INTRACELLULAR INFECTIONS*

JOSEPH E. SMADEL, M.D.

Chief, Laboratory of Virology and Rickettsiology, Division of Biological Standards
National Institutes of Health, Bethesda, Md.

IT is with deep feeling that I again acknowledge my appreciation on receiving the Albert Lasker Clinical Research Award. Earlier today at the time of the award ceremony, I made a few remarks on the coming of age of clinical research in this country. Furthermore, I likened clinical investigation to a bridge between the laboratory experiment and the improved practice of medicine—a bridge on which traffic moved in both directions. Tonight, in presenting a summary of some of the work for which the Clinical Research Award was given, I shall try to illustrate the way in which such a bridge functioned for me and my colleagues in the laboratory and on the ward.

The story begins in the laboratories of associates with the discovery of a new antibiotic which was to become known as chloramphenicol. Our own studies with this substance in the treatment of experimental rickettsial infections led us to examine its effectiveness in patients with rickettsial diseases and, subsequently, in patients with typhoid fever.

Patients with scrub typhus, like those with epidemic typhus, murine typhus and spotted fever, generally run a febrile course of about two weeks' duration. Patient 29,[1] whose findings are illustrated in Figure 1, was given chloramphenicol on the sixth day of scrub typhus at a time when his temperature was 104°F. The fever disappeared promptly and the patient went on to an uneventful recovery. Experience has shown that patients with scrub typhus become afebrile, on the average, in about 30 hours after antibiotic therapy is begun; patients with epidemic or murine typhus respond similarly in about 48 hours and those with spotted fever in about 72 hours. Moreover, in these rickettsial diseases, other broad spectrum antibiotics are as effective as chloramphenicol in arresting the febrile illness.

Typhoid fever is controlled by chloramphenicol but not by the

* The 1962 Albert Lasker Clinical Research Award Lecture, presented at The New York Academy of Medicine, November 14, 1962.

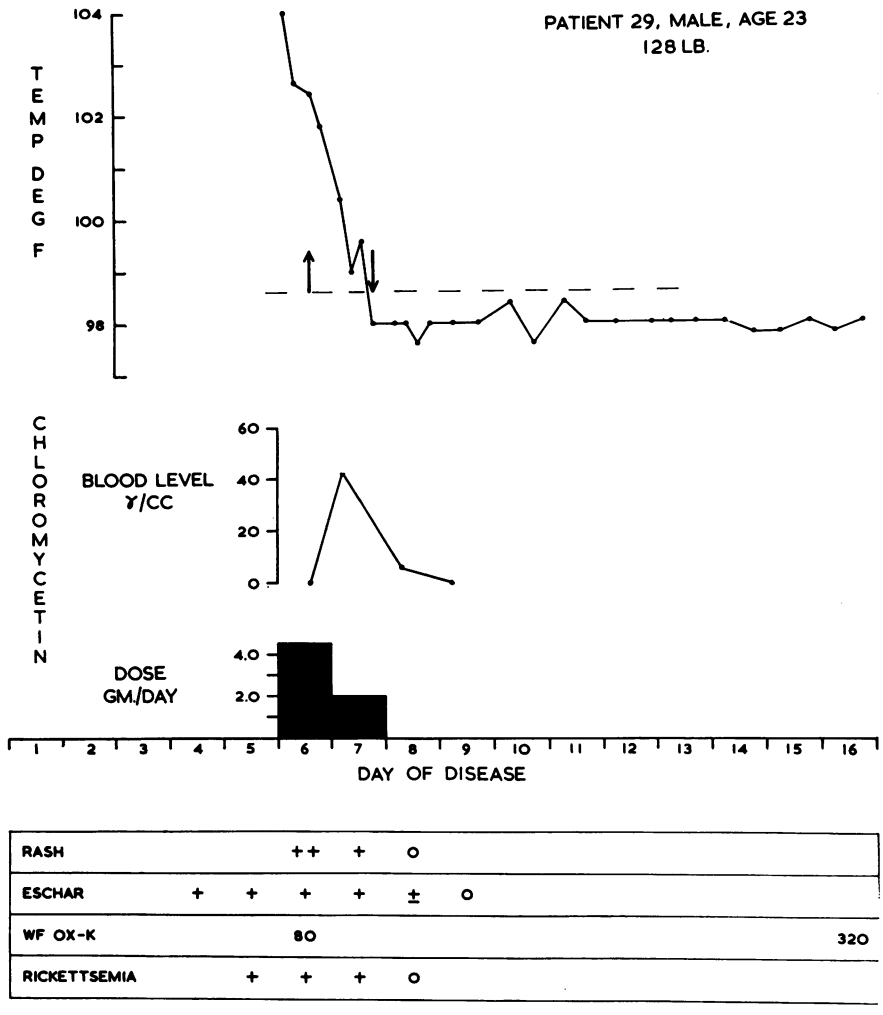

Fig. 1. Clinical response of scrub typhus patient to chloramphenicol therapy begun on the sixth day of disease. Reproduced from *J. Clin. Invest. 28*:1202, 1949 (Smadel *et al.*[1]). By permission.

other broad spectrum antibiotics. Figure 2 illustrates the response of a typhoid patient treated on the ninth day of illness.[2] There was no lessening of pyrexia or toxemia in the first 24 hours, but by the third day these manifestations decreased and the general clinical condition was vastly improved. The patient became afebrile 3.5 days after initiation of therapy.

While practically all patients with scrub typhus or typhoid fever

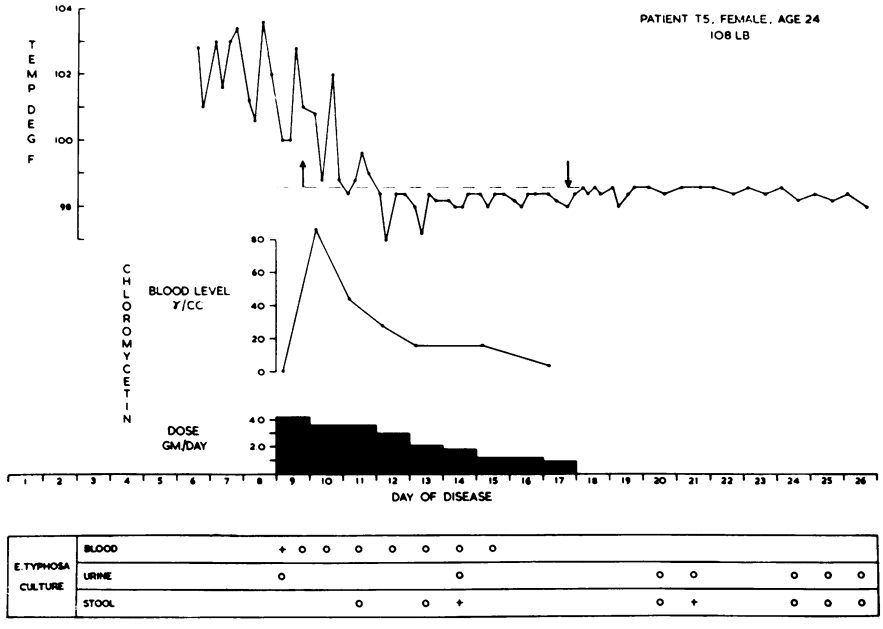

Fig. 2. Clinical response of typhoid fever patient to chloramphenicol therapy begun on ninth day of disease. Reproduced from *Ann. Int. Med. 29*:133, 1948 (Woodward *et al.*[2]). By permission.

respond initially to chloramphenicol in the manner illustrated in the two figures, not all make the uneventful recovery of the two patients just described. A number develop relapses and require further treatment. As information has accumulated, it has become apparent that the usual course of chloramphenicol fails to eradicate the rickettsiae or the *Salmonella typhosa* from the patient even though the antibiotic stops multiplication of the invading organisms and aborts the clinical disease.

Relapse occurs if the antimicrobial effect of the antibiotic is lost before the immunologic defenses of the patient have developed. In scrub typhus, as well as in typhoid fever, such recrudescences are observed most frequently when only a short course of therapy is given early in the disease. Fortunately, such episodes are promptly controlled when treatment is again instituted.

Further evidence for the belief that *S. typhosa* and rickettsiae continue to remain viable *in nidi*, in at least a proportion of recovered treated patients, was obtained from two sources. In the first place, carriers were encountered about as frequently among typhoid patients who

were treated with chloramphenicol as among those who did not have the benefit of specific therapy, i.e., about 2 per cent. In the second place, an occasional chloramphenicol-treated scrub typhus patient had demonstrable viable rickettsiae in his lymph nodes as long as a year after recovery.

I would like to change the point of attack slightly and discuss at greater length the carrier state in typhoid fever and the recurrence of typhus as exemplified by Brill-Zinsser disease. In each of these conditions the individual continues to carry the viable organisms within his tissues for long periods of time without manifesting obvious clinical disease. In other words, to all intents and purposes, the individual is immune but he carries a time bomb. The typhoid carrier is of great public health importance because he continues to excrete *S. typhosa* into his environment and is a potential source of epidemic disease in the community. Similarly, the patient who recovers from epidemic typhus and continues to harbor the causal rickettsiae is a potential hazard to the community. The recovered typhus patient may, within a few years or a few decades, lose his capacity to keep the silent but viable organisms in check. When this happens, clinical disease reappears and the associated rickettsemia can provide a source of infection for body lice, if they are present. Thus, the patient with Brill-Zinsser disease in a lousy community can initiate an epidemic of typhus.

My associates and I have been interested for a long time in the typhoid carrier and in the recovered typhus patient, not only because they present a public health hazard but also because they present a fascinating problem in immunity, as well as a clinical illustration of the importance of continuing intracellular infections. The term "intracellular infections" is introduced here because the most reasonable explanation that I can give for the continued existence of such pathogens as *S. typhosa* and *Rickettsia prowazeki* in the immune person possessing potent serologic and phagocytic cell defense mechanisms, is to assume that the offending organisms have taken sanctuary inside tissue cells where the host's attacking forces cannot reach them. It has been established for a number of decades that both of the organisms under discussion do occur intracellularly in man. Indeed, the rickettsiae are, by definition, obligate intracellular parasites. On the other hand, *S. typhosa* has a free-living existence but it can survive and multiply inside mammalian cells as will be demonstrated later in the presentation.

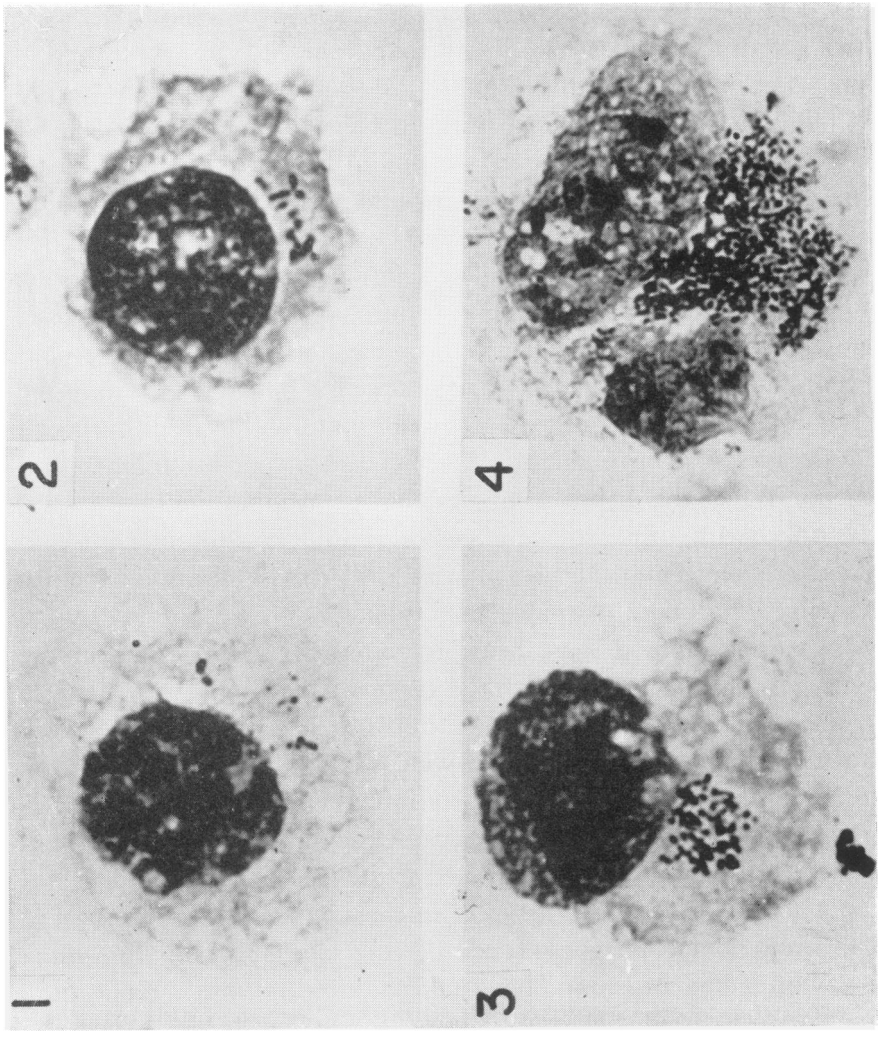

Fig. 3. Photomicrograph of MB III cells infected with *Rickettsia tsutsugamushi* (Karp strain). Reproduced from *J. Immun. 76*:480, 1956 (Bozeman *et al.*[3]). By permission.

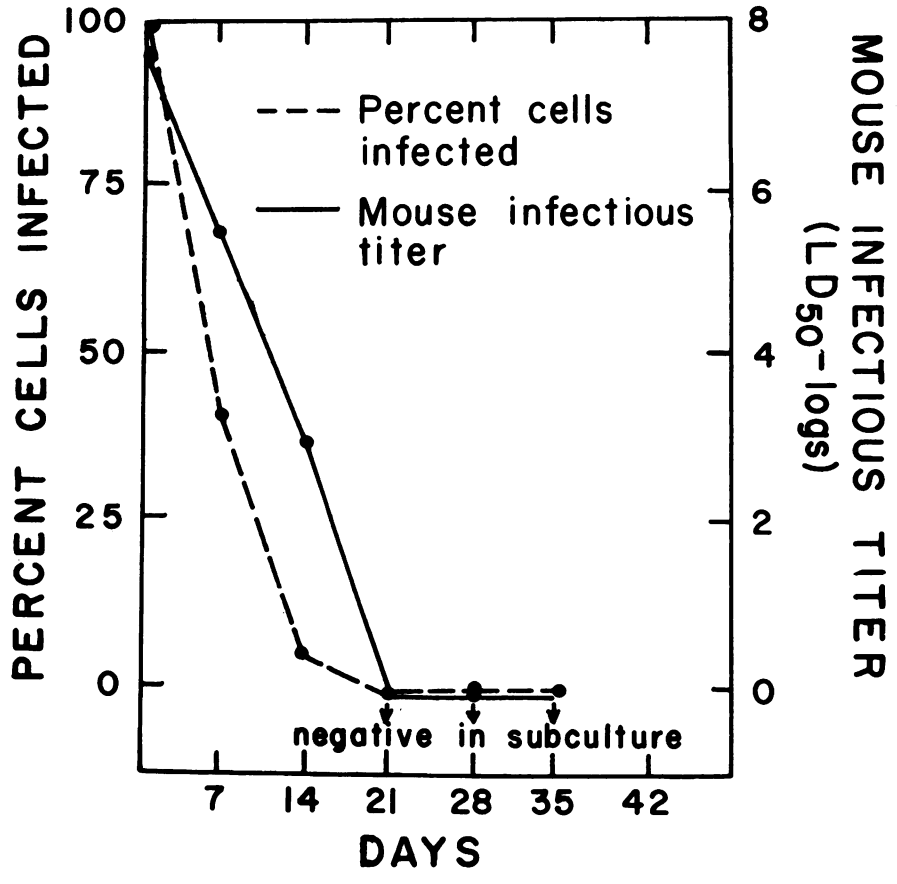

Fig. 4. Disappearance of *R. tsutsugamushi* (Karp strain) from infected L929 cells after prolonged treatment of cultures with chloramphenicol (5μg./ml.). Reproduced from *J. Immun. 82*:173, 1959 *(Hopps et al.[4])*. By permission.

You are familiar with the microscopic appearance of intracellular rickettsiae. Figure 3 will recall this picture for you and also illustrate the growth of rickettsiae in tissue culture cells.[3] A few coccobacillary organisms are present in the cell represented in section 1 of the figure. This cell was stained two hours afer *R. tsutsugamushi* had been added to a culture of mouse lymphoblasts. The other three pictures, each of which shows progressive increase in the number of intracellular rickettsiae, represent photomicrographs of smears prepared from the same infected tissue culture at intervals of 24 hours. Careful quantitative studies using this model revealed that rickettsiae increased about three-

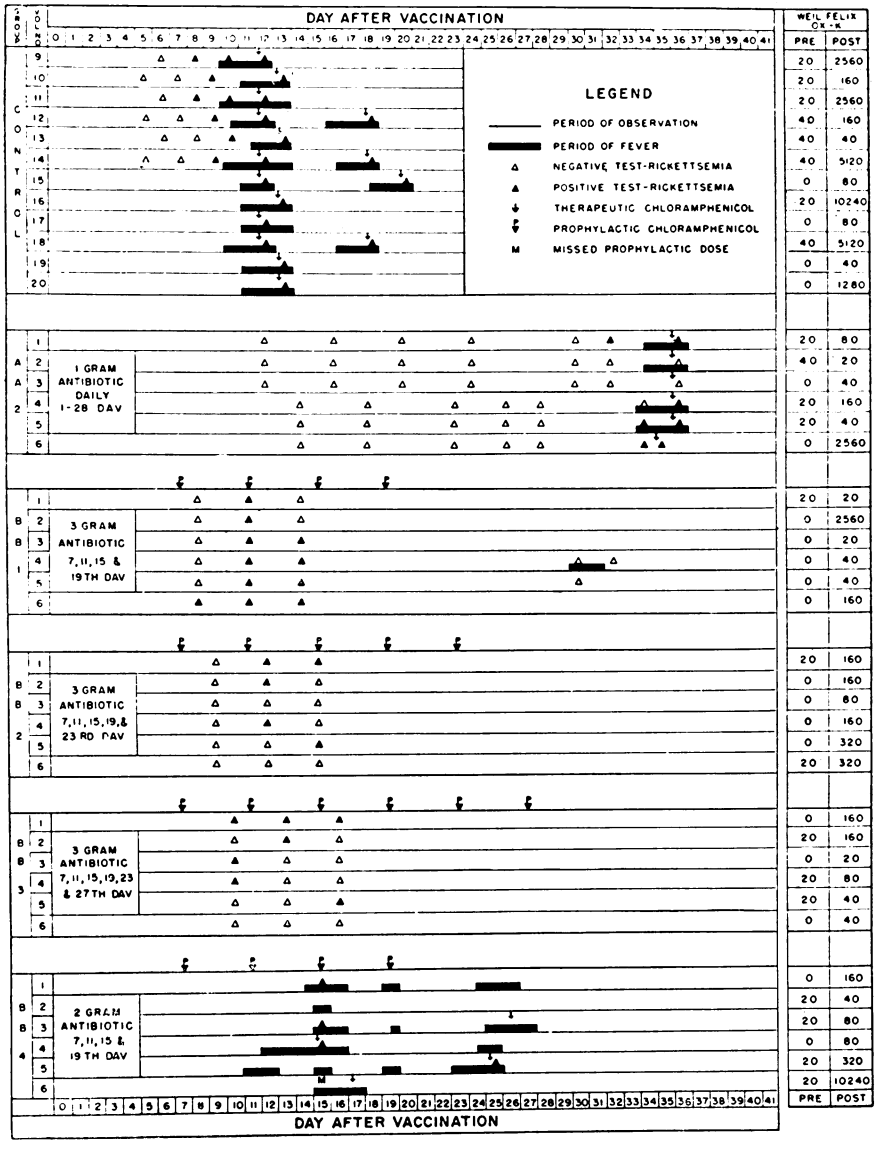

Fig. 5. Scrub typhus vaccine chemoprophylaxis test in volunteers. Daily 1-gm. doses of chloramphenicol given for 4 weeks after inoculation of attenuated living vaccine suppressed infection but clinical disease appeared when the drug was discontinued. Intermittent chemoprophylaxis with 3-gm. doses permitted rickettsemia and ultimate development of immunity without clinical disease. Reproduced from *Amer. J. Hyg. 56*:306, 1952, (Ley *et al.*[5]). By permission.

fold in number every 24 hours. This is an amazingly slow rate of multiplication when compared with that of *S. typhosa* which divides at intervals of about 30 minutes, whether in the intracellular position or in cell-free bacteriologic media.

Figure 4 illustrates the slow rate at which tissue culture cells infected with *R. tsutsugamushi* are freed of organisms when maintained in growth media in which chloramphenicol is present at a concentration of 5 micrograms per milliliter.[4] Even after two weeks a few of the cells still contain recognizable rickettsiae on microscopic examination and the culture contains an appreciable number of infectious rickettsiae. However, after the third week the culture is cured. Rickettsiae are not demonstrable directly in the treated culture or in subcultures maintained without chloramphenicol or by inoculation of the culture into susceptible mice.

It may be noted in passing that higher concentrations of chloramphenicol result in a more rapid initial reduction of rickettsiae in such infected tissue cultures. However, such cultures are not cured even after two weeks of treatment.

The observations on the persistence of *R. tsutsugamushi* in treated tissue cultures helped to explain certain findings made in man some years earlier. Figure 5 summarizes a study done in Malaya on volunteers who were inoculated intradermally with about 100 infectious doses of *R. tsutsugamushi*.[5] The volunteers in the control group developed clinical scrub typhus 10 to 12 days later and their disease was promptly terminated by treatment with one of the broad spectrum antibiotics. Incidentally, the black bars which appear on the graph 15 to 18 days after inoculation indicate that four of the members of the group suffered relapses but again responded to therapy and remained well thereafter. The important section of the figure for the present discussion is that which portrays the results obtained in six volunteers who were given one gram of chloramphenicol daily for a period of four weeks after the intradermal inoculation with living rickettsiae. These persons remained afebrile during the period; furthermore, as indicated by the open triangles on the chart, none of the patients had rickettsemia. Four days after the last chemoprophylactic dose of chloramphenicol, i.e., on the 32nd day after inoculation, the initial evidences of clinical scrub typhus infection appeared in five of the six members. These consisted of mild headache and slight elevation of temperature, but a fever of

100°F. or over did not occur for another two days. At the time these forewarnings of disease were first noted, one of the three volunteers tested had rickettsemia; this is indicated on the chart by the black triangle. At about this point, all six members of the group were hospitalized and were given a course of therapy. Volunteer No. 6 in this group is of particular interest. Although his temperature was not elevated above 100°F. when therapy was instituted, he had rickettsemia at that time. He felt well subsequently and remained asymptomatic for the next several weeks. However, on the 51st day after vaccination and the 16th day following therapy, a febrile episode occurred which was accompanied by rickettsemia and controlled by specific antibiotic.

Thus, the small number of rickettsiae which were put into these men survived a period of four weeks of continuous daily chemotherapy and when the antibiotic was withdrawn, the rickettsiae were still able to induce clinical disease in these persons.

The other part of this figure is not so pertinent to the present discussion. I will take a moment, however, to point out that other volunteers who were inoculated in the same way and then given intermittent chemoprophylaxis with 3-gram doses of chloramphenicol had a suppressed infection which was evidenced by rickettsemia. At no time during the course of chemoprophylaxis did these persons appear sick. Furthermore, when prophylaxis was continued for three to four weeks, none of the volunteers developed disease after the antibiotic was withdrawn. When the chemoprophylactic regimen was shorter than three weeks, some of the volunteers displayed clinical scrub typhus a few days after the last dose of antibiotic. These data, which I have just described, provided the basis for a complex procedure for immunizing people against scrub typhus. Because of its complexity, the procedure has had only limited usefulness; nevertheless, the principle is sound. It is to infect the individual with a partially attenuated organism and, subsequently, to maintain the infection at a sub-clinical level by means of antibiotic until the individual develops his immunological responses.

I would like to turn now for a time from scrub typhus to typhoid fever and to discuss certain of the laboratory observations which appear to bear on the problem of the typhoid carrier.

Several hypotheses have been developed to explain the observation that a variety of antibiotics inhibit the growth of a number of bacteria in ordinary bacteriologic media and kill the organisms within a matter

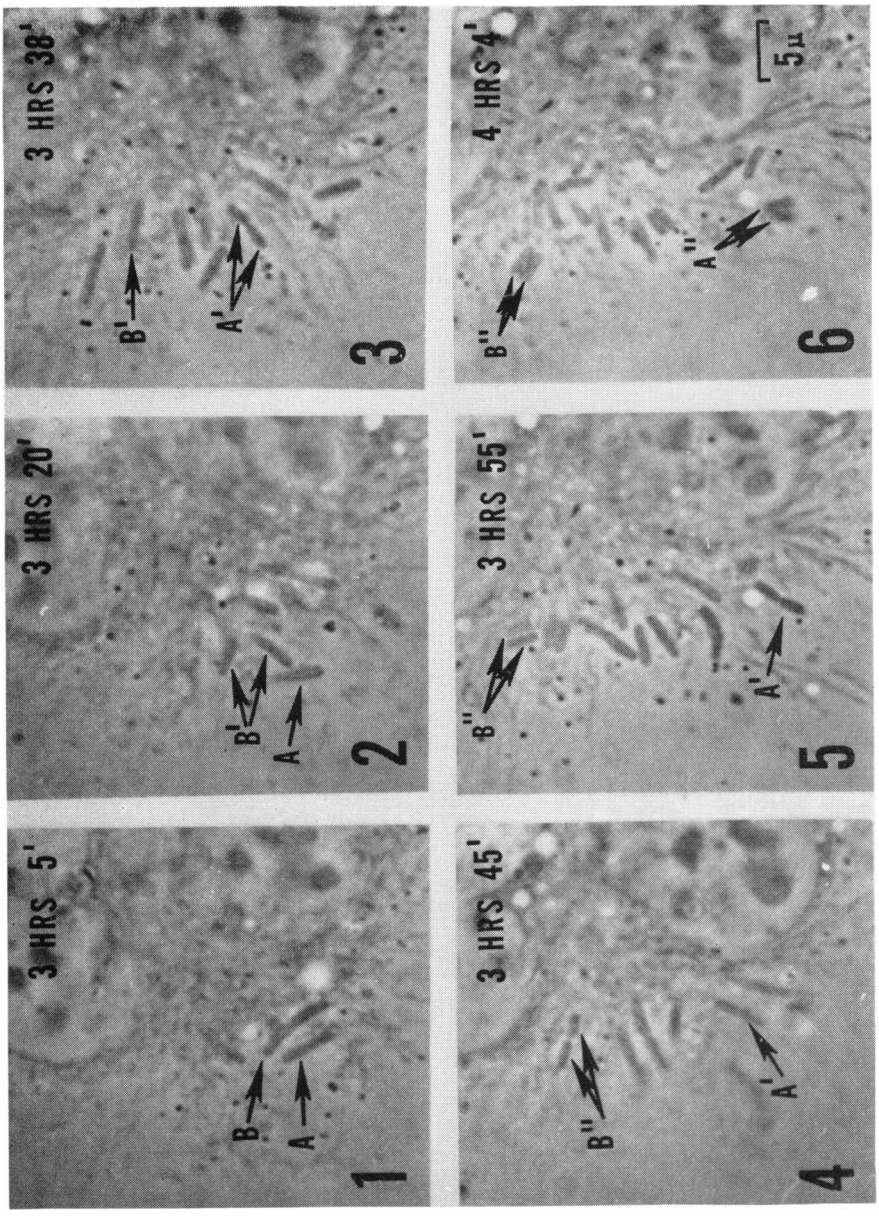

Fig. 6. Intracellular multiplication of *S. typhosa* in tissue cultures. Reproduced from *J. Immun.* 87:156, 1961 (Showacre *et al.*[9]). By permission.

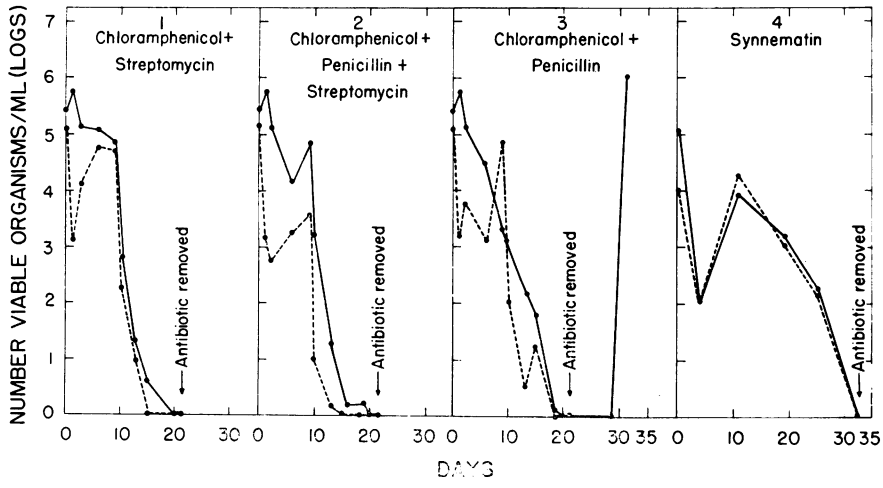

Fig. 7. Response of *S. typhosa* in infected tissue cultures to antibiotic treatment.----, supernatant fluid;———, homogenized cells. Reproduced from *J. Immun. 87*:170, 1961 (Hopps *et al.*[7]). By permission.

of hours, but the same antibiotics appear to be less effective, or are without apparent effect, on the same bacteria when they are located intracellularly. For the most part, these hypotheses have centered around the idea that the antibiotic, if it did indeed get into the cell, was ineffective because it was degraded in some way or, contrariwise, that the intracellular environment protected the antibiotic-damaged organism by supplying the bacteria with metabolites or with physical conditions necessary for survival and growth.

In studies undertaken with several colleagues, it was possible to demonstrate directly that antibiotics promptly penetrate mammalian cells and are as effective in inhibiting growth of bacteria located in the intracellular position as they are against bacteria in the extracellular environment. These studies were carried out with *S. typhosa* in a mouse fibroblast tissue culture system which was observed by phase microscopy over an extended period of time. The photomicrographs presented in Figure 6 illustrate the clarity with which bacteria inside a mammalian cell may be observed to undergo the process of multiplication.[6] Two bacteria were designated in photomicrograph 1. In subsequent pictures it is evident that the B organism divided within 15 minutes after the first photo was taken and its daughter again divided about 25

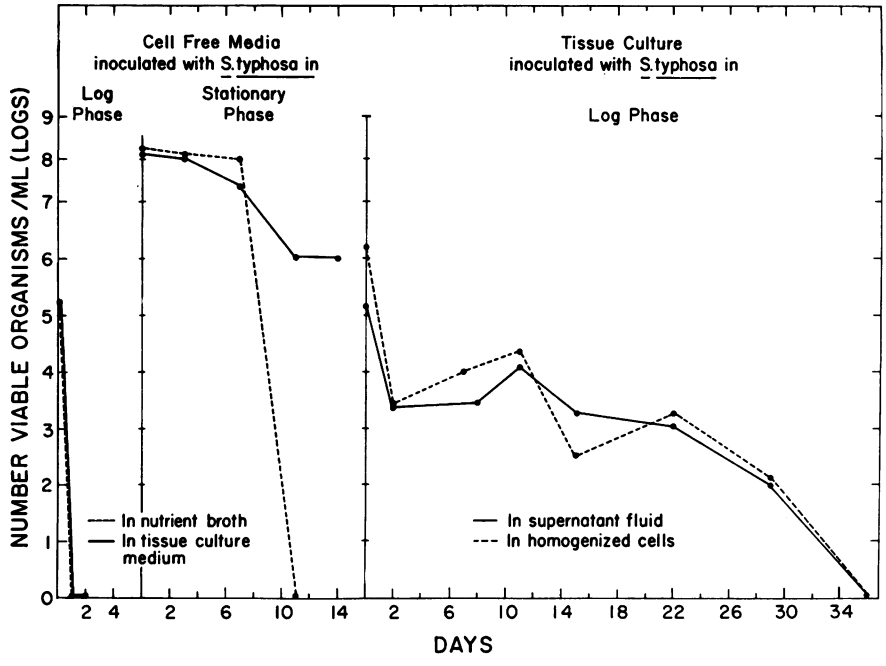

Fig. 8. Comparative inactivation rates of synnematin on *S. typhosa* in different culture media and in tissue cultures. Reproduced from *J. Immun.* *87*:170, 1961 (Hopps *et al.*[7]). By permission.

minutes later. Similarly, organism A underwent two divisions during the 59 minutes of observation.

With a relatively simple modification of this *S. typhosa*-tissue culture model it was possible to perfuse a culture with a selected antibiotic while a given intracellular bacterium was under direct and continuous microscopic observation. Under such circumstances it was found that bacterial multiplication ceased both intra- and extracellularly within a few minutes after the addition of chloramphenicol, penicillin or synnematin and within 40 to 50 minutes after perfusion with streptomycin.

In spite of the prompt inhibition of growth of intracellular *S. typhosa* by antibiotics the number of viable organisms in an infected treated tissue culture remained elevated for a week or so. Figure 7 summarizes four quantitative experiments in which the numbers of culturable *S. typhosa* in the extracellular medium and in the homogenate of tissue cells were determined at intervals after treatment.[7] It is evident from section 1 of the figure that after 10 days of exposure to chlor-

amphenicol and streptomycin, the number of viable bacteria in both the cell brei and the cell-free fluid had been reduced only about 10 per cent. However, during the next 10 days the number of organisms decreased rapidly and none was detectable by the 20th day. Comparable results were obtained in the experiment summarized in section 3. However, in this instance, when chloramphenicol and penicillin were removed from the culture, *S. typhosa* reappeared, multiplied and eventually destroyed the tissue culture. In brief, the data on this slide represent three instances in which our test tube model of the typhoid carrier was cured and one instance of a relapse after an apparent cure. The important point is the long duration of treatment required to obtain a cure, i.e., three to four weeks.

In exploring possible explanations for the time-consuming process required to cure infected tissue culture cells, we examined the effect of antibiotics on *S. typhosa* in the rapidly growing log phase and in the non-multiplying stationary phase. In addition, the effect of the suspending media on the reaction was studied.

Figure 8 summarizes such an experiment in which synnematin was present at a concentration of 20 micrograms per ml. in each of the suspending media.[7] The section on the right of the figure illustrates the results of another test with the tissue culture model and requires no comment other than to note that the culture was not cured until the 36th day of treatment. The section to the far left of the figure shows that all of the several hundred thousand *S. typhosa* in the log phase of activity were rendered nonviable within 48 hours whether suspended in ordinary bacterial culture medium with antibiotic or in enriched tissue culture medium containing synnematin.

In contrast, at least one in ten organisms in the stationary phase survived for a week under similar conditions. Indeed, in the tissue culture medium containing synnematin there were still a million viable bacteria per ml. at the end of two weeks, although by this time all of the organisms in the nutrient broth containing antibiotic were dead. Penicillin, streptomycin and kanamycin elicited similar effects, but chloramphenicol and chlortetracycline were less lethal for *S. typhosa* in either bacterial or tissue culture medium. Others had shown previously that antibiotics were more rapidly lethal for bacteria in the log phase than for those in the stationary phase. However, what had not been emphasized before was the prolonged survival of the stationary phase organism in enriched

acellular medium containing antibiotic.

There is a striking similarity between the response to antibiotic therapy of stationary phase *S. typhosa in vitro* and those organisms which persist in treated tissue cultures. Perhaps some of the actively multiplying bacteria that gained the intracellular position were subsequently damaged by exposure to the antibiotic to the point where they assumed certain characteristics of an ordinary stationary phase organism including that of greater survival under adverse conditions.

It may be noted in passing that spheroplasts were formed by *S. typhosa* in cell-free medium in the presence of penicillin or the closely related antibiotic, synnematin. However, these same antimicrobials did not induce intracellular *S. typhosa* to undergo spheroplast formation. Moreover, this phenonemon was not encountered when chloramphenicol or streptomycin was employed. Hence, it seems unlikely that the long survival but eventual death of nonmultiplying *S. typhosa* in antibiotic-treated tissue culture cells can be specifically related to the spheroplast stage of the organism.

Let us recross the bridge from the laboratory to the ward and discuss one of the more recent studies on typhoid carriers undertaken by my colleague, Dr. Theodore E. Woodward of the University of Maryland, who was the clinical member of the original team that published the reports containing the first two charts shown this evening. On the hypothesis that high levels of antibiotic, i.e., those approaching the bactericidal, must be maintained for relatively long periods of time, he treated typhoid carriers with sufficient penicillin and Benemid to maintain blood levels of the order of 60 units of penicillin per ml. for about two weeks. Four of the six carriers so treated were permanently cured. Another carrier given combined treatment with streptomycin and chloramphenicol for three weeks, in a manner analogous to that employed in the tissue culture model, was also cured. While results of this nature are encouraging, the final solution to the problem of curing typhoid carriers has not yet been obtained. However, it seems reasonable to assume that successful treatment will evolve from the intelligent integration of studies in the laboratory and on the ward—in other words, through the discipline of clinical investigation.

Perhaps my description this evening of a peripatetic series of investigations—which have wandered from man to mice and tissue cultures and back, and from the United States to South East Asia—has impressed

you as a disjointed approach to research. If so, perhaps you are right—but I must say it has been fun!

REFERENCES

1. Smadel, J. E., Woodward, T. E., Ley, H. L., Jr. and Lewthwaite, R. Chloramphenicol (chloromycetin) in the treatment of tsutsugamushi disease (scrub typhus), *J. Clin. Invest. 28*:1196-1215, 1949.
2. Woodward, T. E. and others. Preliminary report on the beneficial effect of chloromycetin in the treatment of typhoid fever, *Ann. Intern. Med. 29*: 131-34, 1948.
3. Bozeman, F. M. and others. Study on the growth of rickettsiae. I. Tissue culture system for quantitative estimations of *Rickettsia tsutsugamushi, J. Immun. 76*:475-88, 1956.
4. Hopps, H. E., Jackson, E. B., Danauskas, J. X. and Smadel, J. E. Study on the growth of rickettsiae. IV. Effect of chloramphenicol and several metabolic inhibitors on the multiplication of *Rickettsia tsutsugamushi* in tissue culture cells, *J. Immun. 82*:172-81, 1959.
5. Ley, H. L., Jr. and others. Immunization against scrub typhus. IV. Living Karp vaccine and chemoprophylaxis in volunteers, *Amer. J. Hyg. 56*:303-12, 1952.
6. Showacre, J. L., Hopps, H. E., DuBuy, H. G. and Smadel, J. E. Effect of antibiotics on intracellular *Salmonella typhosa*. I. Demonstration by phase microscopy of prompt inhibition of intracellular multiplication, *J. Immun. 87*:153-61, 1961.
7. Hopps, H. E. and others. Effect of antibiotics on intracellular *Salmonella typhosa*. II. Elimination of infection by prolonged treatment, *J. Immun. 87*:162-74, 1961.
8. a) Parker, R. T., Fitzgerald, J. C., Perry, H. D. and Woodward, T. E. Treatment of typhoid carriers with penicillin and Benemid (Abst.), *J. Clin. Invest. 32*:593-94, 1953.
   b) Personal communication from these authors.