EXPERIMENTAL SHIGELLA INFECTIONS IN MICE

I. AN ATTEMPT TO ESTABLISH INFECTION WITH THE AID OF ANTIBIOTICS AND RESISTANCE-LOWERING AGENTS

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The failure to establish experimental Shigella infections in smaller experimental animals has been one of the major obstacles encountered when conducting studies on various important phases of dysentery. However, some fairly good results have been obtained using monkeys and chimpanzees. In view of the data on which the authors1,2) demonstrated that even the so-called non-pathogenic fungi, such as Mucor or Rhizopus, were able to produce slight or fatal infections in mice, rats or rabbits by treating them with cortisone and/or ACTH, an initial attempt was made to establish experimental Shigella infections in mice by the use of host-resistance-lowering agents and antibiotics to clear the normal intestinal microbial flora. This paper describes experimental Shigella infections induced in mice in respect to maintenance of the challenged dysentery bacilli in the intestinal tract and, also, histopathological findings on lesions in various organs, including the intestine.

MATERIALS AND METHODS

1. Animals used.

Mice strains ddN and CFW, 4 weeks old male and female, were used. Ten mice each were employed in each group. The animals were reared on “Oriental compressed diet” and water.

2. Strains used.

The following five strains of Shigella flexneri were employed:

1) Sh. flexneri 2b strain #103-S (drug-sensitive)
2) Sh. flexneri 2b strain #103-R (drug-resistant)
3) Sh. flexneri 3a strain MZ-S (drug-sensitive)
4) Sh. flexneri 3a strain MZ-R-1 (drug-resistant)
5) Sh. flexneri 3a strain MZ-R-2 (drug-resistant)
The 3 sensitive strains were isolated by Dr. Koyama, Bacteriology Section, Central Clinico-pathological Department, University of Tokyo Hospital, and the 2 resistant strains were obtained from mix cultures of these sensitive original strains and a multiple-drug-resistant strain of *Escherichia coli* strain C-5, by Dr. Yoshikawa of our department. The first and third strains were sensitive to sulfisoxazole, streptomycin, chloramphenicol and tetracycline; their growth was inhibited by 5 mcg/ml of sulfisoxazole and 1 mcg/ml or less of the antibiotics. The second strain was resistant to all of these four drugs, the fourth was resistant to streptomycin, and the fifth was resistant to tetracycline. The degree of drug resistance was such that all of the test strains grew well in the presence of more than 1,000 mcg/ml of sulfisoxazole, 200 mcg/ml of streptomycin, chloramphenicol and tetracycline following cultivation at 37°C for 24 hours in a nutrient broth at pH 7.0.

3. **Infection of animals.**

One loopful of each of the test strains, which was cultivated in the brain heart infusion broth at 37°C for 18 hours, was inoculated into the same medium and cultivated again under the same condition. The culture was then centrifuged at 3,000 r.p.m. for 30 minutes and about one fifth of the supernatant was discarded. The packed cells were resuspended in the remaining broth and the size of the inoculum was adjusted so as to contain $1 \times 10^9$ cells/ml. Animals were inoculated per os, using a stomach tube, with 0.1 ml of the suspension, that is, approximately $1 \times 10^8$ cells.

4. **Treatment for accelerating infection.**

In order to accelerate the establishment of the challenged organisms in the intestinal tract of mice, the normal intestinal microbial flora, was cleared with chloramphenicol, streptomycin and trichomycin (antifungal agent). The antibiotics were dissolved in water to concentrations of 500 mcg/ml (chloramphenicol), 200 mcg/ml (streptomycin) and 100 mcg/ml (trichomycin), and supplied to the mice through their water bottle at various periods of time in relation to the administration of the challenged organisms and the strains used. When the sensitive strains were used, the antibiotics were administered for only 2 days prior to giving the challenge dose, whereas in the case of the resistant strains, the administration was continued until no organisms could be isolated from the animals' feces.

On the other hand, in order to decrease the resistance of the host against infection, each mouse was infected subcutaneously with 0.15 or 0.25 mg of cortisol acetate (henceforth will be called just cortisone) and/or 1:100 dilution in saline of carbon tetrachloride once per day before the challenge and/or once a day for one to five days singly or in combinations with the above antibiotics. Croton oil (0.1 or 0.2 ml) was, in the same manner, given to the animals orally.
The last two agents were previously dissolved in saline solution, containing 0.1% of tween 80.

5. Observation of the results.

Animals were weighed daily and their feces were collected for bacteriological examination. The collected feces were suspended in sterile saline solution, 1 mg/ml, and homogenized. One tenth of the suspension was inoculated onto Drigalski's and SS agar media with or without chloramphenicol, 30 mcg/ml, and incubated at 37°C. The number of colonies developing on these agar plates were counted after 24 and 48 hours incubation in terms of non-lactose-fermenting, Shigella-type and lactose-fermenting, Escherichia-type colonies. Colonies resembling like Proteus and other enteric bacteria, which appeared in small numbers, were also examined. The Shigella-type colonies were examined in order to determine if they were similar to the challenge organisms. Five colonies were arbitrarily selected from the plates and examined by the slide-agglutination test and cultivated on Kligler's medium. The length of the period during which organisms were excreted along with the feces was followed in each of the experimental group of animals. These animals were treated to accelerate the infection in respect to the difference existing in the sex and strain of animals on the serotype of the Shigella species. Animals which showed negative culture of the organisms were sacrificed in order to determine if the infection was due to the challenge organisms. The heart blood and the intestinal content were cultured in the above two media.

RESULTS

1. Effects of various treatments on the maintenance of the orally inoculated sensitive strain of Shigella in the intestinal tract of mice.

Initially, the orally administered drug-sensitive strain, Sh. flexneri 2b #103-S was tested for its ability to survive in the intestinal tract of ddN strain of mice by culturing the feces of the animals. The animals were divided into the following 10 groups, according to sex of mice and method of treatments:

1) group 1: female, no treatment (control).
2) group 2: female, CM* administered.
3) group 3: female, CM + croton oil × 1 administered.
4) group 4: female, CM + croton oil × 2 administered.
5) group 5: female, CM + CCl₄ × 1 administered.
6) group 6: female, CM + CCl₄ × 2 administered.
7) group 7: male, CM + TrM** + cortisone × 3 administered.
8) group 8: male, SM + TrM + cortisone × 5 administered.
9) group 9: male, CM + TrM + cortisone × 6 administered.
10) group 10: male, CCl₄ × 2 administered.

* CM: chloramphenicol, ** TrM: trichomycin.

The results are shown in Fig. 1. In group 1, control, only 2 of the 10 mice

Fig. 1. Effects of various treatments on the maintenance of the orally inoculated sensitive strain Shigella flexneri 2b in the intestinal tract of mice.

The vertical axes indicate the numbers of mice and the horizontal axes the periods of positive isolation of the organisms from the feces (days). The denominators of the fractions indicate the number of mice showing positive isolation of the organisms, while the numerators indicate the number of animals tested. Parenthesized figures are the average number of days of positive isolation of the organisms. CM: chloramphenicol, SM: streptomycin, TrM: trichomycin. Test strain: Sh. flexneri 2b #103-S. Test animals: mice of ddN strain, male and female.
excreted a small number of the organisms only one day after receiving the challenging dose. The remaining 8 were negative. On the other hand, the groups which were treated, prior to receiving the challenging dose, male or female, showed, more or less, a significantly long period of excretion of the organisms. The female mice seemed to be more susceptible than the male in respect to the number and period of positive culture of the organisms. It was noticeable in group 10 that only one mouse gave a positive culture of the organisms for as long as 4 weeks. No animals succumbed during the observation period.

2. Effects of various treatments on the maintenance of the orally inoculated resistant strain of Shigella in the intestinal tract of mice.

The results of the above experiments suggested that various treatments, including the limited administration of antibiotics prior to the challenge with the organisms of the sensitive strain were, more or less, effective in extending the period at which the organisms could remain in the intestinal tract. As far as the drug-sensitive strains were concerned, the administration of such drugs must, at least be discontinued before inoculating the organisms. Thus, an attempt was made to use resistant strains of Shigella, during a continuous administration of antibiotics for a long period, without being exposed to the inhibitory actions of antibiotics on the growth of the intestinal flora. Therefore, the multiple-resistant strain, Sh. flexneri 2b #103-R was employed. Animals were divided into 16 groups to include some groups which were to receive limited amount of the antibiotics prior to the challenge, this is for comparative purpose.

The resistance-lowering agents, were given to mice in a manner similar to the above experiments.

1) group 11: female, no treatment (control)
2) group 12: female, CM + TrM + cortisone × 3. Antibiotics were administered only prior to the challenge of the organisms.
4) group 14: female, CM Antibiotics was administered throughout the experiment.
5) group 15: female, CM + croton oil × 1. Same as above.
6) group 16: female, CM + croton oil × 2. Same as above.
7) group 17: female, CM + CCl₄ × 1. Same as above.
8) group 18: female, CM + CCl₄ × 2. Same as above.
9) group 19: male, CM + TrM + cortisone × 3. Antibiotics were administered only prior to the challenge with the organisms.
10) group 20: male, SM + TrM + cortisone × 5. Same as above.
11) group 21: male, SM + TrM + cortisone × 6. Same as above.
12) group 22: male, CCl₄ × 2. Same as above.
13) group 23: male, CM + croton oil × 1. Antibiotics were administered throughout the experiment.
14) group 24: male, CM + croton oil × 2. Same as above.
15) group 25: male, CM + CCl₄ × 1. Same as above.
16) group 26: male, CM + CCl₄ × 2. Same as above.

Fig. 2. Effects of various treatments on the maintenance of the orally inoculated resistant strain of Shigella flexneri 2b in the intestines of mice.

See footnote of Fig. 1. Test strain: Sh. flexneri 2b #103-R. Test animals: female mice of ddN strain.
Fig. 3. Effects of various treatments on the maintenance of the orally inoculated resistant strain of Shigella flexneri 2b in the intestinal tract of mice.

See footnote of Fig. 1. Test strain: Sh. flexneri 2b #103-R. Test animals: male mice of ddN strain.

Fig. 2 gives the results obtained using female mice, and Fig. 3 as that of the male.

The six groups (groups 12, 13, 19, 20, and 21, male and female) in which the administration of antibiotics was discontinued, almost the same results as those of the experiments using the sensitive strain were obtained. However, the nine groups (the groups 14, 15, 16, 17, 18, 23, 24, 25, and 26) in which antibiotics
were continuously administered prior to and after the challenge of the organisms, singly or in combinations with the resistance-lowering agents, an increase in the number of animals giving positive isolation of the organisms was noted. The organisms were excreted by the animals for a long period, mostly about a week in both the male and female. Animals of groups 24 and 26, particularly in which all the male mice were treated with croton oil or carbon tetrachloride under continuous administration of chloramphenicol, showed a relatively long-lasting positive isolation of the organisms. Feces of a few of these groups of mice had organisms for as long as 5 weeks or more, rarely did they have diarrhea with exudate. No fatal infection was produced in these experiments.

3. Relationship between serotypes of Shigella flexneri and maintenance of the inoculated organisms in the intestinal tract.

In order to seek a more virulent Shigella strain for inducing experimental infections in mice, two serotypes of Sh. flexneri, 2b and 3a, were tested. For the

![Fig. 4. Difference in the serotypes between Shigella flexneri 2b and 3a in the maintenance of the orally inoculated organisms in the intestinal tract.](image)

See footnote of Fig. 1. Test strain; Sh. flexneri 2b #103-S, 3a MZ-S, MZ-R-1 and MZ-R-2. Test animals; male mice of ddN strain. Treatment; CCl₄ administered twice, once prior to and once after the challenge.
former, the sensitive and resistant strains #103-S and -R and for the latter, the sensitive strain MZ-S and two resistant strains MZ-R-1 and -2, were used. Groups of male mice were treated with carbon tetrachloride prior to and after receiving challenge of these strains.

As can be seen in Fig. 4, the results show a tendency of *Sh. flexneri* 3a to give a somewhat higher incidence of mice with prolonged positive culture of the organisms from their feces when compared with animals injected with *Sh. flexneri* 2b. This case was true for both the sensitive and resistant strains.

It was noted in these experiments that a few mice excreted the organisms for a long period, but no incidence of fatal infection was evident.

4. **Relationships between strains of mice and maintenance of the inoculated organisms in the intestinal tract.**

In an effort made to select a more susceptible strain of mice for experimental infections, the susceptibilities of male and female mice of the CFW strain to the sensitive strain of *Sh. flexneri* 2b (#103-S) were compared with those of the ddN strain. Similar treatment as the above experiments, using carbon tetrachloride, was practiced.

As seen in Fig. 5, mice of the CFW strain appeared to be more susceptible than the ddN strain, in both the male and female. However, this difference was not, if any, too conspicuous.
5. General physiological state of the challenged mice.

Preliminary experiments were carried out to determine if the resistance-lowering agents, cortisone, croton oil and carbon tetrachloride, as well as the antibiotics were toxic for the ddN strain of mice. Also, the effective dose of antibiotics necessary to clear the intestinal flora was also determined.

Following a single or combined administration of these agents in sublethal dosages, the general physiological state of the mice was, more or less, lowered. This state was accompanied sooner or later by the manifestation of a delay in the body-weight increase, dullness of motion, etc. Mice, particularly those which gave a longer period of positive culture of the organisms from their feces and treated with the resistance-lowering agents, showed a remarkable decrease in body-weight. Only a few animals had diarrhea with exudate. Neither severe and characteristic symptoms nor death were, however, recognized in all the animals tested.

In these experiments, a continuous administration of antibiotics did not directly influence the general physiology of the animals. Thus, it seems that the inoculated organisms themselves did not directly and significantly influence the course of infections by their survival and development in the intestinal tract, considering the degree of depression of host-resistance due to resistance-lowering agents in combinations with the antibiotics.

6. Histopathological findings on the challenged mice in regards to the incidence of positive culture of the organisms obtained from the heart blood and intestinal content at autopsy.

All mice, whose feces showed negative culture for dysentery bacilli, were immediately sacrificed to confirm the establishment of infection and survival of the organisms in the intestinal tract. The intestinal content and the heart blood were cultured for the possibility that a systemic infection was established. Macroscopic pathological examinations of the various organs were also performed. Materials obtained upon autopsy were placed in a formaline solution for histological preparations.

No organisms were isolated from all of the heart blood cultured. However, positive cultures were obtained from the intestinal contents of 34 out of 169 mice examined (20%). Positive cultures were obtained particularly from mice which excreted the organisms along with their feces for more than 2 weeks, 8 out of 10 (80%).

Macroscopic pathological examinations revealed that those mice which gave negative culture of the organism within a week showed a mild degree of hemorrhage from the proximal small intestine to the distal large intestine. However, in many of those which excreted the organisms for a longer period and were treated with the resistance-lowering agents, marked and extensive injection or
hemorrhage in the intestinal tract, swelling of the spleen and injection or hemorrhage of the lung, and formation of the foci of infection in the liver and kidneys due to gram-negative cocci and fungi were observed.

Microscopic examination of the histological preparations revealed the same tendency as described above in respect to the grade of lesions and isolation of the organisms. The lesions were very conspicuous in the mice, which were treated with the resistance-lowering agents, especially with carbon tetrachloride in single and combined administrations of antibiotics, and challenged with the sensitive and resistant strains of Sh. flexneri 2b and 3a.

About 10 days after the challenge dose of the organisms was administered, a slight degree of acute colitis was observed. This was manifested by a cellular infiltration in the inner layer of the large intestine, partial desquamation of chorion, and slight or moderate exudation (photo. 1). The spleen showed swelling with hemorrhage, enlargement of lymph follicles and increase of histiocytic reticuloendothelial cells (photo. 2). The lung exhibited an extensive broncho-pneumonitis, which was manifested by the enlargement of the bronchi and blood vessels, including purulent exudate, focal increases in cellularity and congestion and swelling of the lymph apparatus (photo. 3). Microscopic examination of the sectioned and stained kidney preparations revealed a localized, embolic glomerulonephritis with an enlargement of the Bowman’s capsule and cellular infiltration (photo. 4).

In the later stage, about 2 weeks or more after the challenge, severer lesions developed in various organs; the large intestine showed a picture of necrotic colitis, manifested by swelling of the lymph follicles, necrosis of the epithelial layer with partial desquamation and a small amount of exudate (photo. 5). The spleen and lung were also more extensively involved; especially in the latter, extensive and intense purulent and necrotic bronchitis or pneumonitis were noted and atelectasis became conspicuous (photo. 6).

In contrast, even at much later stage, the infections in the animals which had negative culture of the organisms from the intestinal content, as well as the stool, was, more or less, slighter. Throughout all these experiments, it was noted that the damage of the liver was much less when compared with the other organs.

In a few mice which were inoculated with the Shigella, the gram stain preparation of the epithelial layer of the large intestine demonstrated bacterial cells to be present. However, by virtue of this fact only, no decision can be made as to whether or not they resulted from the inoculated organisms.

DISCUSSION

Experimental Shigella infections using larger animals, chiefly chimpanzees or monkeys, have been attempted with unsatisfactory results by many investi-
In regards to other larger animals, for instance, dogs, cats, rabbits, etc., no successful results have been reported. Even if such animals were suitable for establishment of Shigella infection itself, they could not be employed in practical and quantitative experiments.

Smaller animals, such as rats, guinea pigs, mice, etc., had been used in vain for such infections. Recently, Freter reported that a long period of streptomycin, erythromycin and nystatin administrations was useful for the demonstration of Shigella infection in mice. Formal et al. also succeeded in establishing a fatal enteric infection using Shigella flexneri 2b in guinea pigs by depriving the animals of food for 4 days and administering calcium carbonate prior to and opium following an orally administered challenge dose. Diarrhea and fatal infection were induced by subcutaneous injection of carbon tetrachloride in addition to the above treatments. Yasaka confirmed that depriving mice of food prior to the challenge with Shigella was useful for the maintenance of the organisms in the intestinal tract.

The use of a combination of small animals and Shigella in promoting infection is in itself unnatural. Therefore, in order to establish infection, any treatment causing its acceleration must be devised after examining the relationship of host and parasite. In working with parasite, any method employed to protect them from the direct or indirect influence of lowering their resistance is necessary, because it is a well-known fact that when dysentery bacilli are inoculated orally into mice they are easily killed before they reach the large intestine. Therefore, the broth culture of the test strain was concentrated and resuspended in the medium in which it grew. This procedure protects the organisms from the damaging action of saline solution, gastric fluid and other effects.

On the other hand, the elimination of normal intestinal flora and the reduction of defense-mechanism in the hosts may be the important factors in accelerating infections. Antibiotics were employed in this sense by Freter, Yasaka, Ogasahara et al., and others, resulting in extending the survival period of the organisms in the intestinal tract of animals. Such effect was also confirmed by us through the administration of chloramphenicol, streptomycin and trichomycin, singly or in combination. Furthermore, it was noted that when the administration of antibiotic was discontinued, the intestinal flora which first decreased in numbers or disappeared by this treatment appeared again in a higher number as early as the next day. Consequently, the challenge organisms did not appear to exist in the intestine. Thus, the resistant strains which were not affected by the antibiotics were employed with some successes. However, even in such case, the intestinal bacteria which had become resistant to the administered antibiotics, appeared within a few days following the challenge and increased in numbers rapidly. This increase was followed by the decrease or disappearance of the organisms in the intestine (this result will be
reported elsewhere in detail).

Aqueous solutions of antibiotics were given to mice through the water bottles for constant introduction into their intestinal tract. This method eliminated the bacterial flora without causing mechanical injury due to repeated daily oral administration using a stomach tube. However, each mouse did not always drink same amount of water; therefore, the effect due to the difference in the amount of antibiotics taken in respect to establishing an infection should not be neglected.

The combined actions of the antibiotics and the resistance-lowering agents on the establishment of Shigella infections seem to be significant, especially in producing more intense lesions in the intestine as well as in the other organs. However, a single administration of antibiotics also produces an almost similar effects as far as the prolongation of the period of survival of the organisms in the intestinal tract was concerned. Thus, the elimination of the intestinal flora with the aid of antibiotics is considered to play a primary role in establishing Shigella infection. The type of treatment employed to the resistance in the host is, of course, important; radiation by x-ray, starvation technique, administration of resistance-lowering agents, described above, and other treatments appear to be different in their mode of actions.

Formal et al. observed that the tissues of animals, which had been starved, showed a fatty metamorphosis of the liver as the only consistent change. Furthermore, they demonstrated that the amount of complement and properdin of the animals treated with carbon tetrachloride decreased as the result of its hepatotoxic action. Sublethal dose of carbon tetrachloride used in our mice experiments, resulted in neither the production of centrolobular necrosis nor fatty metamorphosis of the liver. Therefore, in order to reproduce fatal enteric infections, the dose which approach the level at which animals succumb should have been applied. This finding is regarded as common to the dose used of other resistance-lowering agents.

Thus, the histopathological changes observed in mice, which were challenged with Shigella, can be regarded as, in themselves, the sequence of maintenance of the organisms in the intestinal tract or the establishment of infection or the influence due to endotoxin of the organisms. This is especially noted in the erosion of the inner layer of the large intestine with desquamation of the epithelial cells and inflammatory exudate which is similar to the ulcerative change noticeable in the large intestine or man with dysentery. In addition, organisms isolated from both the feces and the intestinal content also indicate the establishment of Shigella infection by virtue of the fact that the multiplication of the challenged organisms in the intestinal tract was obvious when compared with the inoculum size, by maintaining the positive culture for about a week or more.

The differences noted in the degree of maintenance of the organisms in the
intestinal tract between the two different serotypes of *Sh. flexneri* 2b and 3a, and between the two different strains of mice, ddN and CFW, are not regarded as significant.

These experiments, however, were performed by treating mice with the resistance-lowering agents in the absence of antibiotics; therefore, such comparisons should have been made under similar conditions as the experiments using the resistant strain in combinations with both agents. By the use of this method the most virulent strains of Shigella and the most susceptible mice to such infections can be determined.

In the event that fatal Shigella infections can not be attained in an experiment, the use of such method as to clear the intestinal flora for the purpose of studying the effect of drug resistant strain is, in itself, an unavoidable limitation for experimental infections. In this sense, the authors do not consider that the methods employed were appropriate; however, it is believed that they may possibly present a possible technique for the establishment of experimental Shigella infections in smaller animals.

**SUMMARY**

A continuous oral administration of antibiotics, mostly chloramphenicol, in combination with resistance-lowering agents, such as carbon tetrachloride, cortisone acetate and croton oil, was very effective in establishing infections in ddN and CFW strains of mice when they were challenged with resistant strains of *Shigella flexneri* 2b and 3a.

The inoculated organisms remained in the intestinal tract for about a week in most of the animals and for as long as 4 or 5 weeks in a few.

Pathologic examination revealed that lesions developed in various organs, especially the intestinal tract, showing acute or chronic colitis.

Infection was not induced in animals which were inoculated with sensitive strains; because of the disruption in the administration of antibiotics to these animals, no organisms could be found in their feces within 1 or 2 days.

Thus, it is concluded that the elimination of intestinal microbial flora with the aid of antibiotics is of primary significance in establishing Shigella infection in mice, and that the resistance-lowering agents seem to play a secondary role in accelerating the infection.

Differences in the serotypes of *Shigella flexneri* (2b and 3a) and in the sexes (male and female) and strains (ddN and CFW) of the test animals did not seem to influence the grade or course of infections as far as the results obtained were concerned.

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