice from which *Shigella* could be isolated from the blood at hourly intervals after oral and

rectal challenge with 0.5 ml. of a saline suspension of *S. flexneri* 3 containing 2 $\times$ 10^8 organisms per ml.

Oral challenge with fewer than 1 $\times$ 10^8 organisms markedly reduced the number of times the *Shigella* could be isolated. Oral challenge with increasing doses had only a very slight effect, and administration of two or three times the oral LD_{50} of the organism increased the culture recovery of *Shigella* from the blood less than 10 per cent. Variation of the volume of the inoculum between 0.1 and 1.0 ml. did not influence the results. In the case of rectal challenge, the administration of fewer than 1 $\times$ 10^8 organisms proportionally reduced the recovery of *Shigella* from the blood. Variation of the challenge volume from 0.1 to 1.0 ml. was without effect on the blood phase after rectal challenge. One hundred per cent of the intraperitoneally challenged animals

TABLE V

Average Number of S. flexneri 3 per Milliliter of Blood after Oral, Rectal, and Intraperitoneal Challenge of Mice with 1 $\times$ 10^8 Bacteria

Time post challenge	No. of *Shigella* per ml. of blood		
	Challenge route		
	Oral	Rectal	Intraperitoneal
hrs.			
1	3.2×10^4	6.2×10^6	3.6×10^6
3	2.6×10^4	5.6×10^6	2.6×10^6
5	3.8×10^3	5.2×10^5	3.0×10^5

showed a positive blood culture for 6 hours regardless of the volume or concentration of the challenge dose.

The number of bacteria present per unit volume of blood was also determined. The average number of *S. flexneri* 3 per milliliter of blood, 1, 3, and 5 hours after oral, rectal, and intraperitoneal challenge is shown in Table V.

The clearance of *Shigella* from the blood stream, as indicated by the data in Table V, begins to occur between 3 and 5 hours after challenge. This observation prompted subsequent work to determine whether a leucocyte reaction occurred during this period.

Fifty mice each were challenged orally or rectally with one-half an LD_{50} of *S. flexneri* 3. Blood was collected periodically from the tail for total and differential leucocyte counts. The average of the counts obtained is presented in Table VI.

Within 4 hours after either oral or rectal challenge of mice with *Shigella* a mild leucocytosis occurred with a concomitant increase in percentage of segmented neutrophiles. The leucocytosis persisted for 24 hours, returning to normal in surviving animals within 72 hours after challenge.

Pathology and Symptoms of Mice Infected wth S. flexneri 3.—

Animals which received a lethal dose of *Shigella* by the intraperitoneal route became listless and apparently ill within 2 hours. Moribund animals were rough in appearance, dull, and cold. Death occurred within 12 to 18 hours after challenge, and the only gross pathology evident at postmortem examination was slight hemorrhagic areas scattered throughout the mesentery and along the wall of the small intestine.

Rectal challenge with a lethal dose of *Shigella* caused the animals to appear ill in 2 to 4 hours. A watery diarrhea with blood and mucus occasionally occurred from 18 to 24 hours post challenge. In moribund animals an area of acute, hemorrhagic, inflammation occurred in the small intestine in the vicinity of the ileojejunal juncture. The mesentery was injected, and the liver, spleen, and mesenteric lymph nodes were enlarged. Death occurred within 18 to 24 hours after challenge.

Oral challenge with a lethal dose of *Shigella* caused mice to appear ill within 4 hours. Although no constant pathology was observed, approximately half the moribund animals presented an enlarged spleen and liver from which *Shigella* could be isolated by culture. Diarrhetic episodes occurred in approximately 30 per cent of the orally challenged mice. Rarely an area of acute, hemorrhagic, inflammation, similar to that produced by rectal challenge, was observed. Death occurred 24 to 36 hours after challenge.

TABLE VI

Mouse Leucocyte Count after Oral and Rectal Challenge with ½LD_{50} of S. flexneri 3

	Time post challenge, *hrs.*											
	0			2			4			24		
	Total	Poly	Lymph	Total	Poly	Lymph	Total	Poly	Lymph	Total	Poly	Lymph
Normal	5,000	34	62	—	—	—	—	—	—	—	—	—
Oral challenge	4,800	43	55	6,000	58	38	10,200	63	36	9,000	61	36
Rectal challenge	4,900	43	53	7,200	64	34	11,000	69	30	13,000	80	20

50 mice per challenge group.

DISCUSSION

Bacillary dysentery in human beings most probably is contracted as a result of the ingestion of food or drink contaminated with *Shigella* or by hand to mouth transfer of *Shigella* from contaminated fomites. The number of organisms necessary to cause human infection is unknown, and non-diarrheal *Shigella* infections occur with sufficient frequency to indicate that the simple administration of *Shigella* to a normally susceptible host does not by itself cause diarrheal disease. The experiments of Shaughnessy *et al.* (9) with human volunteers amply demonstrated that the ingestion of either pure *Shigella* cultures or *Shigella*-infected feces does not necessarily result in clinically recognizable bacillary dysentery. Experiments with primates in which *Shigella*

cultures were intubated into the stomach, and in which *Shigella*-contaminated food was ingested failed to produce shigellosis with reproducible results (10).

In view of the poor results using the natural host of shigellosis as an experimental animal, it is not surprising that the results described for normal mice appear to be only semipositive. While we were able to kill mice by the oral administration of *S. flexneri* 3, the number of organisms required to produce death was massive. The occurrence of a blood phase of *Shigella* after oral challenge, and the pathological changes in the spleen, the liver, and the leucocyte count, indicate that mice are susceptible to *Shigella* infections.

The blood phase in mouse shigellosis suggests that human infection may also be accompanied by a transitory bacteriemia. Such an occurrence could explain the fever and malaise which often occur during the predysenteric period of human shigellosis. While true diarrhea in *Shigella*-infected mice was observed infrequently, this manifestation of the disease is lacking also in many human *Shigella* infections. Diarrhea as a clinical symptom of shigellosis may be a syndrome which reflects other physiological aspects of the *Shigella*-infected host.

The preliminary studies described above have established the feasibility of using the mouse as an experimental animal in the study of shigellosis. Further work to study the effect of natural intestinal flora on resistance to acute bacillary dysentery, and the effect of altering the host's resistance by dietary manipulation, irradiation, physical, and chemical stress will be described in subsequent reports.

SUMMARY

Experimental *Shigella flexneri* 3 infections produced in normal mice have been described. The passage of viable *Shigella* through the stomach of orally infected animals, and the persistence of the organisms in the intestine for periods of 5 to 7 days, with an increase in bacterial numbers, indicated that true infection was produced. Blood culture studies showed that a *Shigella* bacteriemia occurred after either oral or parenteral administration of *Shigella* to normal mice. Mice infected orally revealed mild, but consistent pathological changes, including mesenteric hemorrhage, liver and spleen hypertrophy, and occasional diarrhea.

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