THE EFFECT OF AN ANTIBIOTIC ON THE SUSCEPTIBILITY OF THE MOUSE'S INTESTINAL TRACT TO SALMONELLA INFECTION

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This study grew out of an interest in the so-called "secondary" or complicating infections which develop in the oropharynx or bowel in patients undergoing treatment with antibiotics.[1, 2, 3] Such infections may be caused by a variety of microorganisms, mostly rather non-virulent ones like Pseudomonas or Monilia[4] or other yeast-like fungi, but occasionally by highly virulent strains of staphylococci.[5, 6] Since these microorganisms are all common contaminants of the oropharynx and bowel, and since, with rare exceptions, they produce infection only during antibiotic therapy, it seems reasonable to suppose that they are able to do so only when the normal bacterial flora of these areas has been altered by an antibiotic.[7, 8, 9] Or, to put the explanation the other way around, these common contaminants are unable to initiate infection in the oropharynx or bowel when the normal microflora is intact.

This explanation raises several questions: May not the normal microflora of the mouth and the rest of the alimentary tract play a significant role in the body's defense against bacterial invasion? And, if so, is it the whole flora which is responsible or only certain of its constituents? Lastly, what are the mechanisms involved?

These are some of the questions we had in mind when this experimental study was undertaken several years ago. Most of them are still unanswered, but some promising leads have developed at least in the case of the experimental animal and the test microorganism used in these experiments. Briefly stated, this is an investigation of the effect of streptomycin on the infectibility of the mouse's intestinal tract with *Salmonella enteritidis*.

The streptomycin* was always introduced directly into the alimentary tract by stomach tube, and all inoculations with Salmonella were made

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the same way. The strain of Salmonella was streptomycin resistant because this property made it much easier to recover and identify. Its streptomycin resistance was in no way responsible for the results obtained.

We began by making a quantitative estimate of the numbers of Salmonella required to infect normal mice as compared with mice which had been treated the day before with a single large dose (50 mg) of streptomycin.[10, 11] The "normal" or control mice were given saline at the same time the treated mice were given streptomycin. All injections were made by stomach tube.

The following day, mice in groups of five, were inoculated with 10-fold dilutions of a suspension of Salmonella. The actual numbers of Salmonella inoculated were determined by plate counts. The mice were housed in individual cages to prevent cross infection. Beginning the second day after inoculation, a fresh fecal sample from each mouse was titrated for numbers of Salmonella. After ten days or two weeks, the mice were killed for culture of hearts' blood and spleen.

The results of a series of such experiments are summarized in Table I which shows that between 10^5 and 10^6 Salmonella were required to infect half the normal mice, but in streptomycin treated mice, very small inocula sufficed to establish infection. The criteria of infection were the persistence of Salmonella in the fecal cultures and their recovery at autopsy from hearts' blood and/or spleen. As a rule, mice in which Salmonella persisted in the feces for a week had positive blood or spleen cultures at autopsy indicating that the infection had become systemic.

It is obvious from these results that susceptibility to Salmonella infection was enormously enhanced by a single dose of streptomycin administered the day before inoculation. When this interval (between streptomycin treatment and inoculation) was lengthened, it was found that the increased susceptibility continued through the second day and then gradu-

TABLE I

Incidence of Infection in Control and Streptomycin Treated Mice

Salmonella Inoculated	Controls	Streptomycin Treated*
10^6–10^7	100%	—
10^5–10^6	50%	—
10^4–10^5	33%	—
10^3–10^4	27%	—
10^2–10^3	15%	100%
10 –100	1.5%	83%
1 –10	0%	56%

* = 50 mg. streptomycin by mouth 24 hours before inoculation.

ally diminished. By the eighth day after treatment, it approached but was not quite equal to that of untreated controls.

The fate of the inocula immediately after introduction into the gastrointestinal tract of control and streptomycin treated mice was investigated in the following experiment. Groups of mice were inoculated with approximately 50 Salmonella, killed at intervals for determination of the numbers of Salmonella in homogenates of the whole gut and in the feces which had been excreted between inoculation and sacrifice. It was found that in normal controls no multiplication of Salmonella occurred; within 24 hours the inocula had passed through the gut and been excreted in the feces.

In streptomycin treated mice, the numbers of Salmonella remained constant for three hours, and then increased rapidly to about 10^8 at 24 hours, indicating that they had multiplied at about the same rate as in a broth culture.

The possibility was considered that this effect of streptomycin might have been due to injury to the mucosa of the gut, but no gross or microscopic pathological changes could be observed. Nor was any disturbance in the propulsive motility of the gut demonstrable in streptomycin treated mice. This point was investigated by giving carmine and measuring its rate of transit through the gut.

It was also thought that the resistance of the normal mouse might be due to the presence in the gut of a bacteriophage for Salmonella, or some substance like the colicines, or some growth inhibitor which prevented the multiplication of Salmonella. None of these could be demonstrated. In fact, filtrates of the bowel content of normal mice supported growth of Salmonella *in vitro* almost as well as broth.

It seemed reasonable, therefore, to proceed on the supposition that the effect of streptomycin was due solely to its bactericidal action on the microbial population of the gut. A systematic study was then made of the fecal flora before and after treatment with streptomycin. The results are presented in Table II. The plus marks indicate roughly the relative numbers of microorganisms cultured from the feces. There was no significant change in the lactobacilli, yeasts and yeast-like organisms, or in the Gram positive cocci (mostly enterococci). The only effect demonstrable, by the customary aerobic culture methods, was the elimination of all the Gram negative bacilli. These included the coliforms, *Paracolobactrum*, *Proteus*, and *A. aerogenes*. Of these, the coliforms seemed the most important because only they were present in every mouse and in very large numbers.

These results led to the conclusion that the elimination of the coliforms was responsible for the enhanced susceptibility to Salmonella infection. This conclusion however was insupportable because susceptibility to Salmonella infection was not reduced by inoculation with coliforms, alone,

TABLE II

Various Microorganisms Recovered from Feces Before *and* After *Treatment with Streptomycin*

Micro-organisms	Before	After
Lactobacilli	++++	++++
Yeasts & yeast-like organisms	++++	++++
Gram positive cocci (mostly enterococci)	++++	++++
Gram negative bacilli (Coliforms, *Paracolobactrum*, *Proteus*, *Aerobacter aerogenes*, etc.)	+++	0

Plus marks indicate relative numbers by customary aerobic culture methods.

or in combination with others of the Gram negative bacilli. In other words, the reestablishment of these Gram negative bacilli failed completely to restore the intestinal tract to its normal level of resistance. Moreover, suspensions of bowel content or feces of normal mice did not inhibit growth of Salmonella *in vitro*.

It was found, however, when normal feces were inoculated into streptomycin treated mice, susceptibility to Salmonella infection was lost. This ability of normal feces to counteract the effect of streptomycin indicated that they contained some constituent, other than the bacteria we had isolated, which could be reestablished in the bowel by this procedure.

Since inoculation with bacterial cultures had been so unsuccessful, suspensions of whole feces were used without attempting to isolate this constituent. Fecal suspensions were subjected to various manipulations before inoculation into streptomycin treated mice. The results indicated that the active constituent was inactivated by temperatures above 45° and by the common germicidal agents. It was present in small amount and was not effective immediately after inoculation, but only after an interval of about 16 hours. These facts suggested that living microorganisms of some sort were responsible for the activity described.

Attempts were then made to grow them by culturing fecal suspensions in broth. This was possible only in suitable media and under strictly anaerobic conditions. Such cultures showed a marked increase in effectiveness which was ascribed to multiplication of these microorganisms. It must be presumed, therefore, that it is some of these obligate anaerobes in normal feces which counteracted the effect of streptomycin.

A number of strains of anaerobes have been isolated but they are so difficult to grow in pure culture that it has not yet been possible to test individual strains in mice.

Since they are all sensitive to streptomycin, it seems not improbable that it is the presence of some of them in the intestinal tract of normal mice

which hinders the establishment of Salmonella, and that their elimination by streptomycin is the change in the enteric microflora responsible for the enhanced susceptibility to Salmonella infection which follows the administration of this antibiotic.

REFERENCES

1. KEEFER, C. S.: Alterations in normal bacterial flora of man and secondary infections during antibiotic therapy, Am. J. Med., *11:* 665–666, 1951.
2. SMITH, D. T.: The disturbance of the normal bacterial ecology by the administration of antibiotics with the development of new clinical syndromes, Ann. Int. Med., *37:* 1135–1143, 1952.
3. WEINSTEIN, L.: Alterations in normal bacterial flora of man and animals and secondary infections during streptomycin therapy, Streptomycin, Williams & Wilkins, Baltimore, pp. 546–557, 1952.
4. WOODS, J. W., MANNING, I. H., JR., AND PATTERSON, C. N.: Monilial infections complicating the therapeutic use of antibiotics, J. A. M. A., *145:* 207–211, 1951.
5. JACKSON, G. G., HAIGHT, T. H., KASS, E. H., WOMACK, C. R., GOCKE, T. M., AND FINLAND, M.: Terramycin therapy of pneumonia: Clinical and bacteriologic studies in 91 cases, Ann. Int. Med., *35:* 1175–1202, 1951.
6. FINLAND, M., GRIGSBY, M. E., AND HAIGHT, T. H.: Efficacy and toxicity of oxytetracycline (terramycin) and chlortetracycline (aureomycin), Arch. Int. Med., *93:* 23–43, 1954.
7. USHIBA, D., YUMOTO, M., OHNO, S., AND SASAKI, S.: Infectivity in Oral Infection of Mice with *Salmonella enteritidis:* Effect of Streptomycin on the Infectivity with Special reference to normal intestinal flora, Keio J. Med., *4:* 163–173, 1955.
8. FRETER, R.: Experimental enteric *Shigella* and *Vibrio* infections in mice and guinea pigs, J. Exp. Med., *104:* 411–418, 1956.
9. FRETER, R.: Coproantibody and bacterial antagonism as protective factors in experimental enteric cholera, J. Exp. Med., *104:* 419–426, 1956.
10. BOHNHOFF, M., DRAKE, B. L., AND MILLER, C. P.: Effect of streptomycin on susceptibility of intestinal tract to experimental salmonella infection, Proc. Soc. Exp. Biol. & Med., *86:* 132–137, 1954.
11. MILLER, C. P., BOHNHOFF,, M., AND DRAKE, B. L.: The effect of antibiotic therapy on susceptibility to an experimental enteric infection, Trans. Assn. Am. Phys. *67:* 156–161, 1954.

DISCUSSION

DR. A. MCGEHEE HARVEY (Baltimore, Maryland): I would like to give a brief case report to illustrate a practical aspect of this area of investigation.

A few months ago a patient was brought to the Johns Hopkins Hospital; not a human subject, however, but a chinchilla. There are a number of chinchilla farms around Baltimore and the breeders were faced with a grave problem in that many of their breeder animals were dying of a disease which they had labeled as "Sudden Death."

Such animals, apparently well, suddenly became relatively immobile and would die within a period of twenty-four hours. Dr. Wood, of the Department of Pathology autopsied one of these chinchilla breeders who died, and the only lesions found were more of a typical pseudomembranous enterocolitis. Biological investigation revealed a pure culture of staphylococcus growing in this membrane. A little detective work

concerning this "epizootic" revealed the fact that this disease appeared only in the champion breeders and among the rank and file of the colony there was no disease whatever.

In looking into what difference there was in the way champion breeders and the rank and file were handled, it turned out that the champion breeders got every consideration possible for the maintenance of their health. They even received a very special food in pellet form which was more expensive than the rations handed out to the ordinary members of the colony. Upon further investigation, it turned out that one of the ingredients of this food was a very small addition of an antibiotic not contained in the food that was given to the ordinary chinchillas. With the addition of bacitracin to the water of these animals, the "epizootic" was rapidly eradicated and, with change in the food with elimination of the small amounts of antibiotic, no further cases of staphylococcal infection occurred.

There is an historical note of interest in relation to this investigation. As you will recall, there has been a good deal of discussion in the editorial columns of various medical journals in recent months about the pros and cons of autopsies and the long term preservation of sampled material.

Dr. Bennett and Dr. Wood recalled that one of the earlier, often referred-to, cases of pseudomembranous enterocolitis published was published by Dr. Finney in the early days of the hospital at Baltimore, around 1890, describing this disease in the post-operative period. They descended into the bowels of the hospital and found some of the original material from this case, had sections made, stained a section and found in the membrane organisms morphologically certain to be staphylococci.

Dr. Theodore Woodward (Baltimore): I think that these studies by Doctor Miller have great significance from the point of view of pathogenesis of typhoid fever. His test animal was the mouse. Doctor Harvey has just spoken of the chinchilla. There is also the chimpanzee. Doctor Edsall and others of the Army Medical Service Graduate School has pointed out that the chimpanzee possesses remarkable resistance to *Salmonella typhosa* and that many millions of bacteria are required to infect. With regard to some of the studies designed to infect man preliminary observations indicate that a large infecting dose is required.

It was of interest that Dr. Miller indicated that motility of the intestine was not involved. The carmine particle technique was useful in this regard. One wonders whether motility may serve as a factor in pathogenesis and I should like to ask Dr. Miller how definite he feels concerning this point. Is there not a study in which morphine served to decrease the peristaltic activity of the test animal leading to a higher incidence of infection?

Secondly, I should like to inquire concerning the quantitative bacteriologic observations. In the chimpanzee, Edsall and his group showed that there are fewer *S. typhosa* in the intestinal wall of the infected animal than in the mesenteric lymph glands, the thoracic duct, or other parts of the reticulo-endothelial system. Do you have quantitative data other than those mentioned?

Dr. Chester M. Jones (Boston): I should like to come up from the mouse to the chinchilla to the chimpanzee, and finally arrive at Man.

Actually, it seems to me this paper has very real implications in many of the things that we are seeing following routine surgery on the digestive tract, and over and over again many of the surgeons (particularly the younger ones, I think) are preparing their patients for surgery by giving antibiotics so that they won't get into trouble after the operation. Possibly they are forgetting their technique in good aseptic surgery.

The prophylatic use of antibiotics may, I think, lead to the possibility of enteric infections postoperatively, which may negate the value of good surgery. We have lost patients from enteric staphylococcus infections which may depend upon this sudden change in intestinal flora.

DR. JOSEPH H. HOLMES (Denver): I should like to add to this animal problem. We had a similar experience in our dog colony.

What I really would like to ask is two questions. What Dr. Jones said is very important, I think, and this is particularly true in the renal failure problem where you have the problem of introducing an overdose of many of these agents, and I have often wondered what factor the lack of oral food plays in this, and so I would like to ask Dr. Miller two questions in relation to this.

One is how much difference dosage does make in this problem?

Second, does he have any observations on whether food, or the intake of food, plays a practical part in this?

In other words, one has a definite impression that those who do not get feedings have a definite greater susceptibility than those who are on even two feedings.

DR. H. CORWIN HINSHAW (San Francisco): I should like to ask Dr. Miller if any of these are antibiotic producers, and if not, I hope he searches for antibiotics among these cultures.

DR. MAURICE FREMONT-SMITH (Boston): I should like to make a comment about a clinical problem. This was a young man with an acute salmonella infection who then became a salmonella carrier and who wanted to get into the Navy.

I, not knowing how to manage this situation, conferred with two people in Boston and was advised to give chloramphenicol and streptomycin by mouth. The boy still continued to be a salmonella carrier.

On the basis of Dr. Miller's paper, perhaps the advice to give streptomycin by mouth was the wrong treatment for this condition.

DR. MILLER (closing): To begin with Dr. Harvey's comment: That epidemic among the chinchillas, so well described by Dr. Bennett and Dr. Wood, is very interesting. The addition of antibiotics in some form to animal feeds has become so common that it is difficult nowadays to buy food for laboratory animals which contains none. This has become a matter of concern for those of us who must have antibiotic-free food for our experimental animals. The manufacturers may not add antibiotics as such, but they often add so called "growth stimulating supplements" which are waste products of the manufacture of antibiotics, which do, in fact, contain small amounts of antibiotics.

Dr. Woodward raised the question of the motility of the gut. All I can say is that the method we used showed no disturbance of motility of the mouse's intestinal tract after the administration of streptomycin. What we did was to give a suspension of carmine by stomach tube to streptomycin-treated and control mice, kill them at intervals, and measure the distance traveled by the carmine.

Dr. Woodward also asked a question about morphine and its effect on susceptibility to infection. I omitted from my paper, which was already too long, some experiments in which we gave morphine to mice at different times after inoculation with Salmonella. We found that when normal mice, that is mice which had not been treated with streptomycin, were given morphine immediately after inoculation with very small numbers of Salmonella, they became infected. In other words, treatment with morphine immediately after inoculation markedly enhanced their susceptibility to Salmonella infection. By the use of carmine we found that morphine, administered immediately after inoculation, interrupted peristalsis, and trapped the

inoculated Salmonella in the small intestine, which is relatively free from bacteria and contains an abundant food supply in which the Salmonella could multiply unhindered.

When the administration of morphine was postponed until about 3 hours after inoculation, by which time the Salmonella had passed through the small intestine into the colon, infection did not occur even if large numbers of Salmonella had been inoculated, presumably because the anaerobes which interfere with the multiplication of Salmonella are present only in the large intestine.

Dr. Woodward also asked about the numbers of Salmonella in the viscera of our infected mice. We did not make quantitative determinations, but did find large numbers of Salmonella in liver, spleen and blood by the end of the first week, in those mice in which Salmonella persisted in the feces.

Dr. Holmes raised the question of dosage of streptomycin. We began using 50 mgms. as I indicated. That is a very large dose if you compare it with that used in man. But we later found that 40 mgms. worked as well as 50, and 30 mgms. almost as well—in a single dose. With smaller doses, the results were irregular. In all our later experiments, as a matter of fact, we did work with 40 or 30 mgms.

When the mice were put on continuous intake of streptomycin by giving it to them in their drinking water, the same effect could be produced with as little as 1.0 mgm. per ml. of drinking water.

Dr. Hinshaw asked whether these anaerobes are antibiotic producers. I do not know. These anaerobes are extremely difficult to work with. They are very sensitive to oxygen. When pure cultures on agar plates are exposed on the laboratory table for more than a couple of hours, they die off. They are that sensitive to atmospheric oxygen.

The thing we are working on hardest now is the improvement of our methods of cultivating these anaerobes. I had hoped to be able to tell you more about this aspect of the problem today, but we have run into one difficulty after another.

Dr. Fremont-Smith—what was your question?

DR. FREMONT-SMITH: The treatment of a Salmonella carrier by streptomycin.

DR. MILLER: Streptomycin is a poor drug because all of the ordinary bacteria develop resistance so very rapidly.

I should say that if it were possible to kill off all the Salmonella in two or three days, well and good; but after that resistant individuals are bound to appear in the bacterial population and the drug will no longer be effective.

Dr. Jones spoke about the surgeon's use of antibiotics preoperatively. I have preached against this practice at home for a number of years and I think I have at last begun to make an impression on our surgeons.

In fact, one of them told me not long ago about one of these complicating infections in a patient of his and added, "You know, Phil, I have decided not to give antibiotics unless they are needed."